EVOLUTION AND FUNCTION OF ACOUSTIC AND VISUAL SIGNALS IN AVIAN BROOD PARASITISM

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EVOLUTION AND FUNCTION OF ACOUSTIC AND VISUAL SIGNALS IN AVIAN BROOD PARASITISM

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EDITORIAL
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Editorial: Evolution and Function of Acoustic and Visual Signals in Avian Brood Parasitism

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Keywords: brood parasite, coevolution, host defense, recognition, egg rejection

Editorial on the Research Topic

Evolution and Function of Acoustic and Visual Signals in Avian Brood Parasitism

Avian obligate brood parasites lay eggs in other birds' nests, leaving the host to care for the parasitic young (Soler, 2014). To be successful, parasites must have the ability to evade the frontline defenses and deposit their egg in the host nest, the eggs must be accepted by hosts, and once they hatch, their offspring must elicit provisioning from the host to obtain food and survive until their independence (Davies, 2011). During these stages, the parasites may display acoustic and visual signals which may play a key role to trick, manipulate, or circumvent the hosts defenses and, in response, the hosts may discriminate, reject, or deter the parasitism event. There are numerous examples of these signals in the literature, from host vocalizations serving to recruit conspecifics to repel brood parasites *via* enhanced nest defenses; parasitic chicks producing vocal and acoustic signals during the begging display which tap into host parent-offspring communication pathways; and hosts using these clues to reject parasitic eggs or chicks. These events form a classic coevolutionary process (Davies, 2000; Yang et al., 2019). During these coevolutionary events, brood parasites may evolve visual mimicry of host eggs (e.g., Moksnes and Røskaft, 1995; Spottiswoodea et al., 2011), nestlings (e.g., Langmore et al., 2003; Tanaka and Ueda, 2005), as well as acoustic mimicry of host begging calls (Ursino et al., 2018; Wang et al., 2020; Lama et al., 2022).

Although visual signaling between brood parasites and their hosts has been studied extensively (Davies, 2011; Soler, 2014), less attention has been paid to the coevolution of acoustic signals between brood parasites and their hosts (Wang et al., 2020). As such, this Research Topic fills an important gap in understanding the evolution of adaptations related to visual and acoustic signals between brood parasites and their hosts, which is needed for a complete understanding of how visual and acoustic signals are used in these systems. Here, we provide an overview of this Research Topic and indicate how recent papers on the subject have advanced our understanding of this aspect of brood parasite-host coevolution.

When confronted with a parasitic egg in the nest, egg rejection is the most common host defense and it is generally achieved using visual signals (Spottiswoode and Stevens, 2010; Hanley et al., 2019). The seminal studies on egg rejection behavior demonstrated that hosts utilize the perceivable differences between their own eggs and those of the parasite to recognize the foreign eggs (Rothstein, 1975, 1982). In this section, four of the five papers have focused on the proximate mechanisms of egg rejection. Hanley et al. advocate for using a receptor noise-limited model as a null model for understanding the proximate mechanisms hosts use when deciding to reject foreign eggs in an

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attempt to explain why some hosts appear to make counterintuitive choices. Samaš et al. conducted a meta-analysis of avian egg traits that cue the rejection of brood parasitic eggs and found that hosts rely primarily on eggshell color traits and maculation patterns. Molina-Morales et al. investigated intra-clutch egg variation and egg rejection in the magpie (*Pica pica*), a host of great spotted cuckoo (*Clamator glandarius*), and showed that low intra-clutch variation in the blue-green coloration at the middle region of the eggs was associated with an increased chance of rejection. Weaverbirds are known for their egg variation and Lahti examined the eggs and rejection behavior of the little-known Rüppell's weaver (*Ploceus galbula*), a host of the diederik cuckoo (*Chrysococcyx caprius*; Lahti). He found that weavers used three features to recognize foreign eggs: the brightness of ground color, the brightness of spots, and the spotting pattern at the broad end of eggs. These results suggest this host uses brightness rather than the more distinctive chromatic variation to recognize eggs because of the importance of achromatic contrast in dark enclosed nests. In contrast to studies of common hosts mentioned above, Abernathy et al. examined the red wattlebird (*Anthochaera carunculata*), a host that has been parasitized by the Pacific koel *(Eudynamys orientalis*) in Australia for a relatively short period of time. This host rarely rejected foreign eggs, but populations subjected to higher parasitism rates responded aggressively toward koel mounts indicating the importance of frontline defenses in newly exposed host populations. Collectively, these studies demonstrate that there is no single, universal approach used by all hosts when it comes to the recognition of parasitic eggs, and that different selection pressures can result in the same adaptive behavior by hosts.

Nest desertion is another form of parasitic egg rejection and the only brood parasite host that routinely rejects parasitism by burying parasitic eggs is the yellow warbler (*Setophaga petechia*), a host of the brown-headed cowbird (*Molothrus ater*) (Sealy, 1995). Turcotte-Van De Rydt et al. compared the circulating corticosterone levels in yellow warblers that accepted cowbird eggs to those that deserted clutches and found corticosterone was higher in females who abandoned clutches. Hosts can also defend their nests against parasitism by attacking and mobbing adult parasites (Welbergen and Davies, 2009; Yang et al., 2021), and Tolman et al. showed that a common host like the reed warbler (*Acrocephalus scirpaceus*) does not fine tune its mobbing of cuckoos as the risk of parasitism changes seasonally. In response to these host attacks, some species of cuckoos have evolved to resemble hawks (Davies and Welbergen, 2008) and Go et al. quantified the hawk-like features in four sympatric *Cuculus* cuckoos, finding these traits were widespread in the cuckoos although features varied across species. York reviewed studies to explore the adaptive basis of predator resemblance in avian brood parasites and natural variation in host responses to these stimuli. The author suggested that different modalities of information can have independent effects on hosts and that predator resemblance takes advantage of multiple sensory and cognitive processes (York).

In addition to visual signals, acoustic signals can play an important role in the interaction between brood parasites and hosts (Wang et al., 2020). For hosts, referential alarm calls may encode information to indicate a variety of threats including brood parasites (Bártol et al., 2002; Yang et al., 2014). The yellow warbler emits a "seet" alarm call that serves as a signal to conspecifics that a cowbird is near the nest (Gill and Sealy, 2004; Kuehn et al., 2015). Lawson et al. found that the red-winged blackbird (*Agelaius phoeniceus*) eavesdropped on warbler seet calls and mediated its nest defense based on these alarms and cowbird chatter calls when the risk of parasitism was lower during the nestling stage. Wang J. et al. compared defenses of the Oriental reed warbler (*A. orientalis*) over the nest cycle and in contrast, found no evidence that the alarm calls provided specific information regarding the threat of brood parasitism. Surprisingly, the behavioral response of hosts was stronger in the nestling stage than in the egg stage, which supports the offspring value hypothesis and suggests that cuckoos may act as nest predators.

Some brood parasites have evolved mimetic acoustic signals. Adult male indigobirds (*Vidua* spp.) mimic songs and other vocalizations of their respective hosts. DaCosta and Sorenson examined variation within and among indigobird species in the non-mimetic components of their vocal behavior and found strong species effects for the non-mimetic vocalizations, which may due to phenotypic plasticity, genetic divergence for speciation, or both. Cuckoos also mimic hawks acoustically by producing "bubbling" calls that distract hosts and reduces the likelihood of egg rejection in some populations (York and Davies, 2017). However, Wang Y. et al. found no evidence that these calls affected host response and was insufficient for suppressing bird activities. Unlike female cuckoo vocalizations, male cuckoo calls do not have any apparent role in countering host defenses and instead are sexually selected (Moskát et al., 2017; Moskát and Hauber, 2022). Esposito et al. analyzed acoustic variables and visual display sequences of male common cuckoos to determine whether these signals are multimodal by nature and complement one another as is known to occur in other brood parasites (O'Loghlen and Rothstein, 2010). They found significant variation among calls, but the vocal displays were not associated with visual displays; males either vocalized and remained motionless or displayed visually without vocalizing (Esposito et al.).

Parasitic nestlings must also tap into the host parent-offspring communication system to receive food (Anderson et al., 2009). Nonetheless, Crudele et al. found that shiny cowbird (*M. bonariensis*) chicks reared by the chalk-browed mockingbird (*Mimus saturninus*) begged more intensively to playbacks of conspecific chatter calls than to host calls, while those reared by the house wren (*Troglodytes aedon*) begged with a similar intensity to the two playbacks. This indicates that the shiny cowbirds exhibited preferential begging responses to the conspecific chatter call. Finally, Rojas-Ripari et al. reviewed how parasitic young are able to manipulate hosts to stimulate parental feeding and escape host discrimination. They focused on host chick mimicry, exaggerated begging, host-attuned begging signals, and sensory exploitation. They stressed the importance of considering these adaptations from the host's perspective using experimental manipulations.

The 16 papers in this Research Topic illustrate the diversity of visual and acoustic signals and how the selection pressures imposed by avian brood parasitism may underlie the evolution of them. Many of the contributed papers explore visual signals and show the major advances in the field over the past several decades ranging from the conceptual development of signals during the first line of defense, to egg rejection and begging displays. There is increasingly more work being done on acoustic signals, such as host alarm or mobbing calls toward parasites, and vocal mimicry by parasite adults and chicks. However, the Research Topic also revealed several gaps in our knowledge, which bring the following questions for further studies. (1) What is the stimuli that brood parasites use to recognize conspecifics once they leave host nests? (2) Do the nestling calls the nestling calls of parasites show specific adaptation to their host species or populations? (3) What additional proximate cues do hosts use to reject foreign eggs (4) What are the proximate cues used in the rare cases where hosts discriminate against parasitic nestlings? (5) Does the recognition threshold varies with parasitism pressure in different host populations? (6) Is there a difference between parasites that used visual or acoustical signals to mimic hosts? (7) Is there a negative relationship between acoustic and visual signals? (8) How does auditory perception influence acoustic signals in parasite-host systems? These questions provide new directions and challenges for future research. Overall, we hope that this issue will serve to encourage further studies, which in the end will enhance our understanding of the coevolutionary process of the visual and acoustic signals in parasite-host systems.

AUTHOR CONTRIBUTIONS

CY drafted the manuscript. BP, CU, JY, and JR improved the manuscript. All authors contributed to the article and approved the submitted version.

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An Experimental Test of Defenses Against Avian Brood Parasitism in a Recent Host

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Theoretical studies predict that hosts of avian brood parasites should evolve defenses against parasitism in a matter of decades. However, opportunities to test these predictions are limited because brood parasites rarely switch to naïve hosts. Here, we capitalize on a recent host switch by the brood-parasitic Pacific Koel (*Eudynamys orientalis*) in eastern Australia, to investigate how quickly the Red Wattlebird (*Anthochaera carunculata*), a recent host that has been annexed by the koel within the last 90 years, can learn to recognize and mob adult cuckoos and evolve the ability to eject parasite eggs. Pacific Koel nestlings kill all host young, so there should be strong selection for hosts to evolve defenses. However, low parasitism rates and high egg recognition costs might slow the spread of egg ejection in our study populations, while adult parasite recognition should be able to spread more rapidly, as this defense has been shown to be a learned trait rather than a genetically inherited defense. We tested Red Wattlebirds at two sites where parasitism rate differed. As predicted, we found that the Red Wattlebird showed little or no ability to eject foreign model eggs at either site, whereas two historical hosts showed high levels of egg ejection at both sites. However, Red Wattlebirds responded significantly more aggressively to a koel mount than to mounts of a harmless control and nest predator at the site with the higher parasitism rate and gave significantly more alarm calls overall toward the koel mount. Our results support previous evidence that recognition and mobbing of a brood parasite are learned traits and may be especially beneficial to naïve hosts that have not had enough time or a high enough selection pressure to evolve egg rejection.

Keywords: *Anthochaera carunculata*, avian brood parasitism, egg ejection, *Eudynamys orientalis*, host defenses, mobbing, Pacific Koel, Red Wattlebird

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INTRODUCTION

Brood parasitic cuckoos lay eggs in the nests of other species and provide no parental care. Upon hatching, many cuckoo nestlings eject all host eggs and young from the nest, thereby completely eliminating the host's reproductive success for that particular breeding attempt (Davies, 2000). This cost should select for the evolution of host defenses to circumvent brood parasitism. Mobbing is one type of host defense that has been shown to be highly effective in certain species at deterring parasitism (Feeney et al., 2013) and preventing the parasite from removing or damaging host eggs

(Gloag et al., 2013). In rare cases, mobbing can result in the death of the parasite (e.g., Jackson and Kyne, 2010; Gloag et al., 2013; Abernathy and Langmore, 2016). If mobbing fails to prevent parasitism, egg ejection (removing parasitic eggs from the nest) can be an efficient defense for some hosts because it allows the host to keep its nest and most of its original clutch intact (reviewed in Soler, 2014).

There is evidence that the ability to discriminate and reject foreign eggs is a genetically inherited trait although female hosts may vary in their expression of this trait throughout their lives (Molina-Morales et al., 2014; Martínez et al., 2020). Multiple egg ejection studies have shown that host species tend to retain egg ejection ability even in the absence of parasitism (e.g., Avilés, 2004; Peer et al., 2005; Soler, 2014; Yang et al., 2014) and even through speciation events (Peer et al., 2013). Further, Martín-Gálvez et al. (2006) showed that genetic differences exist between individuals that ejected model parasitic eggs and those that accepted them in the European Magpie (*Pica pica*), although they did not control for age differences known to affect rejection (Martínez et al., 2020). Theoretical models predict that the evolution of egg rejection requires at least 30–100 years and will occur more quickly when parasitism rates and costs of parasitism are high (Rothstein, 1975a; Kelly, 1987; Takasu et al., 1993; Davies et al., 1996). According to one model, egg rejection could evolve within 97 years if parasitism rate is 50%, but would take 1,974 years if parasitism rate is 2% (Kelly, 1987). Similarly, Rothstein (1975a) calculated that under parasitism rates of 19% the proportion of egg rejecters in a population of Eastern Phoebes (*Sayornis phoebe*) would increase from 5 to 95% in about one hundred generations.

While egg ejection is likely to be a genetically inherited trait, the ability to recognize a brood parasite as a nest threat is likely to be a learned trait. Many studies have demonstrated that parasitized populations and individuals with prior experience of parasites respond more aggressively to a parasite near the nest than individuals that have not experienced parasitism (reviewed in Feeney et al., 2012). In one cuckoo host, the Superb Fairy-wren (*Malurus cyaneus*), first-year individuals that had never encountered a cuckoo did not mob a cuckoo mount, but learned to do so after witnessing mobbing of a cuckoo mount by their family members (Feeney and Langmore, 2013). Similar studies were conducted on captive European Blackbirds (*Turdus merula*) that were trained to mob a harmless species by watching other conspecifics mob it (Curio et al., 1978). If brood parasite recognition and mobbing is not dependent on genetic evolution and can be transmitted socially, then this trait has the potential to spread rapidly throughout a host population (Curio et al., 1978; Davies and Welbergen, 2009). This may be especially beneficial for naïve or recent hosts that have not had enough time or a high enough selection pressure to evolve other defenses such as egg ejection.

Because brood parasites rarely switch to naïve hosts, there are only a handful of studies that have quantified the types of defenses that naïve hosts use against brood parasites (Soler et al., 1994; Nakamura et al., 1998; Avilés et al., 2006; Spottiswoode and Stevens, 2012). Understanding what types of defenses are available to naïve hosts is important because it may help us understand how quickly hosts can evolve defenses and can aid us in making conservation decisions in regards to endangered potential hosts, as many brood parasites have been expanding their breeding ranges due to environmental changes (Rothstein, 1975a; Dinets et al., 2015).

The Pacific Koel (*Eudynamys orientalis*) provides a rare case of a cuckoo that has recently adopted a new host. Koels are found along the northern and eastern coasts of Australia and the nestlings are highly virulent, evicting all host young from the nest (Higgins, 1999). In the past, the koel's primary hosts in New South Wales were the Magpie-lark (*Grallina cyanoleuca*) and Noisy Friarbird (*Philemon corniculatus*) (Brooker and Brooker, 1989, 2005). However, in recent decades they have switched to a new host, the Red Wattlebird (*Anthochaera carunculata*) (Abernathy and Langmore, 2017). Evidence suggests that the Red Wattlebird in the SE of Australia (where koel parasitism occurs) was likely to be naïve to brood parasitism, as range expansions of both the koel and wattlebird brought them into contact with one another around the early 1900s and the first time a koel was observed using the wattlebird as a host was in Sydney in 1978 (Abernathy and Langmore, 2017). Further, while wattlebirds in the SW of Australia are a host of the Pallid Cuckoo (*Cuculus pallidus*), they appear to be only rarely used as a host in the SE and there is no evidence that these two wattlebird populations interbreed (Abernathy and Langmore, 2017).

We conducted this study in both Sydney and Canberra. At the time of this study in Sydney (2016), wattlebirds may have been a host of the koel for around 38–86 years and experience a current parasitism rate of 24% (Abernathy and Langmore, 2017), which, as theory predicts, could be enough time and a high enough parasitism rate for egg ejection to have evolved (Rothstein, 1975a; Kelly, 1987; Takasu et al., 1993; Davies et al., 1996). However, parasitism rate in Canberra is very low (4%) and the length of time wattlebirds have been parasitized by koels is also less (around 8–33 years at the time of this study) (Abernathy and Langmore, 2017). Further, while successful parasitism by a koel means the wattlebird will lose its entire clutch (costs of parasitism are high), a previous study found that the impacts of koel parasitism on wattlebird breeding success in Sydney were low (Abernathy and Langmore, 2017). This was partly due to the koel's poor timing of egg laying coupled with the wattlebird's abandonment of koel eggs laid before the host had started laying (Abernathy and Langmore, 2017). Additionally, costs of recognition errors (rejecting a host egg rather than the parasitic egg) could be high, as wattlebird and koel eggs appear very similar in luminance and pattern and their ground colors were found to be nearly indistinguishable from a bird's visual perspective (Abernathy et al., 2017, see also Takasu, 1998; Robert and Sorci, 1999). This high cost could be exacerbated by the fact that wattlebirds typically only lay 1–2 eggs per clutch, potentially making it more difficult to identify the parasitic egg as the "odd one out" in the nest. Because wattlebirds are a recent host, the close match in egg appearance between their eggs and koel eggs is unlikely to be the result of direct mimicry by the koel. Rather, it may be the result of mimicry by the koel of the wattlebird's close relative and a historic host of the koel, the Noisy Friarbird, whose eggs also appear very similar in luminance and pattern and were indistinguishable in ground color from koel and wattlebird eggs (Abernathy et al., 2017). However, this would need to be tested with egg ejection experiments to determine if friarbirds and wattlebirds are capable of ejecting odd-looking eggs from their nests.

We hypothesize that the evolution of egg ejection in the wattlebird has been constrained due to all of the above factors, but wattlebirds are likely to have had enough time to learn to recognize adult koels as a nest threat, especially in Sydney where koel parasitism is higher (24%) than in Canberra (4%, Abernathy and Langmore, 2017). To determine whether wattlebirds exhibit mobbing defenses against adult koels, we exposed nesting pairs to freeze-dried mounts of a harmless species (Crimson Rosella, *Platycercus elegans*), a nest predator (Pied Currawong, *Strepera graculina*), and a female koel and quantified the wattlebirds' behavioral responses toward each mount. Due to the higher parasitism rate in Sydney, we predicted that wattlebirds would be more aggressive and more likely to attack koel mounts in Sydney than in Canberra. Further, we predicted that wattlebirds would exhibit higher aggression toward koel mounts than currawong and rosella mounts in Sydney, but should show similar levels of aggression toward both koels and currawongs in Canberra. Wattlebirds should be able to recognize koels as a nest threat, but may not view them differently from a nest predator, as successful parasitism results in a complete loss of host young. Additionally, koels may depredate nests late in incubation or in the nestling phase in order to force the host to re-lay, as other brood parasites have been shown to do (Elliott, 1999; Davies, 2000; Granfors et al., 2001), though we have no direct evidence of Pacific Koels predating nests. Thus, this type of test was used to determine if wattlebirds viewed koels as a nest threat, but was insufficient to determine whether or not wattlebirds understand the kind of threat koels pose to their nests (brood parasitism, rather than nest predation). As some brood parasite hosts have been shown to exhibit passive nest defense (sitting longer on the nest to prevent the parasite from laying), we also tested wattlebirds for this behavior (Gill and Sealy, 2004; Canestrari et al., 2009; Medina and Langmore, 2016).

We used model eggs to determine the level of egg ejection ability in wattlebirds and compared this to the level of egg ejection in the two old hosts, the Magpie-lark and Noisy Friarbird. Not only did the comparison of the old hosts to the recent host aid us in understanding if our model eggs were useful indicators of egg ejection ability, but it also allowed us to better understand whether koels have evolved egg mimicry with the Noisy Friarbird and/or Red Wattlebird. We presented hosts with either a model egg that appeared somewhat similar to the hosts' eggs (similar spotting pattern and similar ground color) or strikingly different (no spotting and dissimilar, blue ground color). Two different experimental egg morphs were used because discrimination abilities may vary according to the sensory perception of particular species (Lahti, 2015) and we wanted to test if hosts could eject both extremely non-mimetic and somewhat mimetic model eggs. If the evolution of egg ejection has been constrained in the Red Wattlebird, we predicted that the two old hosts would exhibit egg ejection at higher rates than wattlebirds even though they are rarely parasitized in the areas where we conducted our study. This is because many studies show that egg ejection, once evolved, is maintained in the absence of parasitism for most hosts (e.g., Avilés, 2004; Peer et al., 2005; Soler, 2014; Yang et al., 2014). We also predicted that the old hosts would eject the blue eggs at a higher rate than the spotted model eggs because the blue eggs should be easier to distinguish from the host eggs than the spotted model eggs.

MATERIALS AND METHODS

Study Species and Sites

We tested the level of mobbing defenses in Red Wattlebirds and the level of egg ejection defenses in Red Wattlebirds, Magpie-larks and Noisy Friarbirds at two sites, Western Sydney and Canberra. At the time of this study (2016) in Sydney, koels had been parasitizing wattlebirds for 38–86 years with a current parasitism rate of 24%, while in Canberra wattlebirds had been a host of the koel for 8–33 years with a current parasitism rate of only 4% (Abernathy and Langmore, 2017). Abernathy and Langmore (2017) found no cases of parasitism of the Magpie-lark or Noisy Friarbird in Western Sydney or in Canberra. However, there has been one anecdotal report of a Noisy Friarbird raising a koel nestling in Canberra in 2014 (Abernathy and Langmore, 2016). Though no formal studies have been conducted on koel parasitism rates of the two old hosts, Brooker and Brooker (2005) found 20 records of koel parasitism of the Magpie-lark on the east coast of Australia from 1909–2008 and 29 records of koel parasitism of the Noisy Friarbird on the east coast from 1893–2002.

All experiments were conducted during the breeding season. The mobbing experiments on Red Wattlebirds were performed from September–October 2015 in Western Sydney and October–November 2015 in Canberra (**Table 1**). For the egg ejection experiments we searched for Red Wattlebird, Magpie-lark, and Noisy Friarbird nests at several different sites in Canberra from August 2013–January 2014 and August 2015–December 2016, and in Western Sydney from August 2014–January 2015 and August 2015–December 2016 (**Table 1**). In both Canberra and Sydney, wattlebird and Magpie-lark nests were mostly found in residential areas that included a wetland feature, while friarbird nests were only found in high numbers in nature parks or rural areas with native habitat. Koel parasitism was only found in residential areas or urban parks in both Western Sydney and Canberra and wattlebirds were the only host species for which parasitism was detected at our study sites (Abernathy and Langmore, 2016, 2017). For more information on the koel-wattlebird study system, a map of the study sites and evidence of duration of sympatry between wattlebirds and koels in Sydney and Canberra, see Abernathy and Langmore (2017). In addition to testing these three host species, we informally tested the egg ejection ability of a minor host, the Olive-backed Oriole (*Oriolus sagittatus*; Brooker and Brooker, 1989), as we occasionally found them nesting near Noisy Friarbirds and their egg ejection capability was unknown. Due to low sample sizes, we only tested orioles with the non-mimetic blue egg and we did not include these nests in any of our statistical analyses.

TABLE 1 | Location and number of mobbing and egg ejection experiments performed for each host and egg treatment ("M" = mobbing experiment, "B" = blue egg ejection experiment, "S" = spotted egg ejection experiment) in **(A)** Canberra, ACT and **(B)** Western Sydney, NSW.

Name of site	Geographic coordinates	Red wattlebird	Noisy friarbird	Magpie-lark
(A)				
ANBG, ANU	35.28°S, 149.11°E	B: 14	0	B: 1
Lake Ginninderra	35.23°S, 149.07°E	B: 2	B: 1; S: 2	B: 7; S: 5
Belconnen District	35.22°S, 149.06°E	M: 9; B: 1; S: 6	B: 1; S: 2	S: 1
Canberra central district	35.29°S, 149.13°E	M: 2; B: 3; S: 2	0	B: 2; S: 3
Weston creek district	35.34°S, 149.05°E	M: 3; B: 1; S: 4	0	0
Mt. Majura, Mt. Ainslie, Jerrabomberra wetlands	35.27°S, 149.17°E	B: 2	B: 11; S: 3	S: 1
The Pinnacle, Hawker	35.26°S, 149.04°E	0	S: 2	0
Casuarina Sands Reserve, Stromlo	35.32°S, 148.96°E	0	B: 1	0
(B)				
Blacktown; Marayong; Plumpton; Stanhope Gardens; Woodcroft	33.75°S, 150.88°E	M: 1; B: 5; S: 6	0	B: 5; S: 6
Werrington lake	33.75°S, 150.74°E	0	0	S: 1
Blue Hills wetlands and Surveyor's Creek, Glenmore Park	33.79°S, 150.68°E	M: 1; B: 2; S: 4	0	B: 3; S: 3
Chipping Norton lake	33.91°S, 150.95°E	M: 1; B: 2; S: 1	0	B: 2; S: 1
The Crest Sporting Complex, Georges Hall; Kentucky Reserve, Bankstown Aerodrome	33.91°S, 150.99°E	M: 7; B: 2; S: 2	0	0
Tench Reserve, Jamisontown	33.76°S, 150.67°E	0	0	B: 1; S: 1
Cattai and Scheyville National Parks	33.59°S, 150.90°E	0	B: 4; S: 2	0
Glenbrook	33.77°S, 150.62°E	M: 1; B: 1	B: 6; S: 6	0

Mobbing experiments were only performed at Red Wattlebird nests. Geographic coordinates are points near the center of each area obtained from Google Maps. "ANBG", the Australian National Botanic Gardens" and "ANU", the Australian National University.

Mobbing Experiments

We compared the aggressive response of wattlebird pairs in the incubation stage of breeding to mounted specimens of a female koel, a common nest predator (Pied Currawong) and a common, harmless parrot (Crimson Rosella). Each of these species have vastly different plumages, making them easy to distinguish, but similar sizes. Crimson Rosellas are around 35–38 cm long and have mainly crimson and blue-colored plumage (Higgins, 1999). Pied Currawongs are slightly larger, about 48 cm long on average, and have mostly black plumage with some white patches (Higgins et al., 2006). Female koels are around 41 cm long and have a dark cap, off-white to buffy front with dark horizontal barring and a dark brown back with white spotting (Higgins, 1999). We used two rosella and currawong mounts, alternating them for each nest, but only had one available female koel mount. Mounts were placed 2–2.5 m from the nest. For low nests (1.5–3 m high), mounts were fastened to the top of a 2.3 m ladder. For higher nests (4–10 m), mounts were hauled up to the appropriate height using a rope hanging over a nearby branch. While this difference in mount presentation has the potential to cause a response bias, we found no effect on aggressive response based on how the mount was positioned (see section "Results"). Mounts were placed in a mesh cage to protect them from damage by the wattlebirds. Observations were made from a blind or car placed four or more meters from the base of the nest tree.

When possible, each pair was presented with all three mounts and mounts were presented in random order to control for order-effects. We only performed two trials per day at a nest to reduce the amount of disturbance and we waited at least 2 h between trials to reduce the chance of carry-over aggression. Before starting the trial, the wattlebirds were allowed at least 30 min to habituate to the cage and hide setup before the mount was placed in the cage. In all but two trials, if the female was on the nest when the mount was placed in the cage, we flushed her off the nest. Trials commenced when the male or female came within 2 m of the mount and continued for 5 min. Observations of the pair's response to the mount and any vocalizations given by the pair were recorded into a Tascam DR-07 or DR-05 recorder and microphone. We estimated the distances (in meters) of both members of the pair from the mount each time they moved. We counted the number of times they swooped or attacked (physical contact with the cage), the number and type of vocalizations given and we noted whether the female was on the nest.

Male and female wattlebirds are distinguishable in the field by their vocalizations; females give the more musical whistle, while males give harsh clucks and cackles (Higgins et al., 2001, Abernathy personal observation); females give the more musical whistle, while males give harsh clucks and cackles (Higgins et al., 2001). Higgins et al. (2001) mention that males may incubate, but in our study whenever a bird flushed off the nest and vocalized, it gave whistles and never clucks or cackles. Therefore, a bird on the nest was assumed to be a female. For most trials it was possible to distinguish male from female based on these characteristics. We classified wattlebird calls into seven different types by ear and by visual inspection of structural differences in spectrograms generated using Audacity 2.0.5 and Raven Pro 1.4 (alarm calls, growls, cackles, clucks, whistles, contact calls, and single note calls; Higgins et al., 2001). We used Audacity to score the number of each vocalization type. We excluded vocalizations if they were given by a bird not in our view or far away (8–10 m away) or if the bird was clearly responding to another bird.

Egg Ejection Experiments

Studies have shown that many brood parasite hosts use a combination of cues to recognize brood parasite eggs, including ground color, pattern, luminance and size (e.g., Rothstein, 1982; Marchetti, 2000; Spottiswoode and Stevens, 2010; de la Colina et al., 2012). Therefore, to determine how well hosts could discriminate between their eggs and a foreign egg, we created two types of model eggs: a dark blue immaculate egg that differed in two parameters, pattern and ground color, and a spotted egg that was painted to resemble host eggs in pattern and ground color, at least according to the human eye (Longmore, 1991, **Figure 1** and see **Supplementary Materials**). We chose not to test egg size as a variable, so all model eggs came from molds of the same size and were a similar size to all host eggs (see **Supplementary Materials**).

One of these egg types was placed into a host nest during the laying or incubation periods and the nest was monitored to determine if and when the host ejected the model egg. Nests were typically checked every 1–2 days. For high nests we used an extendable mirror pole to check contents. If nests were accessible with a ladder or by climbing the tree, model eggs were typically placed in the nest by hand. For nests that were inaccessible, we created a device to deposit the egg in the nest using an extendable mirror pole (see **Supplementary Figure 1**). Even though koels typically remove one host egg during parasitism (Brooker and Brooker, 1989), we did not remove host eggs from the nest during experimental parasitism, as this has not been found to alter host responses in other studies (Davies and Brooke, 1988; Moksnes and Røskaft, 1989).

In our study, 89% of ejections took place within the first 5 days of the experiment. Therefore, an egg was considered accepted if it remained in an active nest (eggs were warm or bird was observed incubating the clutch or defending the nest) for at least 5 days, though we continued checking after this point for 89% of nests to verify the egg was not ejected after this period (see also Rothstein, 1975b). An egg was considered ejected if the model egg was removed and the nest was clearly still active upon discovery of the ejection event. Of the nests where ejection took place, 75% of nests were checked again at least 1 day after ejection and found to still be active, which suggests the model egg was not taken by a predator. Wattlebird nests that were naturally parasitized during the first 5 days of the experiment were not included in the analyses. There were never any naturally parasitized Magpie-lark or Noisy Friarbird nests. We avoided any potential re-nests or territories of breeding pairs that had already been tested successfully in that particular breeding season or in a previous breeding season to minimize the risk of pseudo-replication. How territories in this study were estimated is explained in a previous study (Abernathy and Langmore, 2017, see "Estimating territories" in the "Materials and Methods" section). It was not possible to record data blind because our study involved focal animals in the field.

In our study, we did not include abandoned nests as rejection events for two reasons. First, all the hosts in our study are large (85–122 g; Higgins et al., 2001, 2006) and were capable of grasp-ejecting a foreign egg (see results in Rohwer and Spaw, 1988). Second, a previous study found nest abandonment by wattlebirds was only related to general nest disturbance by researchers and not to the presence of a koel egg in the nest or the study site (Abernathy and Langmore, 2017).

Model Eggs

Model eggs were made using a two-part silicone mold and polyurethane resin. After removal from the mold, eggs were smoothed with sand paper and painted with acrylic paint. Spotted eggs had a pinkish ground color with dark reddish-brown and violet-gray spots and were meant to appear similar to the host's own eggs (**Figure 1** and see **Supplementary Materials**). Even though koel eggs do appear similar to most of their host eggs in color, luminance and spotting, and there is no evidence they exhibit host egg races, koel eggs are significantly more similar in egg pattern and are closer in ground color to Noisy Friarbird and Red Wattlebird eggs than to Magpie-lark eggs (Abernathy et al., 2017). These differences in spotting pattern and ground color could create confounding variables, making our model eggs easier to distinguish for Magpie-larks than for the other hosts. So instead of making model eggs that resembled koel eggs, we attempted to create model eggs that mimicked each host species' eggs to reduce this potential issue. Spots were created using the pointed end of a plastic dental floss pick, though it was difficult to create the smallest size of spots that occur naturally on the host eggs. Noisy Friarbird and Red Wattlebird eggs are very similar in ground color, luminance and egg pattern, with the majority of spotting at the larger end of the egg, while Magpie-larks typically have a lighter ground color and an obvious ring of spotting around the larger end of the egg (Longmore, 1991; Abernathy et al., 2017, **Figure 1**). Therefore, we created Magpie-lark spotted model eggs with a ring of spotting at the blunt end and with a lighter ground color (extra white paint mixed with pink) and for Noisy Friarbird and Red Wattlebird spotted model eggs, we created eggs without the distinct spotting ring and a darker ground color (**Figure 1**). Many passerine eggs have a spectral reflectance pattern with a peak in the UV range (e.g., Cherry and Bennett, 2001; Honza et al., 2007; Cassey et al., 2010; Abernathy and Peer, 2015), including host eggs in this study (**Figure 1**). In an effort to create more realistic spotted model eggs, we mixed white ultraviolet-reflecting paint (ReelWings) with pink acrylic paint to make the ground color. While this did create a UV-reflecting egg, the spectral pattern of the spotted model eggs did not perfectly match that of a real egg, as there was no peak in the UV range (**Figure 1**). However, the hosts are presumed to have VS rather than UVS opsins, which will have very low sensitivity to these ∼320 nm peaks (Ödeen and Håstad, 2010; Ödeen et al., 2011). To determine how similar our eggs appeared to host eggs from the host's visual perspective, we took objective measurements in size, color, luminance and pattern for a subset of fresh host eggs and each model egg

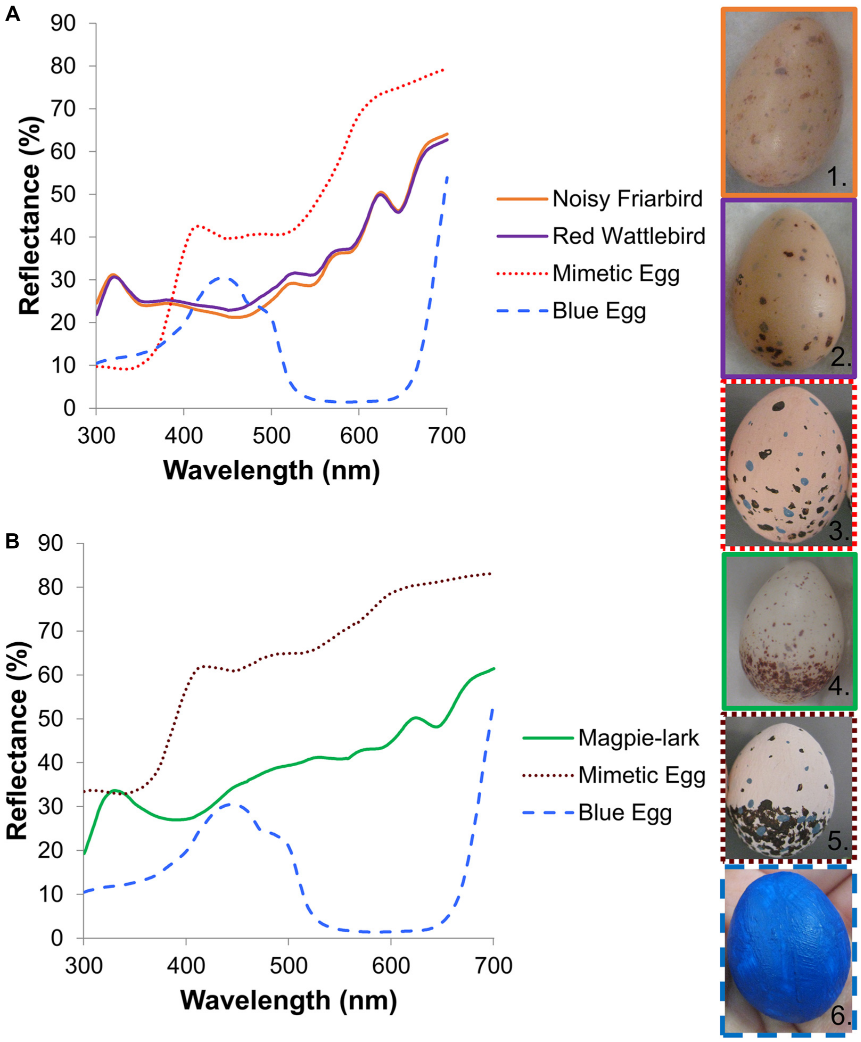

FIGURE 1 | (A) Average spectral reflectance of fresh Noisy Friarbird (*n* = 13) and Red Wattlebird (*n* = 45) clutches and their corresponding spotted model eggs (*n* = 37) and blue eggs (*n* = 10). **(B)** Average spectral reflectance of fresh Magpie-lark clutches (*n* = 15), Magpie-lark spotted model eggs (*n* = 21) and blue eggs (*n* = 10). Images 1–6 are representative real host eggs and model eggs used in the egg ejection experiments: 1, Noisy Friarbird; 2, Red Wattlebird; 3, Wattlebird/friarbird spotted model; 4, Magpie-lark; 5, Magpie-lark spotted model; 6, Blue model. All photographs taken by V. E. Abernathy.

type (see **Supplementary Materials**). While our spotted model eggs did not mimic host eggs perfectly, their ground color was very similar from a bird's visual perspective and their size and all pattern measurements did fall within the natural range found for each species (see **Supplementary Tables 1**, **2** and **Supplementary Figure 2**).

Statistical Analysis

Mobbing Experiments

In order to measure aggressive response during mobbing trials, we used three variables: the proportion of the trial that at least one parent was less than 2 m from the mount, vocalization rate and swoop or attack rate. We suspected that certain vocalizations, such as the alarm call, would be more important in predicting mount type than other vocalizations. However, vocalization function in Red Wattlebirds is poorly understood. Therefore, we first determined which vocalizations were important predictors of mount type by performing restricted maximum likelihood model (REML) analysis for each vocalization. We used the number of calls given during a trial for a particular call type as the response variable, the nest ID as the random effect, and the mount type as the independent variable. We were not able to analyze the growl call type because it was only given during one trial by one pair. These analyses indicated that only alarm calls were significant predictors of mount type (**Table 2** and **Figure 2**; see more in section "Results"). Based on this result, we only used the number of alarm calls given when calculating vocalization rate in our aggressive response analysis.

To determine if females used passive defense to protect their nests from koels we measured the proportion of time females sat on the nest during the trial. Females were present during every trial, while males were only present for some trials. In addition,

TABLE 2 | Results from the best REML testing what predicted wattlebird aggressive response, the GLMM testing if mount type predicted number of attacks, the REML testing if alarm call number was predicted by mount type, and the GLMM testing if females exhibited passive defense during mobbing experiments.

Test	Variable	*F*-value	Degrees of freedom	*P*-value	r^2 adjusted
REML testing predictors of wattlebird aggressive response	Mount type (koel, currawong, rosella)	7.6	2, 39	0.002	0.65
	Site (Canberra, Sydney)	2.5	1, 22	0.13	
	Mount type × Site	5.9	2, 39	0.006	
GLMM testing if mount type predicted number of attacks	Mount type (koel, currawong, rosella)	3.1	2, 43	0.055	0.57
REML testing if mount type predicted alarm call number	Mount type (koel, currawong, rosella)	5.7	2, 41	0.007	0.20
GLMM testing predictors of time females sat on nest	Mount type (koel, currawong, rosella)	9.1	2, 42	<0.001	0.57
	Placement of cage (attached to ladder, hanging from branch)	4.9	1, 23	0.04	
	Whether male attacked mount or not	4.8	1, 53	0.03	

sometimes one member of the pair was present for only part of a trial and distinguishing male from female for the entire duration of every trial was not always possible as the birds did not have colored bands. Therefore, to calculate rates of alarm calling and swooping, we first calculated the total minutes of observation of the two focal birds (e.g., 10 min if both members of the pair were present for the entire trial, 5 min if only one member of the pair was present for the entire trial), and then calculated the number of alarm calls or swoops given over that time period. To be counted as present (aware of the mount) once the trial had begun, the focal bird must have either landed within 5 m of the mount or have clearly shown aggression toward the mount.

In order to test if differences existed in the aggressive responses of wattlebirds based on mount type and other independent variables, we first combined the aggressive response variables using a Principle Components Analysis (PCA) to obtain a single aggressive response score. We checked for high correlation among our three aggressive response variables and found that none had a correlation value above 0.55. The PC1 was the only component with an Eigenvalue above one (1.998) and it explained 67% of the variation.

We treated the PC1 for each trial as our aggressive response score and used this as our response variable in another REML with nest ID as a random effect. Independent variables included mount (koel, currawong, and rosella), the site where the experiment took place (Canberra, Sydney), the Julian date of the trial, whether the cage was hanging from a branch or attached to the ladder and the interaction between site and mount. To determine if pairs physically attacked or swooped based on mount type or if they were equally likely to attack all mounts, we ran a binomial GLMM with a logit link function with nest ID as the random effect and whether the pair attacked (1) or not (0) as the response variable. We used all the same independent variables as in the REML, but were not able to include any interactions due to the few times pairs actually attacked a mount. We also used a Fisher's Exact Test to compare the percentage of pairs that attacked or swooped koels at each site. This test was done in the R Statistical Package (R Core Team, 2014). To determine if females sat on the nest for longer in the presence of a koel mount, we ran a binomial GLMM with a logit link function with nest ID as the random effect and the proportion of time a female sat out of the total time she was present as the response variable. We used the same independent variables in this analysis as in the REML, and also included whether the male attacked or not.

We performed the PCA, REMLs, and GLMMs in JMP Pro 14.3.0. For all statistical models, the most non-significant terms were removed sequentially starting with the interactions until only significant or near-significant terms remained. We used an alpha of 0.05 and the model with the lowest AIC score was chosen. Least square means (LSM) ± 95% confidence intervals are reported for the REMLs and GLMMs.

Egg Ejection Experiments

We used a binomial generalized linear model (GLM) with a logit link function to determine which variables predicted ejection response (1 = ejection, 0 = acceptance). Five of the independent variables in the full model were host species (Red Wattlebird, Magpie-lark or Noisy Friarbird), egg type (blue or spotted), the site where the experiment took place (Canberra or Sydney), the breeding season year (first or second) and laying date based on a Julian calendar. If the actual laying date for a nest was unknown, we estimated laying date by counting back from hatch day (incubation is typically 16 days for wattlebirds and friarbirds, and 18 days for Magpie-larks; Higgins et al., 2001, 2006). We assumed wattlebirds and friarbirds always began incubating after laying the last egg in a clutch and Magpie-larks always began incubating immediately after laying the first egg (Higgins et al., 2001, 2006). For some nests, hatch day or laying date could only be estimated to a range of dates (e.g., August 02–04). In these cases, we estimated laying date by taking the median value of the range and only if the range was from 2 to 3 days.

A final variable in the full model was the number of days left until the clutch would be completed when the model egg was added to the nest ("days until clutch completion"). This variable is similar to the commonly used variable nesting phase (laying or incubating), but it is more precise, breaking down the phases by days. This variable is important in understanding

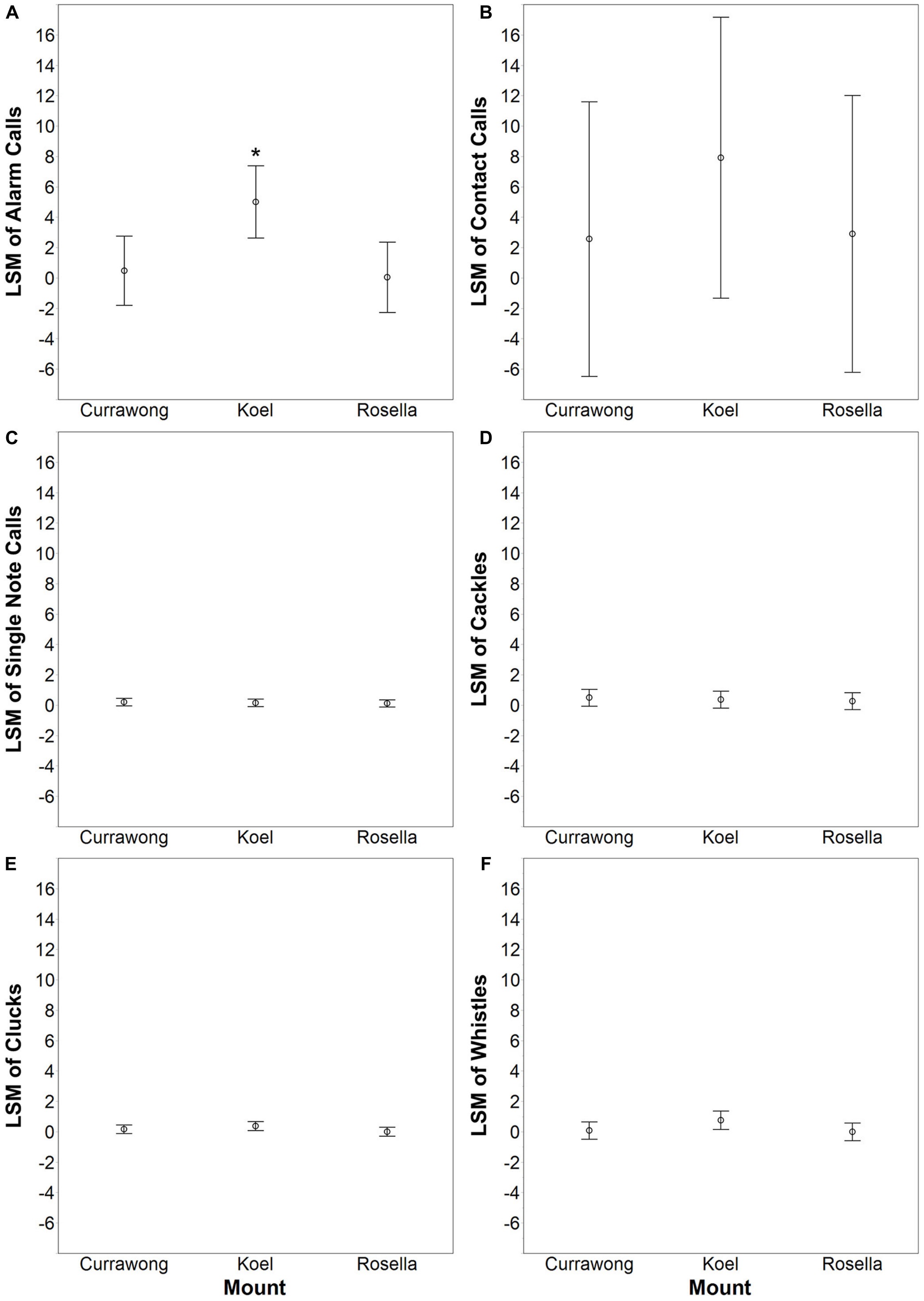

FIGURE 2 | The least square means (LSM) ± 95% CI from six (**A–F**) REMLs where the number of calls given for each call type during a trial was the response variable, nest ID was the random effect and the type of mount (koel, currawong and rosella) was the independent variable. An asterisk indicates a significant difference between treatments (Tukey HSD, $P < 0.05$).

ejection decisions because a host may be less likely to eject an egg if it is placed in the nest at the beginning of the laying period before it has seen its entire clutch, especially if the host is a first-time breeder (Lotem et al., 1995; Rodríguez-Gironés and Lotem, 1999, but see Soler et al., 2013). Days until clutch completion also controls for varying clutch sizes, as a host that lays fewer eggs may be more likely to eject than a host with a larger clutch simply because there is a higher chance of placing the model egg in the nest after the clutch has been completed. We did not include clutch size as an independent variable because each host species tended to lay different clutch sizes. In our study, wattlebirds typically laid 1–2 eggs, friarbirds 2–3, and Magpie-larks 3–4. Therefore, adding the variable "clutch size" might have confounded our results, as clutch size also related to host species.

Laying date was unknown or unable to be accurately estimated for 36 nests. Therefore, we first ran the GLM only including nests where laying date was known (n = 107 nests). However, this variable was not significant or near-significant independently or in interactions. We ran the GLM again, excluding the laying date variable and adding in the remaining 36 nests (n = 143 nests) and only report results for this second GLM.

Generalized linear models were performed using the *rms* (Harrell, 2016) and *multcomp* packages (Hothorn et al., 2008) in the R Statistical Package (R Core Team, 2014) with alpha < 0.05. GLMs were run including all terms and interactions between host and every other term, except for the breeding season year because in Canberra, Magpie-larks were only tested in the first year of the study. The model with the lowest AIC score was chosen, which always only included significant or near-significant variables.

Ethics Statement

This project was approved by and conducted in accordance with the Australian National University Animal Experimentation Ethics Committee: protocol number A2013/20. Permits from the Territory and Municipal Services of the ACT (license number: LT2013678) and the Office of Environment and Heritage of the NSW National Parks and Wildlife Service (license number: SL101349) were obtained to conduct scientific experiments in Canberra and Western Sydney.

RESULTS

Do Wattlebirds Recognize Koels as a Threat?

Overall, we tested the behavioral response of 11 wattlebird breeding pairs in Sydney and 12 pairs in Canberra to the mounts, but two koel trials and one rosella trial conducted on three different pairs were not successfully completed and so these were excluded from the statistical analyses. The best REML for predicting wattlebird aggressive response included mount, site and the interaction between mount type and site (**Table 2**). Overall, wattlebirds responded significantly more aggressively to the koel mount (n = 21 trials, LSM = 0.70 ± 0.55) than to the currawong mount (n = 23 trials, LSM = −0.07 ± 0.53; Tukey HSD: P = 0.03) and the rosella mount (n = 22 trials, LSM = −0.45 ± 0.54; Tukey HSD: P = 0.001), but their response to currawong and rosella mounts was not significantly different (Tukey HSD: P = 0.40). Furthermore, the interaction between mount type and site was important, as wattlebirds in Sydney exhibited a significantly higher aggressive response toward koel mounts than any other group (**Figure 3**; Tukey HSD: $P < 0.03$ for all comparisons). More pairs attacked and/or swooped the koel mount in Sydney (60%, n = 10 pairs), than in Canberra (9%, n = 11 pairs; Fisher's Exact Test, P = 0.02). The type of mount was a borderline significant predictor of whether a pair actually physically attacked the cage containing the mount (**Table 2**), with the koel mount eliciting a significantly higher number of attacks (LSM = −0.97 ± 1.38) than the rosella mounts (LSM = −2.91 ± 1.62; Tukey HSD, P = 0.045), but not the currawong mounts (LSM = −1.46 ± 1.38; Tukey HSD, P = 0.72). The number of attacks on currawongs was not significantly different from the number of attacks on rosellas (Tukey HSD, P = 0.16).

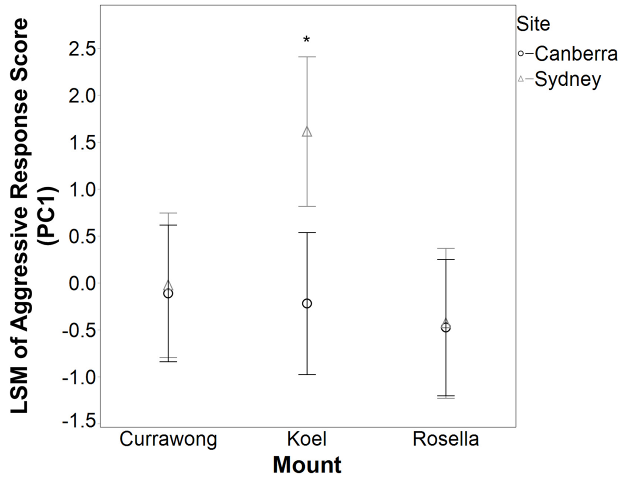

FIGURE 3 | Least square means (LSM) ± 95% CI of the Red Wattlebird aggressive response score (PC1) for each mount type in Canberra and Western Sydney. Wattlebirds in Sydney showed a significantly higher aggressive response score toward koels than any other group, as indicated by the asterisk (REML, Tukey HSD, $P < 0.03$ for all comparisons).

Wattlebirds produced significantly more alarm calls in the presence of the koel (LSM = 5.01 ± 2.38) than the currawong (LSM = 0.48 ± 2.27; REML, Tukey HSD: P = 0.02) and the rosella mounts (LSM = 0.05 ± 2.33; REML, Tukey HSD: P = 0.01) and this was the only call type that was significantly predicted by mount type (**Table 2** and **Figure 2**). There was no evidence of a cuckoo-specific vocalization in wattlebirds (e.g., Langmore et al., 2012). Only one call type, the growl call, was given exclusively during the koel trial by one pair, but this call type was also given by multiple other pairs toward researchers checking nests (Abernathy personal observation).

The best GLMM model for predicting how long female wattlebirds sat on the nest included mount type, whether the cage was hanging or attached to the ladder and whether the male attacked the mount or not (**Table 2**). The results suggest that females do not appear to use passive nest defense against brood parasitism, as females sat for a significantly longer period in the presence of the rosella mounts (LSM = 1.85 ± 1.14) than in the presence of the koel mount (LSM = −0.24 ± 1.01; Tukey HSD, P = 0.001) or currawong mounts (LSM = −0.27 ± 0.95; Tukey HSD, $P < 0.001$). There was no difference in how long females sat in the presence of the koel or currawong mounts (Tukey HSD, $P = 1.00$). Females also sat for longer on the nest when the cage was attached to the ladder (LSM = 1.28 ± 1.38) than when it was hanging from a branch (LSM = −0.39 ± 0.88) and when the male was not attacking the mount (male not attacking: LSM = 1.20 ± 0.83; male attacking: LSM = −0.31 ± 1.32).

Factors Influencing Egg Ejection

The three host species differed in their response to the different egg types (best model: host × egg type: $\chi^2 = 7.4$, df = 2, $P = 0.02$), and this was the only interaction that was significant. Magpie-larks ejected a similar number of blue eggs (91%, $n = 22$) and spotted eggs (86%, $n = 21$; Tukey HSD: $z = -0.2$, $P = 1.00$; **Figure 4**). Friarbirds ejected a similar number of spotted eggs to Magpie-larks (94%, $n = 17$; Tukey HSD: $z = 0.4$, $P = 1.00$), but they ejected significantly fewer blue eggs (38%, $n = 24$; Tukey HSD: $z = -3.6$, $P = 0.003$). Wattlebirds were consistently poor egg ejecters, ejecting significantly fewer blue (3%, $n = 35$) and spotted eggs (4%, $n = 25$) than Magpie-larks (blue: Tukey HSD: $z = -4.9$, $P < 0.001$; spotted: $z = -4.4$, $P < 0.001$) and friarbirds (blue: Tukey HSD: $z = -2.9$, $P = 0.045$; spotted: $z = -4.3$, $P < 0.001$). When all hosts are combined, ejection was more likely to occur when the model egg was added closer to or after clutch completion (best model: Wald's test: $SE = 0.39$, $z = -2.0$, $P = 0.04$). In addition, we successfully tested 7 Olive-backed Oriole nests using the non-mimetic blue model egg (3 in Canberra and 4 in Sydney). Orioles showed 100% ejection of the blue egg and all ejected in less than 4 days.

DISCUSSION

Mobbing Experiments

Wattlebirds recognized koels as a special nest threat, responding significantly more aggressively to the koel mount than to a harmless control and a nest predator, but their aggressive response did not differ between the harmless species and nest predator. Presentations of koel mounts elicited significantly more alarm calls than presentations of the other 2 mounts and this vocalization was the only one that was influenced by mount type. Wattlebirds were also more likely to physically attack koel mounts than rosella mounts. Furthermore, Sydney wattlebirds were more aggressive toward the koel, where parasitism rate is higher (24%) than in Canberra (4%) (Abernathy and Langmore, 2017). This might indicate that brood parasite recognition may take longer to spread throughout an entire population when parasitism rates are low. Overall, our results seem to suggest that wattlebirds in Sydney viewed the threat posed by koels as different from the threat posed by currawongs. Considering the extra energetic cost associated with raising a cuckoo nestling, these results do make sense and similar results have been found in other mount experiments where both an obligate brood parasite and nest predator mount were used (e.g., Gill and Sealy, 1996; Li et al., 2015; Noh et al., 2021).

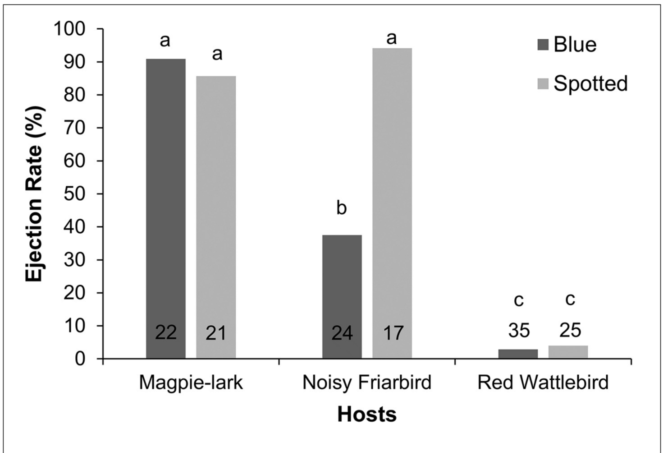

FIGURE 4 | Ejection rates of three Pacific Koel hosts for two model egg treatments from 2013 to 2016 in Canberra, ACT and Western Sydney, NSW combined. Number of nests tested for each treatment are at the bottom of each column and letters indicate significant differences (GLM, Tukey HSD, $P < 0.05$).

Our results are consistent with a learned response because the behavior was acquired rapidly (within 38–86 years of exploitation by the parasite). Moreover, the lower aggressive response and attack rate in Canberra is consistent with learning because a learned response to a threat requires either personal experience of the threat or observation of other species or conspecifics responding to the threat (e.g., Feeney and Langmore, 2013), and this would be less common at the site with lower exposure to koels. Multiple studies have shown that animals can learn anti-predator behaviors toward novel predators over the course of their life through cultural transmission by watching how other individuals respond to those species (reviewed in Griffin et al., 2000; Davies and Welbergen, 2009; Feeney and Langmore, 2013). Anti-predator responses can be further generalized to other novel threatening species that are morphologically similar to the known predator, allowing individuals to respond quickly to potential threats from species they have not previously encountered (Griffin et al., 2001; Ferrari et al., 2007, 2009). The process of cultural transmission and the generalization of anti-predator responses could allow rapid acquisition of the ability to recognize and mob a brood parasite in a naïve host population. This process may be facilitated further by the resemblance of many brood parasitic cuckoos to hawks (Davies and Welbergen, 2008; Welbergen and Davies, 2011). The rapidity of the spread of this defense, however, is likely to be dependent upon how many individuals in the population are actually exposed to the threatening species and the perceived cost of engaging in an aggressive encounter with the threatening species (e.g., Forsman and Mönkkönen, 2001; Davies and Welbergen, 2008; Welbergen and Davies, 2008, 2011). While brood parasites are not a threat to their adult hosts, they will often remove or damage host eggs when laying their own (Sealy, 1992; Davies, 2000; Soler and Martínez, 2000; Gloag et al., 2013) and will sometimes depredate nests late in incubation or in the nestling phase in order to force the host to re-nest (Elliott, 1999; Davies, 2000; Granfors et al., 2001). Therefore, hosts may quickly learn that brood parasites are a threat to their nest and may even view them as nest predators (Mcleod, 1997). Interestingly, our results show wattlebirds may view koels as a greater threat to their nest than a common nest predator, the currawongs. A previous study conducted on wattlebirds and koels at our study sites indicated that predation rate of wattlebird nests does increase once koels have arrived to the breeding area (from 19%, n = 27 before koels arrive to 40%, n = 102 after koels arrive) (Abernathy and Langmore, 2017). This does not necessarily mean koels are the main predator of wattlebird nests, as the currawong is also nesting during this same time period, but it could mean wattlebirds are more vigilant during this time and more likely to mob koels if they view them as a greater threat to their nest.

Contrary to some other studies (Gill and Sealy, 2004; Canestrari et al., 2009; Medina and Langmore, 2016), we found no evidence that wattlebird females exhibit passive nest defense as they were more likely to sit on the nest in the presence of the harmless mount than in the presence of the koel and currawong mounts. They also seemed more disturbed and less likely to sit on the nest when the cage was hanging from a branch, possibly due to more movement of the cage, especially in windier conditions. And male behavior influenced how females responded to the mount, as they were less likely to sit if the male was attacking the mount, which was more likely to happen when the mount was either the koel or currawong.

Factors Influencing Egg Ejection

The two old primary hosts, the Noisy Friarbird and Magpie-lark, as well as the minor host, the Olive-backed Oriole, all ejected eggs at a higher rate than the recent host, the Red Wattlebird, demonstrating that the old hosts have retained egg ejection in the virtual absence of brood parasitism, while this defense has yet to spread throughout the wattlebird population. Indeed, wattlebird egg ejection rates did not differ significantly between Canberra (6%, a site with 8–33 years of parasitism) and Sydney (0%, a site with 38–86 years of parasitism) despite the longer length of sympatry between wattlebirds and koels in Sydney. Many other studies have found that host egg ejection is often maintained after parasitism has ceased (e.g., Avilés, 2004; Peer et al., 2005; Soler, 2014; Yang et al., 2014) and the adaptation may even be retained from a common ancestor after speciation events (Peer et al., 2013). This suggests that once egg ejection evolves and spreads throughout a population, it may pose little cost to the host to maintain it even in the absence of parasitism.

We did not find higher ejection rate of blue eggs compared to spotted model eggs in any of the host species. Surprisingly, friarbirds showed only intermediate ejection of blue model eggs, but ejected almost 100% of spotted model eggs. This could be a consequence of the extremely atypical appearance of the blue model eggs; friarbirds may have failed to associate them with the intended context (Lahti, 2015) and in some species egg recognition is specifically tuned to the natural gradient of eggshell colors such that artificial colors are not rejected in a predictable way (Hanley et al., 2017). Alternatively, friarbirds may have a pre-existing bias toward blue eggs, as several other studies have found that other brood parasite hosts tend to accept bluer eggs over browner eggs (Soler et al., 2012; Hanley et al., 2017; Abolins-Abols et al., 2019; Hanley et al., 2019; Manna et al., 2020). This suggests that, for Noisy Friarbirds, the spotted model eggs provided a better test of egg discrimination ability.

Hosts were more likely to eject model eggs that were added after their clutch was complete. This supports other findings that hosts are more likely to eject an odd egg once they have learned the appearance of their own eggs (Lotem et al., 1995; Rodríguez-Gironés and Lotem, 1999, but see Soler et al., 2013).

Defenses Available to a Recent Host

Even though wattlebirds are a fairly recent host and may be considered naïve to brood parasitism prior to being utilized by the koel as a host (Abernathy and Langmore, 2017), they were able to recognize koels as a nest threat and most pairs in Sydney (60%) exhibited a stronger aggressive response toward the koel than wattlebirds in Canberra. However, wattlebirds showed little to no egg ejection response to model eggs at either site and a previous study showed no evidence that wattlebirds reject naturally laid koel eggs or nestlings, unless the koel eggs are laid in the nest before the wattlebird has started laying (Abernathy and Langmore, 2017). This lack of egg ejection could be due to various factors constraining the evolution of this defense in the wattlebird, despite the high costs of parasitism. A previous study found that, while unparasitized wattlebird nests fledged significantly more young than parasitized wattlebird nests, fledging success rate of koels in parasitized wattlebird nests was relatively low (26%, n = 38) (Abernathy and Langmore, 2017). One reason was due to koels laying eggs at inopportune times (too early or too late to be successful), which may have been exacerbated by the fact that wattlebirds tend to have small clutch sizes compared to the older hosts (1–2 eggs, as opposed to 3–5 eggs) and their incubation period is similar to the koel's, making it more difficult for the koel nestling to hatch out before the wattlebird nestling in order to evict it from the nest. The study also points out that at least 41% of wattlebird pairs in Sydney avoided parasitism of at least one of their nests by initiating nesting before koels arrived to the area. Thus, parasitism rate and the impacts of koel parasitism on wattlebird breeding success appear to be low (Abernathy and Langmore, 2017). This, coupled with the extreme similarity in egg appearance between wattlebird and koel eggs could make egg ejection more costly to evolve in wattlebirds (Davies et al., 1996; Abernathy et al., 2017). We found evidence that the koel's egg phenotype may have evolved as a way to mimic the appearance of the Noisy Friarbird egg, but not likely the Red Wattlebird egg, as 94% of friarbirds ejected spotted model eggs, but only 4% of wattlebirds ejected this same egg type.

CONCLUSION

Our results indicate that 38–86 years of parasitism has been insufficient time for wattlebirds to evolve high egg ejection rates, possibly due to low parasitism rates (Abernathy and Langmore, 2017), the costs of egg recognition errors (Abernathy et al., 2017), and the utilization of generalized defenses that do not require evolution, such as learning the appearance of a koel and chasing it away from the nest (this study and

Abernathy and Langmore, 2016) or abandoning nests that become parasitized before the host has started laying (Abernathy and Langmore, 2017). Our results combined with those from previous studies also indicate that naïve or recent hosts incapable of egg ejection are not completely defenseless. However, egg ejection is considered one of the most efficient defenses against parasitism and could reduce the risk of losing an entire breeding attempt. Thus, it would be interesting to conduct a similar study on wattlebirds after more time has passed to better determine how quickly a recent host population can evolve egg ejection. This knowledge could be helpful in a conservation setting, if a parasite switches to a naïve, endangered host.

DATA AVAILABILITY STATEMENT

The datasets generated during the current study are deposited into the Dryad digital repository (An Experimental Test of Defenses in a Recent Host_Datasets; https://doi.org/10.5061/dryad.dfn2z351b).

ETHICS STATEMENT

The animal study was reviewed and approved by Australian National University Animal Experimentation Ethics Committee.

AUTHOR CONTRIBUTIONS

VA, LJ, and NL conceived and designed the study. VA collected and analyzed the data with assistance from LJ, while NL supervised the research. LJ and NL designed the analysis for the mount experiment, while VA and NL designed the analysis for the egg ejection experiment. VA wrote the manuscript and NL contributed to revised versions and substantially edited the article. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.651733/full#supplementary-material

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Variations of Hawk Mimicry Traits in the Four Sympatric *Cuculus* Cuckoos

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A well-known visual signal, hawk-like features such as yellow eyes and feet, and barred underparts have been recognized as coevolutionary traits obtained against host defense in *Cuculus* cuckoos. However, the variation of these traits within and among species remains poorly understood because empirical studies quantifying these traits are limited in terms of the number of studies and the number of species concerned, and mostly depend on museum collections. In this study, we quantified and compared these traits as well as other new features (e.g., inner wing spot and underpart background color) in the four sympatric *Cuculus* cuckoos (*Cuculus poliocephalus*, *Cuculus micropterus*, *Cuculus optatus*, and *Cuculus canorus*) that were wild-captured in South Korea. We found that the yellow color of the eye ring and feet was fairly consistent across the four species. However, the iris color appeared to vary within a species (e.g., between sexes) and varied more substantially among species from nearly black in *C. micropterus* to bright yellow in *C. canorus*. In addition, there were significant differences among species with respect to the thickness of the underpart bars, from the thinnest in *C. canorus* to the thickest in *C. micropterus*. We also found that the underpart color (pure white versus yellowish brown) and the number of inner wing spots varied within and among species. These results indicate that although hawk-like traits are widely present in *Cuculus* cuckoos, detailed quantitative features of these traits vary across species. We discuss the potential reasons that generate such variations and suggest future directions to increase our understanding of visual signals in avian brood parasitism.

Keywords: brood parasitism, *Cuculus* cuckoos, hawk mimicry, morphology, reciprocal interactions

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INTRODUCTION

Direct, swift flight with a long tail and wings, an elongated body with gray upperparts and pale barred underparts, and yellow eyes and yellow legs, are all features that characterize raptors such as *Accipiter* hawks (Parkes, 1955; Kuroda, 1966; Payne, 1967; Newton and Marquiss, 1982; Duckworth, 1991; Davies and Welbergen, 2008; Newton, 2010; Welbergen and Davies, 2011). Interestingly, however, these features are also observed in the common cuckoo, *Cuculus canorus*, a brood parasite that lays its eggs in the nests of other species, namely the host, which provides parental care such as incubation and feeding of its progeny (Friedmann, 1928; Payne, 1977; Rothstein, 1990; Davies, 2000; Payne and Sorensen, 2005; Erritzøe et al., 2012; Medina and Langmore, 2016). People have been intrigued by such cuckoo-hawk resemblances, and many hypotheses have been proposed to explain the adaptive function of hawk mimicry by cuckoos (Wallace, 1889; Kuroda, 1966; Davies and Welbergen, 2008). For example, Wallace (1889) suggested that hawk mimicry may reduce attacks from predators, such as hawks, on cuckoos that need to spend a substantial amount of time on a perch to observe hosts. Alternatively, Davies and Welbergen (2008) suggested that hawks and cuckoos may have independently evolved such plumage patterns for a cryptic function of countershading, thereby reducing the chances of detection by their prey or hosts. However, the most accepted hypothesis proposed to date is that hawk mimicry is Batesian mimicry that has evolved in cuckoos to increase the chance of accessing host nests while escaping host aggression, by mimicking their potential predators, such as sparrowhawks *Accipiter nisus* and kestrels *Falco tinnunculus* (for the rufous morphs) (Voipio, 1953; Kuroda, 1966; Welbergen and Davies, 2011; Gluckman and Mundy, 2013; Thorogood and Davies, 2013). In other words, hawk mimicry is understood as a coevolutionary outcome of reciprocal interactions between cuckoos and hosts, as best seen in host egg mimicry (Welbergen and Davies, 2011).

Empirical studies to date that have experimentally tested the adaptive function of hawk mimicry, especially like underpart barring, have generally led to inconsistent results with respect to host species and populations, triggering either fear or aggression as a response (Duckworth, 1991; Honza et al., 2006; Welbergen and Davies, 2009, Welbergen and Davies, 2011; Trnka and Prokop, 2012; Trnka et al., 2012; Trnka and Grim, 2013; Ma et al., 2018). For example, some host species, such as the reed warbler *Acrocephalus scirpaceus* and some non-host species, including the great tit *Parus major* and blue tit *Cyanistes caeruleus*, showed escape responses to the exposure of cuckoo dummy and the sparrowhawk dummy, where the presence of underpart barring played a significant role (Davies and Welbergen, 2008; Welbergen and Davies, 2011). However, in the great reed warbler *Acrocephalus arundinaceus*, which is another aggressive host species of the common cuckoo, aggression instead of such escape responses, was observed (Dyrcz and Hałupka, 2006; Honza et al., 2006; Trnka and Prokop, 2012; Trnka et al., 2012). The relative costs and benefits of host responses that are determined, for example, by the presence of model species, that is, dangerous hawks, and/or their local density may generate such variations (Trnka and Grim, 2013). Given that mimicry emerged as a counteracting strategy for host discrimination, individual variation of the degree of hawk mimicry by cuckoos (i.e., intra-specific variation of hawk mimicry) and variations in host responses to such hawk mimicry may be an underlying cause of the generation of mimicry dynamics across cuckoo host races and populations (Davies and Welbergen, 2009; Welbergen and Davies, 2009). In other words, as the degree of host egg mimicry increases in response to increasing host egg rejection, a higher degree of hawk mimicry is achieved in cuckoo host races or populations that undergo higher degrees of host discrimination (Davies and Welbergen, 2009; Welbergen and Davies, 2009). Furthermore, such hawk-like features as underpart barring and yellow eyes occur as a result of Batesian mimicry not only in the common cuckoo but also in other species belonging to different genera (e.g., *Chrysococcyx, Eudynamys, Cacomantis,* and *Cerococcyx*) of old world parasitic cuckoos (Gluckman and Mundy, 2013; Thorogood and Davies, 2013). Through comprehensive comparison with sympatric and allopatric raptors, Gluckman and Mundy (2013) also showed that the appearance of the underpart barring of cuckoos is more similar to that of sympatric raptors than allopatric raptors, suggesting that selection for hawk mimicry may act locally similar to that for egg mimicry.

Although both egg mimicry and hawk mimicry are likely to be based on reciprocal interactions between brood parasites and their hosts, our understanding of hawk mimicry is much less than that of egg mimicry that has placed avian brood parasitism as a representative model system of coevolutionary study (Davies, 2000; Payne and Sorensen, 2005; Trnka et al., 2012). This is partly due to the technical difficulty in collecting sufficient quantitative information on the hawk-like features of wild cuckoos that are extremely shy and secluded. As a result, contrary to the advancement of measurement in egg coloration (Brooke and Davies, 1988; Davies and Brooke, 1989; Moksnes and ØSkaft, 1995; Davies, 2000; Stokke et al., 2002; Payne and Sorensen, 2005; Stoddard and Stevens, 2010, 2011), most descriptions of hawk-like features that have appeared in recent studies or books tend to be qualitative or derived from a small number of museum specimens that were collected haphazardly in space and time, and some traits, including iris color, cannot be measured from these specimens (Payne and Sorensen, 2005; Erritzøe et al., 2012; Lehikoinen and Väisänen, 2020). Furthermore, paralleling Gluckman and Mundy's (2013) study, our knowledge of variations in hawk-like features in sympatric cuckoos of different species needs to be expanded. Such accumulation of quantitative data on hawk-like features across species and how much they vary within and among sympatric cuckoo species, would be a fundamental step to understand and generalize the hawk mimicry of cuckoos from a coevolutionary perspective.

In this study, we quantitatively compared the hawk-like features within and among the four sympatric *Cuculus* cuckoos (the lesser cuckoo *C. poliocephalus*, the Indian cuckoo *C. micropterus*, the oriental cuckoo *C. optatus*, and the common cuckoo *C. canorus*) wild-captured in South Korea (**Figure 1**). Specifically, we first measured a well-recognized hawk-like feature such as eye color (i.e., colors of the eye ring and iris) and the thickness of the underpart barring across species. We also compared other potential hawk-like features that have been rarely quantified in previous studies, such as inner wing spots and underpart background colors, as well as traditional morphometric traits, including body mass and wing length. We then discuss potential reasons that generate variations in hawk-like features within and among species, and suggest future directions to increase our understanding of visual signals in avian brood parasitism.

MATERIALS AND METHODS

Fieldwork

Fieldwork to collect morphological data of wild cuckoos was conducted across the South Korea during the breeding season (May–July) between 2015 and 2020. We captured the four species of cuckoos belonging to the genus *Cuculus* using mist nets with song playback and dummy cuckoos mimicking the respective species (**Figure 1**). We captured 28 oriental cuckoos (21 male and seven female), 68 common cuckoos (56 male and 12 female), and three Indian cuckoos (two male and one female) in and around Yangpyeong-gun (37°29N, 127°29E) between 2018 and 2020. Of the lesser cuckoo, which mostly occurs in the southern part of

FIGURE 1 | Photographs of the four *Cuculus* cuckoos: **(A)** male lesser cuckoo, *C. poliocephalus*, **(B)** male Indian cuckoo, *C. micropterus*, **(C)** male oriental cuckoo, *C. optatus*, **(D)** male common cuckoo, *C. canorus*. ©All photos, J-W Lee.

TABLE 1 | Mean (±s.d.) body size, number of inner wing spots, and thickness of underpart barring of the four *Cuculus* male cuckoos.

	Lesser cuckoo	**Indian cuckoo**	**Oriental cuckoo**	**Common cuckoo**	***F***	***df***	***p***
Mass	59.3 ± 2.75 (38)^a	120.3 ± 8.84 (2)	117.9 ± 7.83 (21)^b	100.1 ± 5.54 (56)^c	1039.0	2, 112	<0.05
Wing	160.2 ± 4.10 (39)^a	215.3 ± 7.42 (2)	201.7 ± 4.49 (21)^b	214.8 ± 5.24 (56)^c	1554.0	2, 113	<0.05
Tail	134.9 ± 4.18 (39)^a	166.5 ± 12.02 (2)	165.5 ± 3.48 (17)^b	170.1 ± 5.80 (44)^c	581.4	2, 97	<0.05
Tarsus	19.2 ± 0.46 (37)^a	23.0 ± 0.78 (2)	21.7 ± 0.66 (21)^b	22.5 ± 0.71 (56)^c	312.9	2, 111	<0.05
Head-bill	45.7 ± 0.85 (39)^a	58.3 ± 2.90 (2)	53.7 ± 1.27 (21)^b	53.2 ± 1.40 (56)^b	511.0	2, 113	<0.05
Bill length	16.8 ± 0.47 (37)^a	23.3 ± 0.85 (2)	20.1 ± 0.57 (21)^b	20.0 ± 0.70 (56)^b	338.3	2, 111	<0.05
Bill Width	8.0 ± 0.31 (39)^a	11.3 ± 0.00 (2)	9.5 ± 0.37 (21)^b	9.5 ± 0.40 (56)^b	212.9	2, 113	<0.05
Bill Depth	7.0 ± 0.24 (39)^a	10.8 ± 0.92 (2)	9.0 ± 0.32 (21)^b	8.6 ± 0.45 (56)^c	285.6	2, 113	<0.05
Wing spots	19.7 ± 1.33 (28)^a	18.0 ± 1.41 (2)	18.9 ± 1.45 (16)^a	24.0 ± 1.74 (49)^b	102.2	2, 91	<0.05
Barring	2.1 ± 0.21 (19)^a	4.1 ± 0.15 (2)	2.4 ± 0.26 (18)^b	1.5 ± 0.24 (48)^c	122.2	2, 84	<0.05

Length and thickness are measured at millimeter level (mm) and mass at gram (g). Numbers in the parentheses represent sample size (i.e., the number of individual measured). The results of the one-way ANOVA test from which the Indian cuckoo is excluded are provided. Different superscript letters on the values indicate statistically significant differences from post hoc tests.

TABLE 2 | Mean (±s.d.) body size, number of inner wing spots, and thickness of underpart barring of the four *Cuculus* female cuckoos.

	Lesser cuckoo	**Indian cuckoo**	**Oriental cuckoo**	**Common cuckoo**	***F***	***df***	***p***
Mass	59.2 ± 3.02 (17)^a	85.5 (1)	87.2 ± 2.70 (7)^b	94.5 ± 4.96 (12)^c	350.1	2, 33	<0.05
Wing	156.8 ± 3.09 (18)^a	211.5 (1)	186.4 ± 5.47 (7)^b	205.6 ± 6.94 (12)^c	345.0	2, 34	<0.05
Tail	130.8 ± 4.87 (18)^a	169 (1)	151.4 ± 4.61 (7)^b	158.3 ± 2.71 (10)^c	148.1	2, 32	<0.05
Tarsus	19.6 ± 0.42 (18)^a	22.8 (1)	20.6 ± 0.44 (7)^b	22.0 ± 0.71 (12)^c	75.4	2, 34	<0.05
Head-bill	45.6 ± 0.87 (18)^a	59 (1)	51.3 ± 1.24 (7)^b	51.3 ± 1.49 (12)^b	110.8	2, 34	<0.05
Bill length	17.0 ± 0.59 (18)^a	23.4 (1)	19.0 ± 0.75 (7)^b	19.1 ± 0.58 (12)^b	55.9	2, 34	<0.05
Bill Width	8.0 ± 0.26 (18)^a	10.6 (1)	9.0 ± 0.20 (7)^b	9.2 ± 0.43 (12)^b	56.3	2, 34	<0.05
Bill Depth	6.9 ± 0.20 (18)^a	10 (1)	8.6 ± 0.29 (7)^b	8.4 ± 0.41 (12)^b	120.4	2, 34	<0.05
Wing spots	20.9 ± 1.34 (16)^a	19 (1)	20.1 ± 2.97 (7)^a	25.4 ± 1.83 (12)^b	24.5	2, 32	<0.05
Barring	2.3 ± 0.16 (16)^a	3.6 (1)	2.7 ± 0.30 (7)^b	1.3 ± 0.24 (12)^c	103.8	2, 32	<0.05

Length and thickness are measured at millimeter level (mm) and mass at gram (g). Numbers in the parentheses represent sample size (i.e., the number of individual measured). The results of the one-way ANOVA test from which the Indian cuckoo is excluded are provided. Different superscript letters on the values indicate statistically significant differences from post hoc tests.

Korea, we caught a total of 57 individuals (39 male and 18 female) in Jeju-do (33°29N, 126°29E) between 2015 and 2016. The sex of the individual was determined using vocal cues confirmed in the field. After capturing, we metal-ringed each individual and measured the morphological traits related to body size, including body mass, wing length (primary feathers), tail length, tarsus length, head-bill length, and bill size (length, width, and depth from/on nostril) using standard methods (Eck et al., 2011).

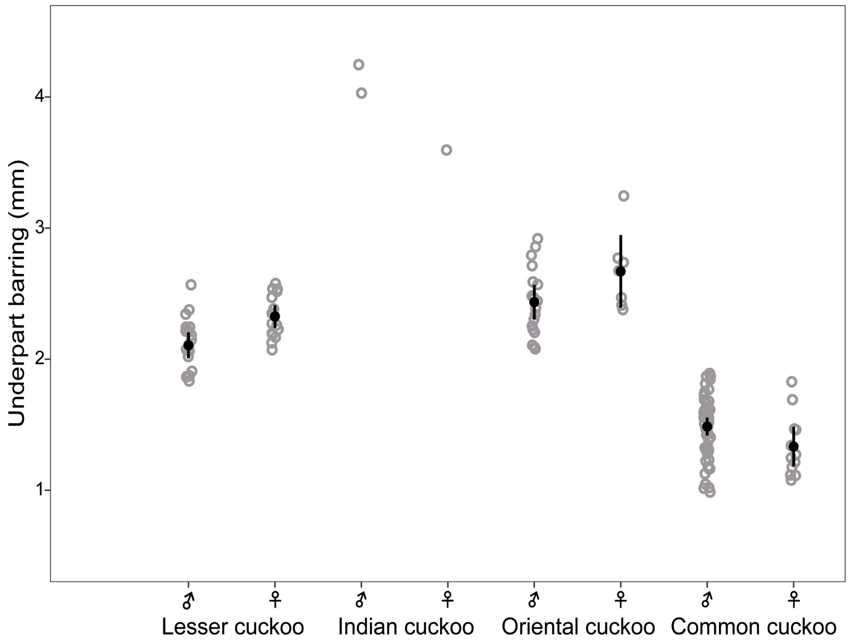

FIGURE 2 | The thickness of underpart barring of males and females of the four *Cuculus* cuckoos. Each gray open dot represents the thickness of different individuals, for which the thickness of three randomly selected barring were averaged. Black dots with vertical bars indicate mean values with its 95% confidence intervals.

TABLE 3 | Eye colors (iris and eye-ring) according to right/left eye, sex and species among the four *Cuculus* cuckoos.

Parts	**Codes**	**Lesser cuckoo**		**Indian cuckoo**		**Oriental cuckoo**		**Common cuckoo**	
		Male (16)	**Female (15)**	**Male (2)**	**Female (1)**	**Male (15)**	**Female (6)**	**Male (33)**	**Female (9)**
Right eye									
Iris	RGB code	(85, 67, 47)	(91, 72, 46)	(46, 37, 32)	(41, 31, 24)	(132, 90, 26)	(143, 119, 76)	(184, 134, 22)	(185, 148, 64)
	Hex code	#55432F	#5B482E	#2E2520	#291F18	#845A1A	#8F774C	#B88616	#B99440
Eye-ring	RGB code	(236, 199, 7)	(235, 203, 19)	(230, 197, 10)	(234, 206, 4)	(229, 180, 6)	(216, 189, 26)	(236, 194, 3)	(234, 209, 8)
	Hex code	#ECC707	#EBCB13	#E6C50A	#EACE04	#E5B406	#D8BD1A	#ECC203	#F3D108
Left eye									
Iris	RGB code	(88, 72, 50)	(89, 70, 46)	(50, 36, 31)	(41, 32, 24)	(131, 91, 26)	(132, 112, 67)	(180, 129, 25)	(181, 144, 58)
	Hex code	#584832	#59462E	#32241F	#292018	#835B1A	#847043	#B48119	#B5903A
Eye-ring	RGB code	(241, 205, 15)	(242, 208, 28)	(254, 218, 18)	(247, 213, 0)	(228, 180, 11)	(207, 180, 30)	(240, 197, 8)	(231, 203, 6)
	Hex code	#F1CD0F	#F2D01C	#FEDA12	#F7D500	#E4B40B	#CFB41E	#F0C508	#E7CB06

Representative colors are presented by RGB and Hex color code, for which the RGB values of individuals in the parentheses were averaged.

We also took images of each cuckoo using a digital camera (Canon EOS 70D) with a standard color chart (X-rite ColorChecker Passport) placed beside each individual for color or size calibration, from which we obtained data on eye color, underpart barring thickness, the number of inner wing spots, and underpart color. Using the Capture One 20 software from Phase One (Erni, 2017), we quantified eye colors from photos, for which we extracted the RGB color values from five randomly selected points in the area of the iris (excluding the iris flecking area, see Yoo et al., 2017) and eye ring, respectively, after which an average of the values was taken. We also provided hex color code (e.g., #2E231D) for the corresponding RGB values to facilitate color checking, where the first two digits after # represent the *R* value, the next two and the last two digits represent the *G* and *B* value, respectively. Although ultraviolet (UV) vision in birds are well recognized and thus measuring colors including UV light as well as visible lights is common place (Finger and Burkhardt, 1994; Hausmann et al., 2003; Stevens and Cuthill, 2007; Stoddard, 2012; Tedore and Nilsson, 2019), the application of these technologies was limited in this study due to logistical constraints. We also measured the thickness of the underpart barring using ImageJ ver. 1.52a (Schneider et al., 2012), for which we randomly selected three barrings located on the upper belly. The number of inner wing spots was determined by counting all spots observed on the three out primaries (p9 = second-out primaries, p8 = third-out primaries, p7 = fourth-out primaries) of the right wing. The

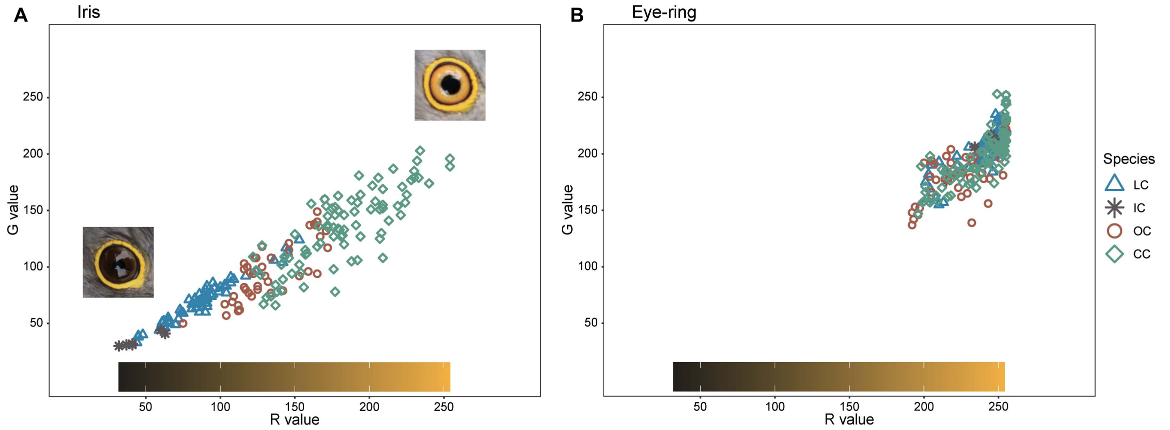

FIGURE 3 | The *R* and *G* values of iris **(A)** and eye-ring color **(B)** in the four *Cuculus* cuckoos. Among the RGB color code, for visual understanding, only *R* and *G* values are represented. The color bar in the box represents the approximate color of iris and eye-ring of the observed combination of *R* and *G* values. The shapes and colors of dots are different according to species. LC, lesser cuckoos; IC, Indian cuckoos; OC, oriental cuckoos; CC, common cuckoos.

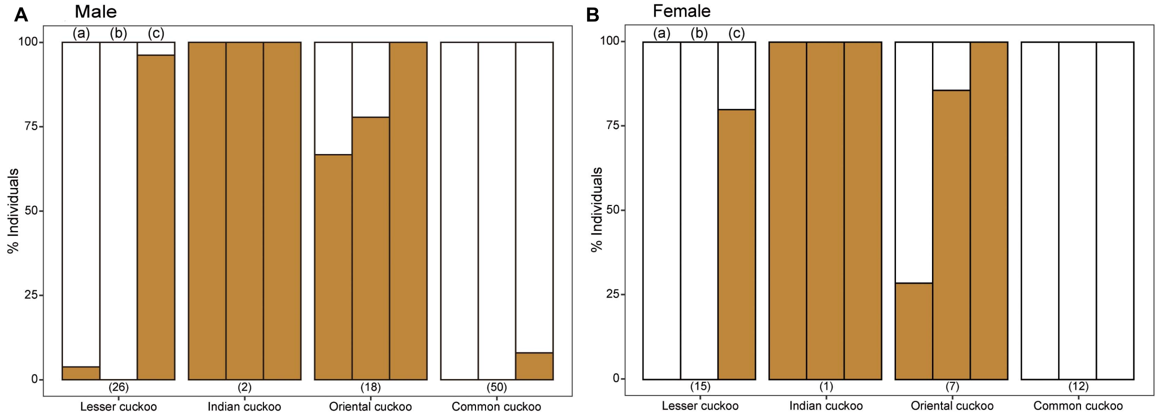

FIGURE 4 | The relative proportion of the four *Cuculus* cuckoos according to their underpart background colors: either pure white or yellowish-brown tinge. The colors of different areas of underpart [i.e., (a) belly, (b) underwing covert, and (c) undertail covert] were accessed separately. The numbers in the parentheses represent the sample size. **(A)** Male *Cuculus* cuckoos, **(B)** Female *Cuculus* cuckoos.

background colors of the underpart (i.e., belly, underwing covert, and undertail covert) were scored binomially either 0 (white) or 1 (yellowish brown) from the photo.

Statistical Analyses

All data except those of the Indian cuckoo met the condition of normality. We first conducted classical tests (e.g., the Student's *t*-test for sexual comparison, ANOVA for species difference) to provide information on the quantitative comparison of morphology data in the four *Cuculus* cuckoos. Due to the small sample size, however, the Indian cuckoo was excluded from those analyses. Tukey HSD test was applied as a *post hoc* test for significant differences from the ANOVA. Secondly, to test morphological differences more statistically, we carried out a principal component analysis (PCA) where body mass, wing length, tail length, bill length and head-bill length, the thickness of the underpart barring, the *R*, *G*, *B* value of the iris, and number of inner wing spots of the four species of cuckoos were included. We then adopted the first two principal components (PCs) with eigenvalues >1, with which we constructed a linear model (i.e., ANOVA). PCs were used as response variables and sex and species were included as explanatory variables in the model. We achieved a minimal adequate model by removing non-significant terms until all terms in the model were significant (Crawley, 2013). All statistical tests were conducted using the R version 4.0.2 (R Core Team, 2020).

RESULTS

Among the four *Cuculus* cuckoos that regularly breed in South Korea, the Indian cuckoo was the largest in general and the lesser cuckoo was the smallest. However, the order could be changed depending on the traits measured and sex (**Tables 1**, **2**). For example, although male common cuckoos were larger than male oriental cuckoos and similar to Indian cuckoos when it came

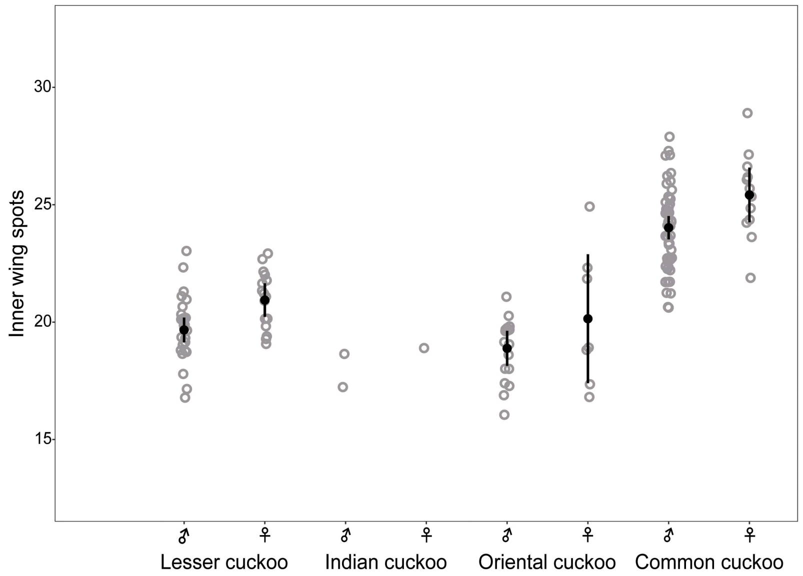

FIGURE 5 | Comparison of the number of inner wing spots according to sex and species among the four *Cuculus* cuckoos. Each dot indicates the total number of white spots located on the three primaries (p9, 8, 7) of the right wing. Black dots with vertical bars indicate mean values with its 95% confidence intervals.

to wing length, these two latter cuckoos were much heavier than male common cuckoos (**Table 1**). In females, however, common cuckoos were heavier and at the same time had longer primaries than oriental cuckoos (**Table 2**). In fact, female common cuckoos were heaviest among female *Cuculus* cuckoos, resulting in smaller sexual dimorphism with respect to body mass, than those in the Indian cuckoo and oriental cuckoo (**Tables 1**, **2**).

Barred underparts are one of the key hawk-like features, and we found that the thickness of the underpart barring varied among species (**Tables 1, 2** and **Figure 2**). In males, for example, the Indian cuckoo had the thickest barring (on average *ca.* 4.1 mm), followed by the oriental cuckoo (2.4 mm) and the lesser cuckoo (2.1 mm), and the common cuckoo had the thinnest barring (1.5 mm). In lesser cuckoos, females had thicker barring than males, but such significant sexual differences were not observed in other species. The iris color of the four *Cuculus* cuckoos also varied significantly among species, ranging from nearly black (average hex color code: #2E231D) in Indian cuckoos, dark (#58462F), and light (#856227) brown in lesser cuckoos and oriental cuckoos, respectively, to yellow (#B68720) in common cuckoos (**Table 3**). However, there were also substantial variations in the iris color among individuals of the same species, irrespective of sex, making it difficult to define a single representative color for the iris (**Figure 3**). For example, some common cuckoos had bright yellow irises, while others were nearly dark brown. Likewise, some oriental cuckoos had deep dark brown iris, in contrast, others were deep yellow. As a result, these individual variations caused large overlaps in iris color between species, as shown between oriental cuckoos and common cuckoos (**Figure 3**). In contrast, the eye-ring color of the four *Cuculus* cuckoos generally represented a yellow color with a relatively small variation between species as well as between individuals of the same species (**Figure 3**). The brightest yellow (#FFFC23) was observed in the common cuckoo, and the darkest (#C08901) was observed in the oriental cuckoo (**Figure 3**).

The four *Cuculus* cuckoos also varied in the background color of their underparts, including belly, underwing, and undertail coverts (**Figure 4**). Both male and female common cuckoos represented a pure white color across their underparts, whereas most oriental cuckoos and Indian cuckoos had a brownish tinge as a background color. The lesser cuckoos also represented a pale yellowish-brown tinge in their undertail coverts. The total number of inner wing spots presented in the second, third, and fourth out primaries were approximately 20 across four *Cuculus* cuckoos (**Figure 5**). However, the number of spots was significantly larger in the common cuckoo than in the other three

TABLE 4 | Results of the principal component analysis for the 11 morphological traits of the four *Cuculus* cuckoos.

Variables	PC1 (72.5%)	PC2 (19.3%)
Mass	0.20	**−0.42**
Wing	0.27	**−0.42**
Tail	0.19	**−0.31**
Tarsus	0.02	−0.02
Wing spots	0.02	0.00
Bill	0.01	−0.03
Head to bill	0.03	−0.07
Barring	−0.01	−0.00
R value of iris	**0.73**	0.08
G value of iris	**0.56**	0.37
B value of iris	−0.01	**0.63**

The percentages in parentheses indicate the amount of variation explained by each PC, and the components that were loaded most highly for each parameter are in bold. Total explanation power 91.8%.

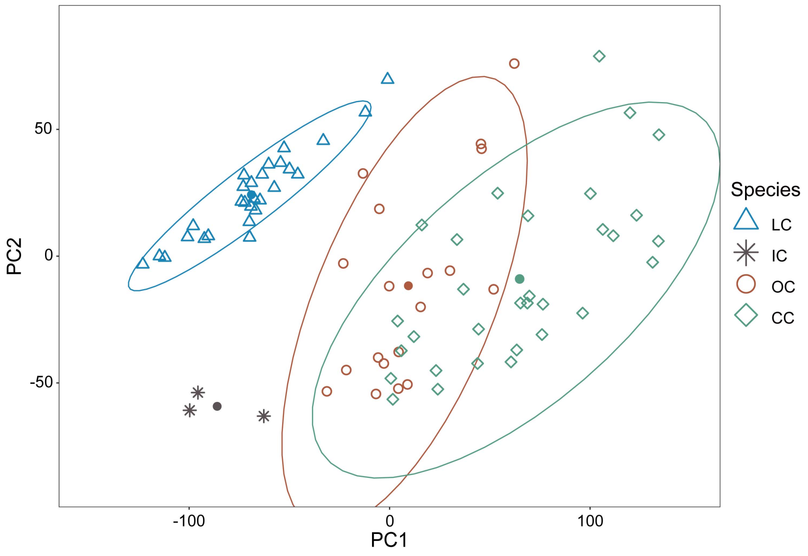

FIGURE 6 | The score plot of PC1 versus PC2 of the principal component analysis for the 11 morphology features of the four *Cuculus* cuckoos. PC1 explains 72.5% of the total variations while PC2 explains 19.3% of the total variations in the data. The shapes and colors of the dots are different according to species. LC, lesser cuckoos; IC, Indian cuckoos; OC, oriental cuckoos; CC, common cuckoos. Close circles are centroid vale (PC1 and PC2) of each species.

TABLE 5 | Results of the linear models for PC1 and PC2 extracted from 11 morphological traits of the four *Cuculus* cuckoos.

PCs	Variables	Estimates	Std. error	*p*
PC1 (72.5%)	Intercept (Lesser cuckoo)	−68.959	6.444	<0.0001
	Indian cuckoo	−17.051	20.714	0.413
	Oriental cuckoo	78.319	10.135	<0.0001
	Common cuckoo	133.830	8.890	<0.0001
	Sex	9.068	8.221	0.273
PC2 (19.3%)	Intercept (Lesser cuckoo)	22.614	5.303	<0.0001
	Indian cuckoo	−84.539	15.462	<0.0001
	Oriental cuckoo	−55.902	7.783	<0.0001
	Common cuckoo	−44.718	6.760	<0.0001
	Sex	3.460	7.783	0.658
	Indian cuckoo: sex	4.665	26.332	0.860
	Oriental cuckoo: sex	64.997	12.781	<0.0001
	Common cuckoo: sex	54.599	11.765	<0.0001

Response variables are species, sex, and their interactions. The results of interaction terms for PC1 which are all non-significant are not presented for simplicity. Sex refers to the difference from male.

species, and females had more spots than males across species with variable statistical significance (**Figure 5** and **Tables 2**, **3**).

Collectively, the results of PCA showed that the morphological variations of cuckoos were explained by the first two PCs, which explained 91.8% of the total variance in the data (**Table 4**). PC1 best explained the variation of a hawk-like feature, the iris color (R and G value), which explained 72.5% of the total variation in the data. PC2 was associated with body size, including mass, wing length, tail length, and iris color (B value), which accounted for 19.3% of the total variation. Increasing PC1 and PC2 values represented more yellow eyes and smaller body sizes. Meanwhile, the explanatory powers of other features, such as tarsus, wing spots, bill, head to bill, and barring thickness were low in this dataset (**Table 4**). The score plot for the first two PCs showed that PC1 was spread widely along the axis according to species (**Figure 6**) and the linear model showed that those of species differences were statistically significant (**Table 5** and **Figure 7**). In PC2 the values showed small but distinct variations among species as well as sex in the Oriental and common cuckoos (**Table 5** and **Figures 6**, **7**). Overall, the plot showed that the lesser cuckoo and Indian cuckoo were clearly distinct from each other and from the common cuckoo and oriental cuckoo. The oriental cuckoo and common cuckoo almost overlapped in PC2 and some overlapped in PC1, making them most similar in appearance among the four species (**Figures 6**, **7**).

DISCUSSION

Our study shows that the four sympatric *Cuculus* cuckoos share an overall hawk-like appearance, such as yellow eyes and feet, gray upperparts, and pale barred underparts, which often make it difficult to distinguish them in the field (Lehikoinen and Väisänen, 2020). However, the detailed attributes of these features varied considerably between species. First, previous experimental studies proposed the importance of underpart barring as a hawk-like feature in the common cuckoo (Davies and Welbergen, 2008; Welbergen and Davies, 2011). However, this species had the thinnest barring among the four *Cuculus* cuckoos considered in this study. Second, the yellow eyes (yellow iris and eye-ring) of the common cuckoo have been considered as hawk-like features (Thorogood and Davies, 2013), as well as cues for species recognition in some host species (Trnka et al., 2012). However, such bright yellow eyes were seldom observed in the other three species. These species had nearly black or brown

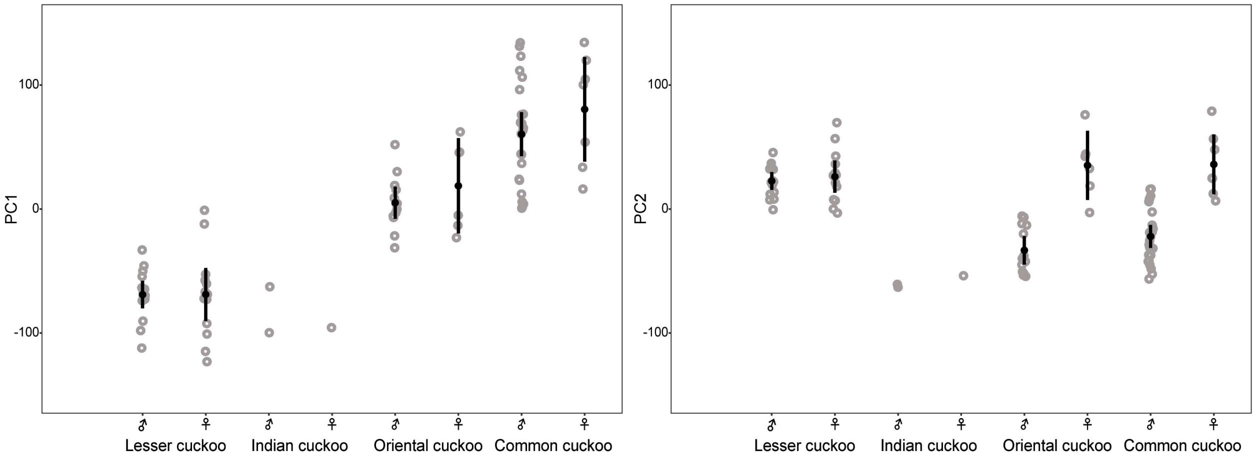

FIGURE 7 | Comparison of the PC1 and PC2 among the four *Cuculus* cuckoos. Each dot indicates the value of PC1 and PC2. Black dots with vertical bars indicate mean values with its 95% confidence intervals.

iris, although the color of the eye ring was yellow with small variations, indicating that the color of the iris and eye-ring may be determined by different biochemical and genetic pathways. For example, in some species, the eye-ring color is known to be carotenoid-based, so their color is largely affected by the food they consume (Bortolotti et al., 2003; Pérez-Rodríguez and Viñuela, 2008) and similarly the eye-ring color of cuckoos reared in captivity become paler, probably due to change in food (Meshcheryagina and Opaev, 2021). In contrast, the iris color may be determined by multiple agents, including various types of pigments, such as melanin and purines (Oliphant, 1987; Waldvogel, 1990; Sweijd and Craig, 1991; Gill and Prum, 2007), patterns including flecking, superficial blood vessels, and eye structures, irrespective of pigmentation, and also vary according to age, sex, and social status (Newton and Marquiss, 1982; Sweijd and Craig, 1991; Scholten, 1999; Bortolotti et al., 2003; Volpato et al., 2003; Guzzetti et al., 2008). In this study, however, we did not measure UV light which birds including host species are able to see. Future studies that measure eye color under UV vision are necessary to fully generalize the species difference in eye color in cuckoos. Third, although the common cuckoo has an elongated body with a long tail and wings and a lighter body mass, generating a hawk-like flight shape, such flight features seem to be less clear, at least in the Indian cuckoo, which has a shorter tail but heavier body mass, leading to fast but waddling wing flapping compared to the common cuckoo. Overall, these results show that although their respective host species meet the same dangerous raptor species in the area, the hawk-like features of sympatric cuckoos of different species could vary in their attributes. Whether these interspecific variations in hawk-like features are derived as a result of different host responses remains to be tested in future studies.

Although the four cuckoo species could occur in the same area, specific habitat preferences may differ among species (Lee et al., 2014). For example, both the oriental cuckoo and Indian cuckoo appear to prefer mountainous areas, whereas the common cuckoo and the lesser cuckoo have broader habitat preferences, including open areas such as grasslands, reedbeds, and mountains (del Hoyo et al., 1997; Payne and Sorensen, 2005; Allen et al., 2012; Erritzøe et al., 2012; Lee et al., 2014; Yun et al., 2020). Therefore, these differences in habitat preferences may alternatively generate variations in their morphology (Linsdale, 1938; Hamilton, 1961; Norberg, 1979, 1990). For instance, dark iris, thick barring, and brownish underpart background color that are observed in the oriental cuckoo and Indian cuckoo may effectively increase their degree of camouflage in dark habitats such as forests, thus increasing the chance of accessing host nests, and also protecting them from predatory attacks (Lindholm and Lindén, 2003; Speed et al., 2005; Rowland, 2009; Welbergen and Davies, 2011; Barnett et al., 2017). However, clear differences in eye color and barring thickness between the common cuckoo and the lesser cuckoo that share a similar habitat preference, may indicate that habitat structures and light conditions therein may not be the only factors causing morphological variations among species. Morphological features, including body mass and the thickness of the underpart barring, also differ among subspecies of the common cuckoo (del Hoyo et al., 1997; Payne and Sorensen, 2005; Erritzøe et al., 2012; Lehikoinen and Väisänen, 2020). As shown in the score plot, among the four *Cuculus* species, the common cuckoo and the oriental cuckoo that are phylogenetically closest are also most similar in appearance (Payne and Sorensen, 2005; Wang et al., 2016). Therefore, regional and/or phylogenetic effects need to be considered to comprehensively understand their morphological variations (Linsdale, 1938; Hamilton, 1961; Norberg, 1990, 1995; Thorogood and Davies, 2013; Wang et al., 2016; Qiu et al., 2019).

The *Accipiter* hawk generally has dark spots on white-background inner wings, so one looks at dark inner wing linings from the bottom (Parkes, 1955; Kuroda, 1966; Newton, 2010). In contrast, the cuckoos show a reverse pattern with white spots on dark-background inner wings (i.e., white inner wing linings). Such inner wing patterns may increase the cryptic function of camouflage while approaching prey or hosts (Newton, 2010; Welbergen and Davies, 2011). In addition, Lehikoinen and Väisänen (2020) showed that the common cuckoo had more spots on the primary wing than the oriental cuckoos, suggesting that the number of wing spots could be used to distinguish the common cuckoo from the oriental cuckoo. Our study further shows that the common cuckoo has more spots on the primary than the lesser cuckoo and the Indian cuckoo as well as the oriental cuckoo, thereby distinguishing the common cuckoo from the other three cuckoos by the wings, albeit not among those three. However, their number also seems to vary among individuals within a species, and likely between sexes. Further studies revealing the biological meaning of these variations and their role in hawk mimicry would be worthwhile.

Thus, this study has shown the quantitative variations in morphology including hawk-like features among the four sympatric *Cuculus* cuckoos. Our results suggest that comparing body size in, for example, the meta-analysis should be conducted with caution because the outcome could be changed according to traits (e.g., body weight, wing length) and sex. For hawk-like features, our results also indicate that the detailed attributes of hawk-like features such as eye color, underpart barring, and flight shape could be different among sympatric cuckoo species that share the same model species locally. Comparing these inter-specific variations with those from other assemblages of sympatric cuckoos that have different model species, would be necessary to elucidate the effect of model species on the evolution of hawk mimicry among sympatric cuckoos. Furthermore, experimental studies testing the adaptability of hawk mimicry across diverse cuckoo-host systems would help generalize the hypothesis that the hawk-like features in brood parasitic cuckoos evolve as a result of reciprocal interactions between the cuckoos and their hosts to maximize their respective fitness.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The capture of the four *Cuculus* cuckoos was authorized by the local governments and the cultural heritage administration of Korea. The fieldwork procedures were compiled with the national law of Korea, and the animal study was reviewed and approved by the Kyung Hee University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

J-WL conceived and designed the study. J-SG and J-WL analyzed the data. J-SG wrote the manuscript with assistance from J-WL. All authors performed the fieldwork, and reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.702263/full#supplementary-material

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Reed Warbler Hosts Do Not Fine-Tune Mobbing Defenses During the Breeding Season, Even When Cuckoos Are Rare

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Hosts of brood parasitic cuckoos often employ mobbing attacks to defend their nests and, when mobbing is costly, hosts are predicted to adjust their mobbing to match parasitism risk. While evidence exists for fine-tuned plasticity, it remains unclear why mobbing does not track larger seasonal changes in parasitism risk. Here we test a possible explanation from parental investment theory: parents should defend their current brood more intensively as the opportunity to replace it declines (re-nesting potential), and therefore "counteract" any apparent seasonal decline to match parasitism risk. We take advantage of mobbing experiments conducted at two sites where reed warblers (*Acrocephalus scirpaceus*) experience (in Italy), or do not experience (in Finland), brood parasitism. We predicted that mobbing of cuckoos should be higher overall in Italy, but remain constant over the season as in other parasitised sites, whereas in Finland where cuckoos do not pose a local threat, we predicted that mobbing should be low at the beginning of the season but increase as re-nesting potential declined. However, while cuckoos were more likely to be mobbed in Italy, we found little evidence that mobbing changed over the season at either the parasitized or non-parasitized sites. This suggests that re-nesting potential has either little influence on mobbing behavior, or that its effects are obscured by other seasonal differences in ecology or experience of hosts.

Keywords: frontline defense, seasonal change, re-nesting potential, parental investment, avian brood parasitism

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INTRODUCTION

When encountering an intruder at the nest, many parent birds use noisy vocal and visual displays to defend their brood. However, indiscriminate mobbing behavior is rarely optimal (Montgomerie and Weatherhead, 1988) and depends on the benefits gained by protecting the survival of the current brood outweighing the risks it entails for a parent's future reproduction (Trivers, 1972) or continued survival (Montgomerie and Weatherhead, 1988). Taking such an optimality approach to parental risk-taking behavior has advanced understanding of the ecology and evolution of predator-prey interactions (Caro, 2005), and inspired extensive research into quantifying the ecological factors that shape the costs and benefits of parental behavior (e.g., Oteyza et al., 2021). Although mobbing to protect the nest is also a widespread "frontline" defense behavior

(Welbergen and Davies, 2009) employed by hosts against avian brood parasites (Feeney et al., 2012), it has rarely been investigated explicitly in terms of trade-offs against a parent's future reproduction or continued survival. Therefore, and despite receiving increasing research interest over the last decade (Feeney, 2017), we still lack a comprehensive understanding of the ecological factors that shape the costs and benefits determining optimal mobbing responses.

During mobbing attacks, hosts attempt to prevent the brood parasite from laying its egg in their nest (e.g., Welbergen and Davies, 2009), or reduce the collateral damage of parasitism for host fitness (e.g., mobbing prevents shiny cowbirds from damaging their host's eggs during laying, enabling more mockingbird young to survive: Gloag et al., 2013). Mobbing a brood parasite, however, also involves potential risks for hosts: noisy mobbing displays can attract attention and reveal the nest's location to eavesdropping predators (Krama and Krams, 2005) or other brood parasites (Marton et al., 2019), and inadvertently contribute to the eventual loss of the reproductive attempt (Campobello and Sealy, 2018). Furthermore, many brood parasite species have evolved mimicry to thwart host detection. For example, female cuckoo finches (*Anomalospiza imberbis*) appear to be aggressive mimics of a harmless heterospecific (Feeney et al., 2015), whereas several *Cuculidae* cuckoos are Batesian mimics of dangerous hawks (Welbergen and Davies, 2011; Thorogood and Davies, 2013a). In the case of hawk-mimicry, recognition errors can be fatal for adults (if a hawk is misidentified as a cuckoo and approached), whereas misidentifying a virulent cuckoo as a hawk can lead to elimination of the current brood. Together these costs mean that, as in models of antipredator mobbing, hosts should adjust their mobbing to match local variation in parasitism risk.

Evidence that hosts match their mobbing behavior to parasitism risk is, however, mixed. In their landmark paper, Moksnes and colleagues systematically compared mobbing responses among hosts in Norway and found that of the 14 species parasitized by Common cuckoos (*Cuculus canorus*) in their study, the *least* aggressive species were most commonly parasitized (Moksnes et al., 1991). In a follow-up study, however, they detected a strong positive correlation between the suitability of a host species and its propensity to show aggression toward cuckoos (Røskaft et al., 2002). Nevertheless, intensive experiments with reed warblers (*Acrocephalus scirpaceus*) indicate fine-scale matching of mobbing to parasitism risk can occur. Mobbing occurs less often and at lower intensity at sites where parasitism is absent (Lindholm and Thomas, 2000; Thorogood and Davies, 2013b), and also matches temporal changes within sites: as parasitism rates declined dramatically over three decades at Wicken Fen in England, the proportion of reed warbler pairs that invested in mobbing also declined (Thorogood and Davies, 2013b). Furthermore, the parasitism rate of the population within 3 days of focal birds' laying predicted the likelihood that reed warblers would mob a cuckoo at their nest, suggesting that small scale adjustments of mobbing propensity shape patterns at larger temporal and spatial scales (Thorogood and Davies, 2013b). However, there was no evidence that reed warblers adjusted their mobbing to seasonal reductions in parasitism of a similar magnitude to annual changes in parasitism risk (Thorogood and Davies, 2013b), despite becoming less likely to reject experimental cuckoo eggs (also see Brooke et al., 1998). Indeed, of the four studies investigating mobbing behavior in reed warblers that factored season into their analyses as a covariate (**Table 1**), only one found an effect: aggression decreased linearly through the season (Čapek et al., 2010). While positive correlations between season and mobbing behavior have been detected in other host-brood parasite systems (e.g., Japanese bush warbler, *Cettia diphone*, vs. little cuckoo, *Cuculus poliocephalus*: Hamao, 2011), this occurred when parasitism increased as the season progresses. How can we reconcile evidence for optimal mobbing at fine and coarse temporal and spatial scales, but not with season?

One possible explanation may come from considering how trade-offs of protecting current against future reproduction vary during the season. Parental investment theory predicts that parents should defend their current offspring aggressively as they increase in value (Trivers, 1972), but only as long as the fitness benefit gained from mobbing is greater than the cost it imposes on a parent's potential for reproduction in the future (i.e., residual reproductive value; Williams, 1966). Opportunities for future reproduction decline rapidly during the breeding season ("re-nesting potential"; Barash, 1975), especially when parents face uncertainty about their survival to the following year (Montgomerie and Weatherhead, 1988). If diminishing re-nesting potential increases the relative benefit of mobbing behavior, then parents might therefore continue mobbing cuckoos, even as the risk of parasitism declines. Depending on the relative strength and seasonal patterns of changing parasitism risk and re-nesting potential, this could result in either no apparent overall seasonal change in mobbing behavior, or a curvilinear relationship at parasitized sites (**Figure 1**). However, when parasitism risk is absent, a seasonal pattern in response to re-nesting potential should become easier to detect. Although re-nesting potential has received much attention in how it might shape optimal anti-predator mobbing (e.g., Shew et al., 2016), it remains virtually unexplored in the context of brood parasitism, and all but one of the previous studies that incorporated an effect of season into their analyses tested mobbing behavior at parasitized sites (**Table 1**).

Here we use a non-parasitized population of reed warblers in Finland to investigate if host mobbing toward cuckoos changes as re-nesting potential declines through the breeding season. Finland lies at the northern range margin for reed warblers, and although cuckoos are common, the gens that parasitizes reed warblers is absent. Reed warblers migrate to sub-Saharan Africa to overwinter, and their adult survival is ∼50% (Stolt, 1999). Furthermore, their breeding season is several weeks shorter in Finland than in the core of the European breeding range (Halupka et al., 2008). Together this means that re-nesting potential should diminish rapidly through the season, and parents will face uncertainty about surviving to future breeding seasons (i.e., main criteria for re-nesting potential to affect mobbing; Montgomerie and Weatherhead, 1988). As models evaluating the effects of re-nesting potential emphasize changes in mobbing intensity (Montgomerie and Weatherhead, 1988),

TABLE 1 | Summary of studies with reed warblers, *Acrocephalus scirpaceus* that included seasonal effects in analyses of mobbing toward cuckoos (other models presented are also shown), or investigated effects of reproductive value *via* breeding stage.

References	Accounted for season	Measurement period	Site	Model/s	Result
Campobello and Sealy, 2010	Covariate, egg laying date; compared egg and chick stages	Apr–Jul	Modena, Italy	Cuckoo, Pigeon, Magpie	No significant effect of date on mobbing, but gave more churr calls when chick rearing than when incubating
Čapek et al., 2010	Covariate, date of trial	15 May–30 June	Hodonín to Mutěnice, Czechia	Cuckoo	Parasitism rate did not decline with date, but mobbing intensity decreased
Thorogood and Davies, 2013b	Covariate, week of egg laying	9 weeks, 3 years	Wicken Fen, England	Cuckoo	No significant seasonal declines in mobbing behavior
Welbergen and Davies, 2012 #	Covariate, date of trial	May–July (Wicken) May–Jun (Llangorse)	Wicken Fen, England; Llangorse, Wales &	Cuckoo, Parrot, Teal, Sparrowhawk	No significant effect of date on mobbing of any model, at either location
Duckworth, 1991	Compared egg-laying, incubation and chick-rearing stages	Not given	Wicken Fen, England	Cuckoo, Sparrowhawk, Jay	Mobbing propensity to cuckoo increased after egg-laying

#Not including previously reported data, &non-parasitized site.

here we use an information theoretic approach to investigate seasonal changes in reed warblers' latency to approach their nest, their propensity to mob, and the intensity of their mobbing displays. If mobbing behavior is based on parasitism risk alone, we predicted reed warblers in Finland to show low aggression overall toward cuckoos (similar to Lindholm and Thomas, 2000; Welbergen and Davies, 2012). However, if re-nesting potential informs mobbing behavior, then we predicted aggression toward nest intruders to increase as the breeding season progressed. Finally, all previous studies that included season in their analyses of mobbing behavior did so as a linear term (**Table 1**). As the shape of seasonal change in a parasitised population may be curvilinear (e.g., **Figure 1**), we re-examined a published dataset from a parasitized population of reed warblers in Italy (Campobello and Sealy, 2010) to explore whether mobbing showed a non-linear relationship with date (e.g., declining with parasitism risk until increasing later in the season as re-nesting potential declined). In both populations we compared mobbing responses to a magpie (*Pica pica*), a common nest predator that preys on passerine eggs and young (Birkhead, 1991). If seasonal patterns in mobbing are a function of reduced re-nesting potential, then we predicted that mobbing of both cuckoos and magpies would increase over the season in Finland, but in Italy mobbing would increase only toward magpies.

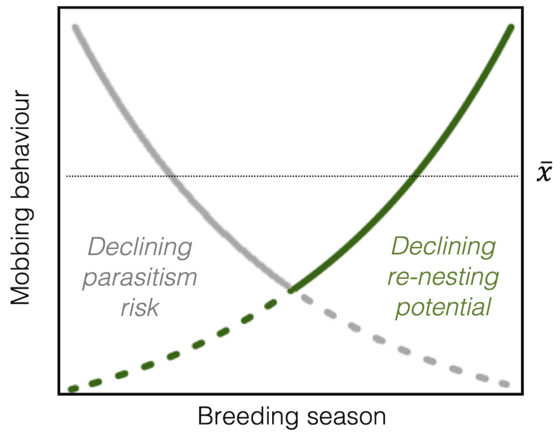

FIGURE 1 | Conceptual example of how changes in re-nesting potential (dark green line) over the breeding season could explain no overall seasonal change ($\bar{x}$, horizontal dotted line), or a quadratic change (solid lines), in mobbing behavior of hosts toward cuckoos, despite declining parasitism risk (dark gray line). Dashed lines indicate where the benefit of reducing mobbing is outweighed by the positive effect of either factor. Note that the shape of the detected seasonal relationship will depend on the relative strength and seasonal patterns of different factors.

MATERIALS AND METHODS

Study Area and Host Populations

We studied reed warblers nesting in 30 reed-lined bays along 100 km of the southern coast of Finland, near Helsinki (from 60.19848N 24.07305E to 60.34039N 25.71162E, EPSG:3857). From mid-May until mid-July in 2019 and 2020, we searched potential nesting sites by locating singing males and observing behavior. The majority of nests were found during building or egg-laying so their exact date of clutch initiation was known (2019: 50 nests, 2020: 62 nests), however, a small number of nests were found during incubation and we used the hatching date of the eggs to back-date their clutch initiation. Presentations of cuckoos at these latter nests were only included in the main dataset if they occurred within 7 days of clutch initiation (8 nests, all in 2019).

Reed warblers breed at low density in Finland and their territories are distributed sparsely (shortest distances among nests measured using GPS coordinates, median (lower quartile, upper quartile) 2019: 57 m (33, 95), 2020: 49 m (27, 77); range, 2019: 15–573, 2020: 9–398), meaning that we could be confident that each pair was tested only once each year. Despite attempts to color-ring as many adults as possible at the end of each breeding season, a small proportion may have been resampled in subsequent years. Nevertheless, annual survival rates of reed warblers are low (Thaxter et al., 2006), we recorded a return rate of 14.3% between 2019 and 2020 for color-ringed adults at our sites), and we expanded our study area in 2020. Therefore, replication of individuals during the study was likely to be low. For details of the study area in Italy, see Campobello and Sealy (2010). In brief, data was collected over 2 years (2004, 2005) from a single site in northern Italy (Natural Reserve of the Valli di Mortizzuolo and surrounding area, Modena, 44.89498N, 11.18025E) where 16% of reed warblers were parasitized during the study (Campobello and Sealy, 2010).

Measuring Mobbing Behavior

At each nest we presented a model of a cuckoo, and at half of the nests this was followed by presentation of a magpie, with at least 1.5 h between presentations. The model was placed adjacent to the nest rim, and we recorded the number of bill snaps and rasp calls within 5 min (intensity) after the arrival of the first reed warbler within 3 m of the nest (latency, s). Bill snaps and rasp calls are correlated with close approach, threat postures and direct attack of cuckoo models (Welbergen and Davies, 2008), and differ according to the threat posed by the model (Duckworth, 1991; Welbergen and Davies, 2008; Campobello and Sealy, 2010). We used the same conservative measure as previous studies (Welbergen and Davies, 2009; Thorogood and Davies, 2012, 2013b) to estimate mobbing propensity (at least 20 combined rasp calls and bill snaps) as this reduces the risk of erroneously identifying the sound of drying and cracking reed as bill snaps, or some "churr" warning calls with rasp calls (Welbergen and Davies, 2008). Repeating our analyses using a less conservative threshold of five combined mobbing calls did not alter our conclusions (see **Supplementary Material**). We aimed to assess defensive mobbing at each nest on the day the 4th egg was laid. However, reed warblers typically lay 3–5 eggs, so some nests were tested either the day before ($N = 35$ cuckoo presentations, $N = 15$ magpie presentations) or the day after ($N = 1$ for both cuckoo and magpie presentations) clutch completion (with $N = 84$ cuckoo presentations and $N = 41$ magpie presentations on day of clutch completion). As incubation usually starts once the penultimate egg is laid (Cramp, 1992) any differences in nest attendance among nests were nevertheless likely to be small. In total, we presented a cuckoo at 120 nests (2019: 58 nests, 2020: 62 nests), and a magpie at 57 nests (2019: 26 nests, 2020: 31 nests).

We used commercially-available plastic models; the cuckoo was 3D-printed ABS plastic (3D QuickPrinting United Kingdom) painted with acrylics by an artist, and the magpie was a hunting decoy (Live Decoys). Our cuckoo models were produced using the same template as independent studies by other research groups (e.g., Marton et al., 2019), and elicited a similar range of mobbing behaviors as studies using taxidermy models to assess mobbing behavior in reed warblers (e.g., Welbergen and Davies, 2008). The threat presentations were conducted as part of a larger experiment (unpublished) where the experimental design did not alternate the order of presentation of cuckoo and magpie models. To check if this could bias our conclusions, we used data available from a further 27 nests where magpies were presented either before or after a cuckoo using an otherwise identical protocol. There were no significant differences in mobbing responses according to the order in which the magpie was presented (propensity: binomial glm, estimate $= -0.406 \pm 1.008$, $z = -0.402$, $p = 0.687$; intensity: negative binomial glm, estimate $= -0.278 \pm 0.703$, $z = -0.395$, $p = 0.693$). Furthermore, at another 17 nests where we had presented a magpie twice (without presentation of a cuckoo), there was also no difference in response according to the order of the magpie's presentation [intensity: paired $t(16) = 1.033$, $P = 0.317$]. Therefore, it is unlikely that our approach of comparing mobbing responses toward cuckoos and magpies without accounting for order influenced our results.

Nest presentations in Finland were carried out by three observers, each using a different set of models. To account for potential differences and biases among observers, we filmed each nest presentation with a small action camera mounted on a green pole 1 m from the nest. After the field season we selected a random set of 10 presentations and each observer recorded data from the videos, blind to the data collected in the field. We then calculated the consistency of the observers in their detection of the number of rasps and bill snaps from the videos, using the Intraclass Correlation Coefficient (ICC) in the package "irr" (Gamer et al., 2019) (using R statistical computing environment, see Data analysis methods). All three observers were consistent in detecting the number of rasps [ICC: 0.98 (95%CI 0.95–1.0), $p < 0.001$] and bill snaps [ICC: 0.92 (95%CI 0.80–0.98), $p < 0.001$].

Mobbing data were collected differently in Italy (see Campobello and Sealy, 2010 for details). In brief, mobbing behavior toward cuckoos and magpies was elicited using taxidermy models fixed to wooden poles painted green to match the vegetation, both models were presented to nests with at least 20 min between presentations, and alternated randomly. Mobbing intensity was measured in terms of occurrence of rasps and/or bill snaps within 10 s intervals (rather than call rate) and data on the birds' latency to approach was not available. Therefore, we limited our analyses of the Italian dataset to the birds' propensity to mob cuckoos and magpies (i.e., either rasps or bill snaps occurred). Although it is unlikely that these methodological differences could generate large differences among sites [e.g., presentations using wooden models elicit similar responses to taxidermy cuckoos when tested within (e.g., Welbergen and Davies, 2008) and among populations at similar risk from parasitism (e.g., Thorogood and Davies, 2013b)], we did not combine Finnish and Italian datasets for formal statistical comparisons. None of the nests included in either the Finnish or Italian datasets were parasitized or depredated at the time mobbing behavior was measured.

Data Analysis

All data analyses were conducted in R (v.3.6.2; R Core Team, 2019). We used generalized linear mixed effects models (GLMMs), implemented using the lme4 package

(Bates et al., 2015) and generalized linear models (GLMs) to estimate the relationship between Julian date and each of the mobbing behavior measures (latency to approach, propensity to mob, and mobbing intensity) in Finland and Italy, respectively. For data from Finland, we included a random effect term to account for ecological variation among the different bays within each year (for example, some bays are more impacted by human activity, have larger areas of continuous reed, or are closer to forest areas with cuckoo activity). We checked for the most appropriate model error structure for each mobbing measure (i.e., the response variable) by using QQ-normality plots, residuals vs. predicted plots, and dispersion and outlier tests (as implemented in the DHARMa package (Hartig, 2020) on the most complex model (see below). Propensity to mob (yes/no) was modeled using a binomial error distribution, while latency to approach and mobbing intensity (number of rasps and bill snaps per 5 min) were modeled using a negative binomial error distribution (implemented with the glmer.nb function in lme4). Models comparing reed warblers' propensity to mob magpies versus cuckoos in Finland included a random effect term for nest identity that accounted for repeated presentations, and in analyses of seasonal change of mobbing propensity toward magpies, we included a covariate to account for variation in the number of days elapsed between the mobbing trial and clutch initiation (magpies were occasionally presented later in incubation than cuckoos, see above).

We used a model selection approach to evaluate support for predictions of mobbing behavior to increase during the season in Finland (either linearly or exponentially) but remain constant overall (or vary quadratically) in Italy. For each measure of mobbing, we built a candidate model set where the relationship with date (mean-centered and scaled by standard deviation) was described using a third-order polynomial (Italian data only), a second-order polynomial (i.e., increasing exponentially during the season), or a linear term, as well as a null model. We then ranked these models using their corrected Akaike information criterion (AICc) values (using the AICcmodavg package; Mazerolle, 2020), and accepted strong support for (or against) a relationship if the model was at least 2 AICc values smaller (or larger, respectively) than the next best model, or if the null model had the smallest AICc we accepted inconclusive evidence if the model of interest was no more than 2 AICc values larger (Burnham et al., 2011). We repeated these analyses for data available for mobbing propensity toward a model magpie to check whether behavior varied toward an avian nest predator familiar to reed warblers at both the parasitized and non-parasitized sites. We present the estimated effect sizes and *p*-values of the best-supported relationships for comparison with previous analyses that included date as a covariate (see **Table 1**). Summaries of model outputs and analysis codes are available in the **Supplementary Material**.

RESULTS

As predicted, reed warblers in Finland showed low aggression toward cuckoos: mobbing occurred at 30.8% of nests (37/120) whereas in Italy mobbing occurred at 78.6% of nests (81 out of 103). However, we found little evidence of mobbing behavior changing over the season (**Figure 2**). Latency to approach the cuckoo showed little change over the season; the model containing the linear term for date was 1.2 AICc units larger than the null model (**Table 2A**) and the relationship was not significant (negative binomial GLMM, linear term: estimate $= 0.103 \pm 0.104$, $z = 0.991$, $p = 0.322$; **Figure 2A**). A linear relationship with date was also within 1.6 AICc of the null model for mobbing propensity (**Table 2B**), but again the relationship was not significant (binomial GLMM of linear term: estimate $= 0.169 \pm 0.245$, $z = 0.691$, $p = 0.490$; **Figure 2B**). Finally, at the nests where reed warblers mobbed the cuckoo ($N = 37$), there was no change in their intensity of mobbing calls: no model was within 2 AICc of the null model (**Table 2C**, negative binomial GLMM of linear term: estimate $= 0.012 \pm 0.164$, $z = 0.076$, $p = 0.940$; **Figure 2D**).

The lack of significant relationships with season was not because reed warblers in Finland failed to recognize our model cuckoo as a threat to their reproductive success. Reed warblers were more likely to mob the cuckoo than a magpie nest predator (of 57 nests tested with both models, 33.3% mobbed cuckoos vs. 22.8% mobbed magpies; binomial GLMM, estimate $= -8.103 \pm 2.446$, $z = -3.313$, $p < 0.001$), and by comparison their propensity to mob magpies showed some weak evidence to decline through the season (**Table 2D**; binomial GLMM of linear term: estimate $= -0.516 \pm 0.402$, $z = -1.284$, $p = 0.199$; **Figure 2C**). However, this difference among model types was not significant (GLMM of linear term in interaction with model type: dAICc = 2.0 greater than model without interaction, estimate $= 1.083 \pm 2.617$, $z = 0.414$, $p = 0.679$).

Parasitized reed warblers in Italy showed a similar declining propensity across the season to mob magpies, with all three relationships with date being within 2 AICc of the null model (**Table 2D**; GLM of simplest linear term: estimate $= -0.919 \pm 0.509$, $z = -1.808$, $p = 0.071$; **Figure 2F**). They were also less likely to mob magpies than cuckoos (of 30 nests tested with both models, 83.3% mobbed cuckoos vs. 23.0% mobbed magpies; GLM: estimate $= -3.080 \pm 0.694$, $z = -4.438$, $p < 0.001$). However, in Italy there was no evidence of any seasonal change in their propensity to mob cuckoos as any model containing a relationship with date was > 2.1 AICc larger than the null model (**Table 2B**; GLM of linear term: estimate $= -0.0209 \pm 0.237$, $z = -0.121$, $p = 0.904$; **Figure 2E**).

DISCUSSION

While theory predicts that mobbing might increase toward brood parasites and nest predators through the season due to the diminishing opportunities for a pair to re-nest (Barash, 1975; Montgomerie and Weatherhead, 1988), here we found little evidence that this occurs in reed warblers. Our information theoretic approach suggested that seasonal change in the propensity to mob cuckoos was more ambiguous in non-parasitized sites in Finland (within 2 AICc of the null model, Burnham et al., 2011) than at a parasitized site in Italy. However,

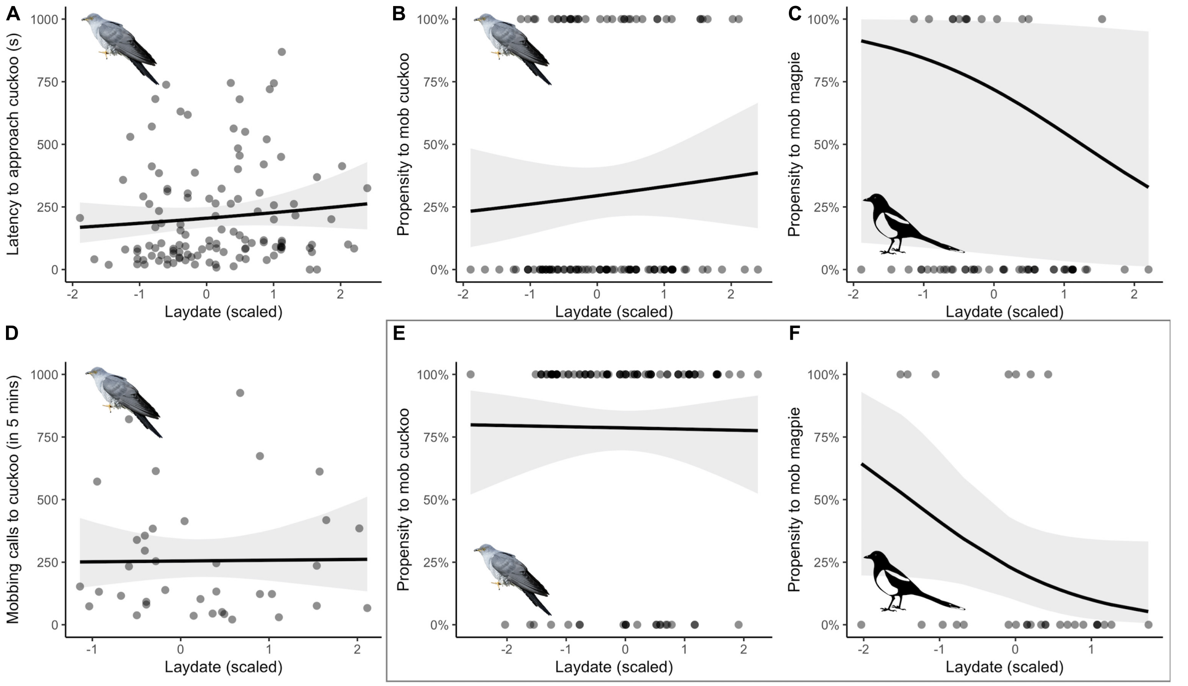

FIGURE 2 | Investigating potential effects of re-nesting potential (estimated using laying date scaled relative to breeding season) on reed warblers' **(A)** latency to approach (s) and **(B,E)** propensity to mob (using bill snaps and rasp calls) a cuckoo model presented at the nest, and **(C,F)** propensity to mob a model nest predator (magpie). **(D)** presents the intensity of mobbing (number of calls per 5 min) at nests with positive propensity in panel **(B)**. Panels **(A–D)** represent behavior at non-parasitized sites in Finland, **(E,F)** (in box) show data from parasitized sites in Italy (previously published in Campobello and Sealy, 2010). Solid lines (±95% confidence intervals, shaded) indicate the relationship from generalized linear mixed effect models **(A–D)** and generalized linear models **(E,F)**; no relationships were significant (see **Table 2**).

at both sites reed warbler's remained as likely to mob a cuckoo at the end of the breeding season as they did when their potential to re-nest was high at the start. Interestingly, mobbing toward magpies also showed little change, and in fact tended to decline rather than increase over the season.

We ceased experiments at both non-parasitized and parasitized sites as nests became scarce later in the season. However, some clutches could possibly have been laid after our study period and this might have reduced our ability to detect a trend associated with re-nesting potential. Nevertheless, our results were similar to the five previous studies of reed warblers' mobbing where date was included in analyses (**Table 1**), and to several studies of mobbing by the more aggressive great reed warbler (*Acrocephalus arundinaceus*) that is also often used as a host by the common cuckoo [(e.g., mobbing of cuckoos: Honza et al., 2010; Trnka et al., 2012; Trnka and Grim, 2014); mobbing of magpies: Trnka and Požgayová, 2017)]. Furthermore, the propensity of reed warblers to mob in Finland was similar to previous studies at locations where cuckoo activity is absent (e.g., 31% compared to 25% at Llangorse Lake, Wales United Kingdom.: Welbergen and Davies, 2012). Since our results are analogous to many other studies of mobbing by reed warblers, and that responses to painted models are similar to taxidermy versions (Welbergen and Davies, 2008) as well as responses to live cuckoos (Tryjanowski et al., 2018), we can assume that our model cuckoos and magpies were sufficiently accurate stimuli to detect variation. Why then do reed warblers not modify their mobbing behavior through the season?

Re-nesting potential is not the only determinant of the value of a current reproductive attempt for parents. For example, parents are also predicted to decide whether to risk mobbing an intruder based on the probability that their current brood will survive to breed themselves (Trivers, 1972). Although this theory is generally interpreted to represent increasing reproductive value as offspring progress toward independence (Shew et al., 2016), many bird species have a greater probability of fledging young when breeding earlier in the season (Grant et al., 2005; Grüebler and Naef-Daenzer, 2010). If offspring survival decreases through the season then this could make eggs laid earlier more valuable for parents to defend, regardless of the nest intruder's identity. Indeed, one of the few studies to consider potentially opposing forces of selection found that variation in nest defense by red-winged blackbirds was best explained by an interaction between re-nesting potential and parental investment (Shew et al., 2016). For example, increasing predation risk or worsening rearing conditions could counter-act high re-nesting potential and elevate mobbing early in the season (i.e., increasing mobbing away from the green dashed line in **Figure 1**). This would then generate more consistent patterns of mobbing across the season than we would otherwise expect.

Besides altering the value of the brood, changing predation risk may also modify mobbing behavior *via* informational

TABLE 2 | Akaike Information Criterion (corrected for small sample size) values (AICc) for generalized linear mixed effects models investigating relationship between laying date (scaled relative to breeding season) and reed warblers' (A) latency to approach (s), (B) propensity to mob (using bill snaps and rasp calls), and (C) intensity of mobbing (number of calls per 5 min) toward a cuckoo model presented at the nest at (i) non-parasitized sites in Finland and (ii) parasitized sites in Italy.

	(i) Non-parasitized (Finland)			(ii) Parasitized (Italy)		
Candidate models	**AICc (d.f.)**	**dAICc (rank)**	**weight**	**AICc (d.f.)**	**dAICc (rank)**	**weight**
(A) Latency to approach						
2nd order Polynomial	1531.4 (5)	2.6 (3)	0.15			
Linear	1529.9 (4)	1.2 (2)	0.31			
Null	**1528.7 (3)**	**0.0 (1)**	**0.55**			
Sample size	*N* = 120 nests					
(B) Propensity to mob (cuckoo)						
3rd order Polynomial	–	–	–	114.0 (4)	5.1 (4)	0.05
2nd order Polynomial	155.1 (4)	3.6 (3)	0.10	113.1 (3)	4.2 (3)	0.08
Linear	**153.1 (3)**	**1.6 (2)**	**0.28**	111.0 (2)	2.1 (2)	0.23
Null	**151.4 (2)**	**0.0 (1)**	**0.62**	**108.9 (1)**	**0.0 (1)**	**0.64**
Sample size	*N* = 120 nests			*N* = 103 nests		
(C) Mobbing intensity (cuckoo)						
2nd order Polynomial	495.2 (5)	5.1 (3)	0.06			
Linear	492.6 (4)	2.5 (2)	0.21			
Null	**490.1 (3)**	**0.0 (1)**	**0.74**			
Sample size	*N* = 37 nests					
(D) Propensity to mob (magpie)						
3rd order Polynomial	–	–	–	36.5 (4)	3.2 (4)	0.08
2nd order Polynomial	**66.3 (5)**	**0.9 (3)**	**0.26**	**34.0 (3)**	**0.6 (2)**	**0.30**
Linear	**65.8 (4)**	**0.4 (1)**	**0.34**	**33.4 (2)**	**0.0 (1)**	**0.41**
Null	**65.4 (2)**	**0.0 (2)**	**0.41**	**34.7 (1)**	**1.4 (3)**	**0.21**
Sample size	*N* = 57 nests			*N* = 40 nests		

Top models (within 2 AICc) are shown in bold and the ranks of the models are in parentheses. For comparison, (D) presents models investigating propensity to mob a model nest predator (magpie). Data from Italy were only available for mobbing propensity. See methods for further details of model composition.

constraints. Nest defense is traditionally considered costly only to the parent, but mobbing calls can, paradoxically, endanger the nest by attracting other predators and brood parasites to the nest (Marton et al., 2019). If predator abundance increases through the season, warblers may be less prone to mob to avoid alerting other threats to the location of their brood, as opposed to favoring their own survival. In other words, this would suppress the predicted effects of declining renesting potential later in the season (i.e., reducing mobbing away from the solid green line in **Figure 1**). Measures of mobbing might thus not fully reflect parental investment in a brood since there is a trade-off between deterring a current threat and avoiding further threats in the future (in addition to the trade-off between current and future investment; Trivers, 1972). Parents might also use less detectable nest defense behaviors, such as by increasing nest attendance (Samelius and Alisauskas, 2001) rather than noisy vocal displays, to mitigate against the risk of attracting attention to the nest. Here we assessed attentiveness through latency to approach the nest, but this can be affected by the perceived risk of the nest intruder [e.g., reed warblers are slower to approach a sparrowhawk at the nest (Duckworth, 1991); including after warnings by neighbors (Thorogood and Davies, 2012)]. Unfortunately, seasonal patterns of predation incurred by reed warblers in Finland are largely unexplored, and elsewhere show variation among sites and years (e.g., no seasonal trend: Honza et al., 1998; three seasons decreasing, two increasing, and one with no seasonal trend of predation: Halupka et al., 2014). More in-depth investigation of other aspects of nest defenses against cuckoos, combined with analyses incorporating changing predation risk (e.g., Lima, 2009), may therefore help to uncover why mobbing behavior does not appear to change over the season.

Finally, we may not have detected seasonal changes in mobbing behavior if re-nesting potential was experienced unequally among individuals. Theoretically, re-nesting potential should shape nest defense according to (1) the time before a parent can make another breeding attempt and (2) the parent's probability of survival (Montgomerie and Weatherhead, 1988; Shew et al., 2016). However, individual differences in intrinsic behavior, condition, age and experience are unlikely to be consistent across the season, and each of these could influence mobbing behavior directly (e.g., Avilés and Parejo, 2011), as well as timing of breeding and probability of survival. For example, personality can influence nest defense and survival (Vrublevska et al., 2015), including mobbing (Trnka and Grim, 2014) and egg rejection (Zhang et al., 2021) by hosts, and breeding phenology (Abbey-Lee and Dingemanse, 2019) and survival to breed again (Smith and Blumstein, 2008) can covary with differences in boldness and exploration behavior. Older individuals are also expected to take more risks as the value of their current brood increases relative to potential future breeding attempts (e.g., asset-protection principle, Clark, 1994; de Jong et al., 2021), even in relatively short-lived passerines (e.g., Class and Brommer, 2016). Indeed, older birds are more likely to reject cuckoo eggs (e.g., magpies, *Pica pica*: Martínez et al., 2020;

great reed warbler: Lotem et al., 1992). Although reed warblers arrive on the breeding grounds in successive waves, with earlier arriving birds being typically older and more familiar with local conditions (as assessed by intensive bird ringing and monitoring, Chernetsov, 1999), it is impossible to age adult reed warblers accurately by plumage or morphometrics to incorporate age-specific survival probabilities into our study, and unfortunately it is not feasible to track individuals through multiple breeding attempts across years. Nor do we have data available on seasonal variation in personality. Integrating individual differences among breeders into studies of re-nesting potential is, however, likely to be an important next step in explaining why some studies find support for this theory (e.g., Hollander et al., 2008) while others do not (e.g., our study; Weatherhead, 1979, 1989; Thornhill, 1989).

CONCLUSION

Although reed warblers do adjust mobbing behavior adaptively according to reproductive value in terms of breeding stage (Duckworth, 1991; Campobello and Sealy, 2010), and fine-tune mobbing based on local variation in parasitism (Thorogood and Davies, 2013b), here we find no evidence of seasonal change in either a parasitised or a non-parasitised population. While this might suggest that re-nesting potential cannot explain the lack of seasonal trends previously described in parasitised populations (**Table 1**), there is still a need for quantitative modeling with realistic parameters, informed by field data, to explore the relative magnitude of the different effects of re-nesting potential, parasitism risk, and offspring survival on parental investment in nest defense. Climate change is altering both the onset and length of breeding seasons for birds, and these effects appear to vary across species (Halupka and Halupka, 2017; Hällfors et al., 2020). Reed warblers in particular have lengthened their breeding season (Halupka et al., 2008), potentially contributing to mismatches in phenology with cuckoos (Saino et al., 2009) while increasing hosts' opportunities to re-nest. Further work is therefore required to determine whether ecological factors and individual traits interact with the length of the breeding season to shape the expression of defenses and the consequent effects on both host and cuckoo population dynamics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Project Authorization Board of the Regional State Administrative Agency (ESAVI/7857/2018) and conducted under permission of Centre for Economic Development, Transport and the Environment (VARELY/758/2018 and VARELY/799/2019).

AUTHOR CONTRIBUTIONS

EK, DT, KR, and RT conceived and designed the study. EK, KR, and RT collected the data in Finland. DC contributed previously published data from Italy. DT analyzed the data with assistance from EK and RT. DT and RT wrote the manuscript with contributions from EK, KR, DC, and RT supervised the research. All authors contributed to the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.725467/full#supplementary-material

Supplementary Data Sheet | Code for statistical analyses and results.

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Behavioral and Acoustic Responses of the Oriental Reed Warbler (*Acrocephalus orientalis*), at Egg and Nestling Stages, to the Common Cuckoo (*Cuculus canorus*)

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Cuckoo nest parasites lay eggs in host nests and thereby transfer all reproduction costs to the hosts. This greatly reduces host fitness. Parasitism has selected for the evolution of anti-parasitic strategies in hosts, including nest defense. The dynamic risk assessment hypothesis holds that nest parasitism only threatens the nests during the egg stage, so hosts should reduce the level of defense against nest parasites after the egg stage. We studied the behavioral and acoustic responses of oriental reed warblers (*Acrocephalus orientalis*), during both the egg and nestling stages, toward the common cuckoo (*Cuculus canorus*), sparrowhawks (*Accipiter nisus*) and oriental turtle doves (*Streptopelia orientalis*). *A. orientalis* can visually distinguish cuckoos from sparrowhawks and doves, indicating that hawk mimicry did not work for the cuckoos. The behavioral response of hosts in the nestling stage was stronger than in the egg stage, which supports the offspring value hypothesis and suggests that cuckoos may also act as nest predators. However, there was no difference in the alarm calls *A. orientalis* produce in response to different invaders, indicating that different types of alarm calls may not contain specific information.

Keywords: alarm calls, brood parasite, hawk mimicry, nest parasitism, nest predator

INTRODUCTION

Nest parasitism is a special reproductive behavior in which parasitic birds such as common cuckoos (*Cuculus canorus*) do not build nests themselves but lay their eggs in the nests of other birds (hosts). This transfers all of the reproductive costs to their hosts, who incubate the parasite eggs and raise parasite young (Davies, 2011; Soler, 2014). Successful nest parasitism severely reduces host fitness and compels them to invest time and energy in caring for alien eggs or nestlings, while reducing their chances of re-nesting and reproducing (Rothstein, 1990; Yang et al., 2019). In addition to being nest parasites, these birds are also potential nest predators. Many cuckoos remove or eat at least one of the host eggs before laying their own eggs during parasitism (Davies, 2000; Soler, 2014). Moreover, many adult parasites (including some cuckoos, *Cuculus* spp., and cowbirds, *Molothrus* spp.) destroy entire eggs or nestlings in host nests at advanced breeding stages that are unsuitable for parasitism. This forces the hosts to rebuild nests and increases their chance of parasitism

(Arcese et al., 1996; Swan et al., 2015; Soler et al., 2017). In addition, parasites may revisit host nests after parasitism and destroy the eggs or nestlings of the hosts who have rejected parasite eggs (Tate, 1967; Soler et al., 1995, 2017; Ponton et al., 2006; Hoover and Robinson, 2007). Finally, there are also nest predation cases involving brood parasites without parasitism intent (Su et al., 2017; Šulc et al., 2020).

Hosts have evolved responses to parasitism with a series of countering strategies. Nest defense is the first line of defense, and successful nest defense can greatly improve host fitness (Moore, 2002; Welbergen and Davies, 2009; Feeney et al., 2012). Some hosts recognize the parasites as a particular threat, and exhibit aggressive behavior that successfully prevents the parasites from approaching their nests (Duckworth, 1991; Welbergen and Davies, 2008; Trnka and Prokop, 2012; Yang et al., 2014b; Li et al., 2015; Ma et al., 2018a). Some hosts adjust their nest defense strategies according to the species of intruder and at different stages of reproduction (Patterson and James, 1980; Montgomerie and Weatherhead, 1988; Redondo and Carranza, 1989; Caro, 2005; Welbergen and Davies, 2009; Campobello and Sealy, 2010, 2018). The hypothesis of dynamic risk assessment (Kleindorfer et al., 2005) assumes that nest parasitism only poses a threat to the hosts during the egg stage, so the level of defense against parasites should be reduced after the egg stage. However, the response to predators should be the opposite. For example, Duckworth (1991) found that the reed warbler (*Acrocephalus scirpaceus*) showed a strong aggressive response to the common cuckoo during the egg stage, but the cuckoo was ignored by the host after the chicks had hatched. In addition, many species make specific alarm calls in response to different threats (Robertson and Norman, 1977; Briskie and Sealy, 1989; Gill and Sealy, 1996; Lawson et al., 2020). For example, the yellow warbler (*Setophaga petechia*) makes specific "seet" calls toward the brown-headed cowbird (*Molothrus ater*) in order to warn intraspecific or interspecific individuals of the danger (Gill and Sealy, 1996; Lawson et al., 2020) so that they can take corresponding defensive measures.

Most studies have focused on the behavioral response of hosts to the presence of brood parasite individuals (Smith et al., 1984; Honza et al., 2004; Welbergen and Davies, 2009; Neudorf and Sealy, 2012; Feeney et al., 2015). Fewer studies have documented quantitative analyses of the alarm calls (Feeney et al., 2013; Yu et al., 2017b) due to their complexity (Marler, 2004). Alarm calls are an important part of the defense of nest owners against intruders (Marler, 2004) because they may contain information about the type of intruder. For example, the barn swallow (*Hirundo rustica*) or great tit (*Parus major*) showed no behavioral response differences to cuckoo and sparrowhawk models (Liang and Møller, 2015; Yu et al., 2017b), but acoustic playback revealed that the alarm calls carried information about the types of threat (Yu et al., 2016, 2017b). Therefore, it is helpful to understand the coevolution of acoustic communication between hosts and parasites if they reveal the specific meaning of the alarm calls emitted by hosts. We studied both the behavioral and acoustic responses of oriental reed warblers (*Acrocephalus orientalis*) to nest intruders (including common cuckoos) across egg and nestling stages by investigating a variety of host traits.

MATERIALS AND METHODS

Study Site and Species

The research was performed in Yongnianwa National Wetland Park (36°40′–36°41′N, 114°41″–114°45′E) in Handan city, Hebei Province of China from May to August 2019. Yongnianwa has a temperate sub-humid continental monsoon climate and is 40.3 m above sea level. The annual average rainfall and annual average temperature are 527.8 mm and 12.9°C, respectively. The low-lying land is dominated by a large area of reed, calamus and lotus (Ma et al., 2018b). The Oriental reed warbler (*Acrocephalus orientalis*) belongs to the Acrocephalidae, Passeriformes and breeds in the reeds (Zheng, 2017). *A. orientalis* is a host of the common cuckoo in Asia, and the interaction between them has reached a high level of intensity during their coevolution (Yang et al., 2014a, 2016, 2017; Li et al., 2016). In the population studied in Yongnianwa, 14.8% of the nests were parasitized by the common cuckoo (Ma et al., 2018b).

Measure of Behavioral Response

Mounted specimens of nest intruders were presented in the incubation stage (3rd day of incubation, $n = 22$) and nestling stage (ca. 4-day-old nestlings, $n = 14$) to investigate the behavioral response of *A. orientalis*. Due to the high predation rate, only three nests were tested at both egg and chick stages. Each observed nest was exposed to three species (common cuckoo: native parasitic bird, sparrowhawks *Accipiter nisus*: unusual predator and oriental turtle doves *Streptopelia orientalis*: native harmless bird species and often encounter hosts) during experiment, with an interval of at least 60 min between them. To avoid pseudo-replication, two specimen replicates of each intruder were randomly selected for the experiment. Each specimen was presented at a distance of 0.5 m from the host nests, with the bill of the specimen toward the nest. A digital video recorder (HDR-PJ510E, Sony Corporation, Tokyo, Japan) was placed at a distance of 5 m from the nest to record *A. orientalis* behavior. An observer (JW), dressed in camouflage and wearing a camouflage hat, squatted or stood 5 m away from the host's nest, so that reed bushes could shade the observer, and host responses were recorded for 5 min after the hosts returned to the nests while alarm calls from the hosts were recorded using a tape recorder (Lotoo L300E, Infomedia Inc., Beijing, China) connected to a gun microphone (MKH418, Sennheiser Inc., Wiedmark, Germany) with a sampling frequency of 44.1 kHz and a sampling resolution of 24 bits (Yu et al., 2016). Neighbor nests were not tested on the same day (Yu et al., 2019a). The following parameters of host response were recorded: (1) response intensity, which was classified to watching (the host was only observed around the specimen without any other apparent response; score = 1), alert (birds produced alarm calls when they saw a specimen, but they had no physical contact with the specimen and did not appear to be in an aggressive posture; score = 2), mobbing (birds made alarm calls and flew past the specimen in a feint of aggression; score = 3) or attack (birds produced alarm calls when they attacked the specimen and had physical contact with the specimen; score = 4); (2) number of attracted individuals (the

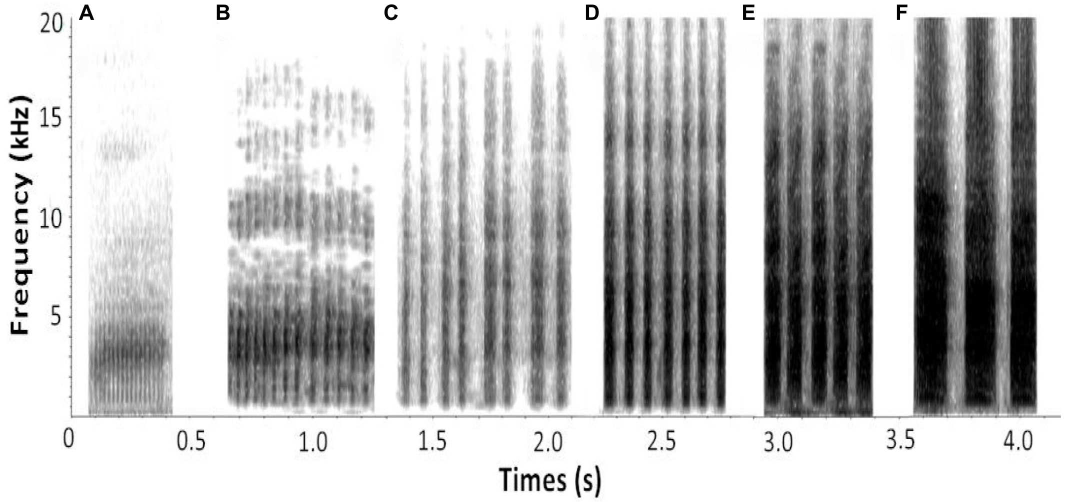

FIGURE 1 | Alarm calls spectrogram of six note types produced by *A. orientalis* (aggressive behavior is escalated from A to F, where F is related to the attack behavior).

largest number of conspecific individuals attracted during the experiment); (3) number of responsive individuals (the number of attracted individuals showing alarm and above-mentioned response intensity); (4) response time (the time from hosts arrival to the strongest reaction they produced), (5) attack frequency (recorded within the first 1 min from the attack initiated to avoid host fatigue); (6) the alarm duration of 5 min.

Measure of Acoustic Response

The alarm calls recorded (egg stage: n = 17; nestling stage: n = 10) in the specimen experiment were imported into the Raven Pro (version 1.4; Cornell Lab of Ornithology, Ithaca, NY, United States) sound analysis software, and were divided into six types according to the different note types presented in the spectrogram (**Figure 1** and **Table 1**). Only the non-overlapping alarm calls with low noise were analyzed (Courter and Ritchison, 2010; Suzuki, 2014). Referring to the relevant literature (Butchart et al., 2003; Madden and Davies, 2006; Samaš et al., 2020), we selected several parameters commonly used in song measurement. Because it was difficult to define the low frequency and the high frequency in the alarm calls of *A. orientalis*, these two parameters were excluded from the measurement, along with the bandwidth. Moreover, to cover the characteristics of different note types, the sound parameters measured included (1) the number of note types, (2) the longest duration of a note, (3) the average duration of a note, (4) the fastest note rate, (5) the average note rate, (6) the highest peak frequency, and (7) the average peak frequency (Suzuki, 2014).

Statistical Analyses

Principal component analysis (PCA) was used to extract the important principal components (PC) from the behavioral or acoustic variables, and generalized linear mixture models (GLMMs) were used to analyze the components. In GLMMs for either behavioral or acoustic analyses, the PC were the response variables while the fixed effects included nest intruder (cuckoo, sparrowhawk, or dove), breeding stage (egg or nestling stage), intruder order (presented order of specimens to each nest during experiment), and intruder replicate (identity of two replicates for each specimen type). The interaction between nest intruder and breeding stage was also tested and the nest ID was included as random effect while controlling for clutch size and egg laying date. Pairwise comparisons were conducted by the least significant differences (LSD) test. Statistical analyses used IBM SPSS 25.0 for Windows (International Business Machines Inc., Armonk, NY, United States). All the tests were two-tailed, and data are presented as mean ± SD, and the P-value significance level was 0.05.

RESULTS

One principal component (PC1) with a characteristic value > 1.0 was extracted that explained 70.14% of the total variation of the behavioral response data, while two principal components (PC1 and PC2), both with characteristic values > 1.0, which explained 80.06% of the total variance, were extracted for the acoustic response (**Table 2**). The results of GLMMs showed that the responses of *A. orientalis* to different nest intruders were significantly different ($F_{2,80} = 14.532$, $P < 0.001$, GLMMs), and the interaction between the nest intruder and breeding stage also had a significant effect on the behavioral response ($F_{2,80} = 4.250$, $P = 0.018$, GLMMs; **Table 3**). The results of LSD showed that the behavioral response in the nestling stage contributed to the significant difference of total response toward intruders in the breeding stage (**Figure 2**). For the egg stage, the response intensity to cuckoo was slightly higher than that to the sparrowhawk and dove, but it was not significant ($P > 0.05$, LSD; **Figure 2**). The response to the cuckoo in the nestling stage was more aggressive than that to the sparrowhawk and

TABLE 1 | Descriptive statistics of alarm calls parameters in *A. orientalis*.

Note types	Number of notes measured	Peak frequency (Hz)	Note duration (s)	Note rate (note/s)
A	35.67 ± 11.15	3447.87 ± 230.01	0.01 ± 0.00	49.41 ± 5.07
B	45.3 ± 12.75	3682.35 ± 464.14	0.02 ± 0.00	24.84 ± 5.66
C	42.16 ± 14.90	3988.75 ± 498.99	0.03 ± 0.01	15.58 ± 2.63
D	35.88 ± 12.82	4188.13 ± 398.37	0.05 ± 0.01	11.37 ± 1.74
E	34.90 ± 11.28	4218.22 ± 776.05	0.08 ± 0.01	7.60 ± 1.37
F	31.09 ± 21.88	4365.16 ± 582.60	0.14 ± 0.02	4.65 ± 0.93

dove ($P < 0.001$ for both, LSD), and there was no significant difference between the sparrowhawk and dove ($P > 0.05$, LSD). The maximum numbers of conspecific individuals recruited by *A. orientalis* to specimens of cuckoo, sparrowhawk, and dove were 2.56 ± 1.42, 1.82 ± 0.73, and 1.88 ± 1.02 at the egg stage, and 2.79 ± 1.19, 2.08 ± 0.79, and 1.83 ± 0.72 at the nestling stage, respectively, with no significant differences between dummies. All types of alarm calls appeared as an acoustic response to different nest intruders in *A. orientalis*, except for type A that was not present in the response to the cuckoo. The note type F was related to attacking behavior and was most frequently used by hosts toward the cuckoo (**Figure 3**). However, there was no significant difference in acoustic response toward different nest intruders, neither for PC1 ($F_{2,51} = 0.702$, $P = 0.500$, GLMMs) nor for PC2 ($F_{2,51} = 0.302$, $P = 0.741$, GLMMs). The breeding stage also had no significant effect on the acoustic response PC1 ($F_{1,51} = 1.031$, $P = 0.315$, GLMMs) and PC2 ($F_{1,51} = 1.160$, $P = 0.286$, GLMMs; **Table 3**).

DISCUSSION

Our results revealed that *A. orientalis* responded to nest intruders in a similar way at the egg stage; however, they were more aggressive to the cuckoo than to the sparrowhawk and dove at the nestling stage, suggesting that they can visually distinguish the parasite from the sparrowhawks and doves, suggesting that hawk mimicry did not work for the cuckoos. In addition, they were able to adjust their nest defense strategies at different stages of breeding. However, there was no difference in the alarm calls they produced to different specimens, indicating that *A. orientalis* makes general alarm calls in response to different nest intruders without specific information of each one.

TABLE 2 | Components, extracted by principal component analysis, for behavioral and acoustic responses in *A. orientalis*.

	PC 1	PC 2
Behavioral response		
Number of responsive individuals	0.939	–
Number of attracted individuals	0.861	–
Responsive intensity	0.861	–
Alarming time	0.836	–
Attacking frequency	0.691	–
Response time	−0.817	–
Acoustic response		
Longest duration of note	0.931	0.262
Number of note types	0.867	0.009
Average duration of note	0.783	0.443
Highest peak frequency	0.376	0.784
Average peak frequency	0.118	0.901
Fastest note rate	−0.116	−0.802
Average note rate	−0.662	−0.657

The large breeding cost of nest parasitism provides strong selection on the host to evolve anti-parasitism strategies. Among these, nest defense is the first response. Many other hosts have evolved aggressive nest protection behaviors to prevent cuckoos from approaching their nest (Welbergen and Davies, 2009), and they can also use social information to better tune their responses to various threats (Davies and Welbergen, 2009; Campobello and Sealy, 2011). This study found that the response intensity of *A. orientalis* to different nest intruders varied with the breeding stage. There was no difference in their responses to the three types of intruders at the egg stage, which may be because the *A. orientalis* is a highly territorial species, with an extremely high response to any intruders that come close to the nest during the egg stage, whereas the responses of *A. orientalis* to different intruders differed at the nestling stage, and the birds reacted more strongly to the cuckoo than to the sparrowhawk and the dove, which may be because cuckoos themselves are harmless to adult birds, while sparrowhawks, though adult predators, are uncommon in the study site. Therefore, our study suggested that *A. orientalis* could visually distinguish the cuckoo from the sparrowhawk and the dove. This was consistent with the results of other studies (Duckworth, 1991; Trnka and Prokop, 2012; Li et al., 2015; Ma et al., 2018a). In addition, our study also suggested that the visual simulation of cuckoo to sparrowhawk may not be successful for *A. orientalis*, which was different from the conclusions of some studies (Davies and Welbergen, 2008; Welbergen and Davies, 2011). The response intensity of *A. orientalis* to cuckoo and sparrowhawk was stronger in the nestling stage than in the egg stage. This supports the offspring value hypothesis that adult birds invest more in offspring during the nestling stage than the egg stage (Smith, 1977). However, previous studies on the closely related great reed warbler (*A. arundinaceus*) did not find any difference between the breeding stages (Briskie and Sealy, 1989; Moskát, 2005; Avilés and Parejo, 2006). In addition, Trnka and Prokop (2012) found that the aggressive behavior of great reed warbler to cuckoos decreased as the breeding stage progressed.

Contrary to the hypothesis of dynamic risk assessment, this study found that the response of *A. orientalis* to the cuckoo was

TABLE 3 | Generalized linear mixed model for behavioral and acoustic responses in *A. orientalis*.

Effects	PC 1 for behavioral response				PC 1 for acoustic response				PC 2 for acoustic response			
	F	***df1***	***df2***	***P***	***F***	***df1***	***df2***	***P***	***F***	***df1***	***df2***	***P***
Nest intruder	14.532	2	80	<0.001**	0.702	2	51	0.500	0.302	2	51	0.741
Breeding stage	1.449	1	80	0.232	1.031	1	51	0.315	1.160	1	51	0.286
Intruder order	2.066	2	80	0.133	0.821	2	51	0.446	0.032	2	51	0.968
Intruder replicate	0.343	1	80	0.560	0.002	1	51	0.961	1.935	1	51	0.170
Nest intruder × Breeding stage	4.250	2	80	0.018*	1.989	2	51	0.147	1.095	2	51	0.342

**P* < 0.05; ***P* < 0.01. The models include nest id as a random effect and control for clutch size and egg laying date.*

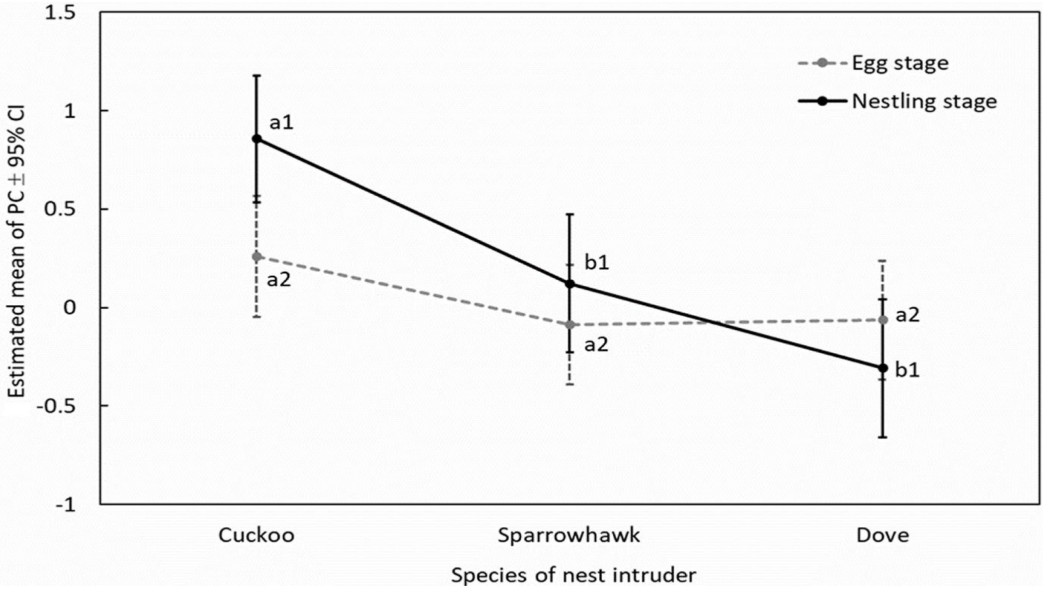

FIGURE 2 | Pairwise comparisons for behavioral responses between nest intruders by least significant difference in *A. orientalis*. Significant differences are indicated by different letters; a1 and b1 refer to nestling stage, and a2 refers to egg stage.

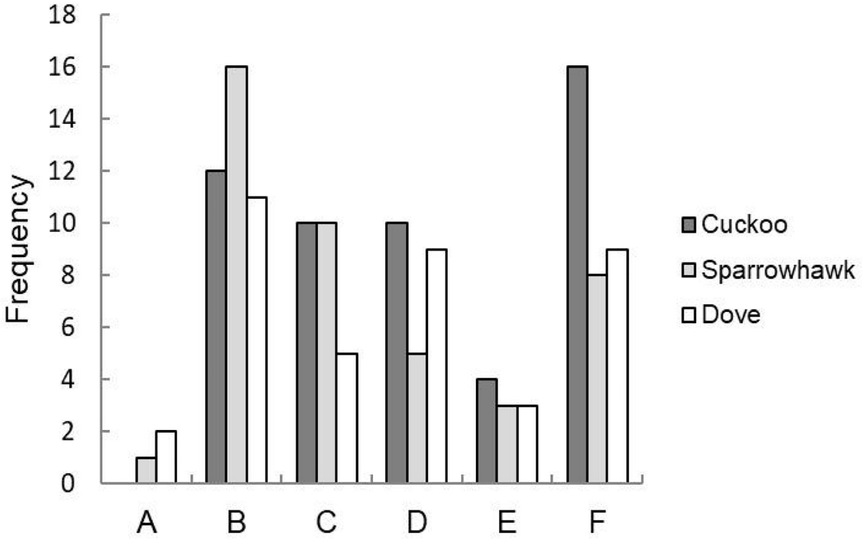

FIGURE 3 | Frequency of note types in alarm calls produced by *A. orientalis* toward cuckoo, sparrowhawk, and dove.

stronger in the nestling stage than in the egg stage. Two mutually non-exclusive explanations may contribute to this result. First, this behavior may reflect the possibility that the cuckoo is also an important nest predator, and this explanation is supported by recent research by Lawson et al. (2021). Many studies have found that cuckoos may kill host nestlings, and the amount of killing varies from a single chick to the entire brood (Kinoshita and Kato, 1995; Briskie, 2007; Kawaji, 2009; Soler et al., 2017; Šulc et al., 2020). There are two hypotheses to explain the behavior of destroying host nests by brood parasites including the mafia hypothesis and the farming hypothesis (Soler et al., 2017). The mafia hypothesis suggests that the parasites will return to the host nests after laying eggs. If their eggs are rejected by the hosts, they will destroy the host nests as a punishment so that the host will be more willing to accept their eggs in the future. According to the farming hypothesis, when the parasite finds a host nest that is not suitable for parasitism (i.e., nest in late incubation or nestling stage), they will destroy it, forcing the host to build a new nest, and thus increase the chance of parasitism in the future (Soler et al., 2017). The mafia hypothesis seems only applicable to non-evicting parasitic birds because the hosts can benefit from raising their own offspring without rejecting the parasitic eggs or nestlings (Zahavi, 1979; Soler et al., 2017). The farming hypothesis, however, is suitable for any parasitic bird (Soler et al., 2017). Therefore, the cuckoos in our studied population may play an important role as nest predators, predating host nests so as to manipulate their breeding progress for suitable parasitism. Second, *A. orientalis* may be a general defender that shows similar aggression to different intruders. They exhibited higher aggression to cuckoos in the nestling stage than in the egg stage because they have invested more time and energy in this stage.

When many species encounter intruders, they produce alarm calls, which carry information about the size, type and speed of intruders (Suzuki, 2012, 2014; Book and Freeberg, 2015; Yu et al., 2016, 2017a,b, 2019a,b; Cunningham and Magrath, 2017; Dawson Pell et al., 2018; Kalb and Randler, 2019; Kalb et al., 2019; Walton and Kershenbaum, 2019). Given that species may differ in their behavioral and vocal responses to intruders (Liang and Møller, 2015; Yu et al., 2017b), it is necessary to conduct quantitative analysis of alarm calls. For example, yellow warbler studies found that the host can send out specific alarm calls responding to the parasitic cowbird (Gill and Sealy, 1996; Grim, 2008; Lawson et al., 2020). However, in this study we found that *A. orientalis* did not produce specific alarm calls in response to different nest intruders. This result was consistent with our previous study, which played back the alarm calls against different nest intruders to *A. orientalis* but these did not trigger specific responses (Wang and Yang, 2020). Here the main intention of alarm calls may be to attract intraspecific neighbors (Wang et al., 2020) so that they can join to expel intruders from their territories more effectively (Goodale and Ruxton, 2010). This was supported by a previous study on *A. orientalis* which found that neighboring conspecifics would assist the nest owner to defend against nest intruders. Nests located far from neighbors were more likely to be parasitized by cuckoos (Ma et al., 2018b).

In conclusion, we found that *A. orientalis* can visually identify the common cuckoo, indicating that the hawk mimicry of the cuckoo was not working in this parasite–host system. In the nestling stage, the host increased its response intensity to the cuckoo, which may be related to the possibility that the cuckoo is also a nest predator.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research Ethics Committee of Hainan Provincial Education Centre for Ecology and Environment, Hainan Normal University.

AUTHOR CONTRIBUTIONS

CY designed and carried out laboratory and statistical analyses. JW, LM, and XC performed the field experiments. CY and JW wrote the first draft of the manuscript. All authors approved the final submission.

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REVIEW
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A Meta-Analysis of Avian Egg Traits Cueing Egg-Rejection Defenses Against Brood Parasitism

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The capability of hosts to reject the odd egg from their nest is one of the key defenses against avian brood parasitism. Considerable research effort has been devoted to exploring which phenotypic traits of eggshells facilitate to cue the recognition of the parasitic egg. Here we have reviewed studies addressing salient egg traits involved in the rejection of foreign eggs and used a formal meta-analysis to quantify their relative importance. Hosts appear to rely to a large extent on eggshell color traits, followed by maculation patterns. Hosts respond with similar rates of egg rejection to natural vs. model eggs and when breeding in both closed and open nests. Analyses of experiments on hosts of *Cuculus* and *Molothrus* parasites, the two best studied brood parasitic lineages with different co-evolutionary histories, yield similar conclusions. We also identify several poorly studied potential egg recognition cues, such as odor or weight, and recommend exploring even the visual traits in more detail, including chromatic and achromatic contrasts or experimentally manipulated egg maculation characteristics. Recent technological and sensory ecological advances open many new research avenues to experimentally examine the role of diverse egg characteristics in antiparasite defenses.

Keywords: meta-analysis, brood parasitism, egg rejection, egg traits, egg color, egg maculation

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INTRODUCTION

The capability to perceive, recognize, and reject the parasitic egg(s) is a critical defense in hosts of avian brood parasites, which lay their eggs into the nests of other birds (Davies, 2000). To combat host defenses, some lineages of brood parasites have evolved sophisticated eggshell mimicry to fool the hosts, which in turn, have evolved fine-tuned abilities to discriminate and reject the foreign egg. This suite of antiparasite defense behaviors has attracted considerable observational, comparative, and experimental research attention in the last decades (e.g., Grim, 2007; Medina and Langmore, 2015), albeit the first such experiments had been performed by naturalists already more than a century ago (reviewed in Sealy and Underwood, 2012). Experiments usually involve adding to or exchanging one or more foreign eggs in the host nest and observing the host's reaction. Stephen Rothstein was a pioneer of egg rejection experimentation (e.g., Rothstein, 1970), and his methods are still used by many researchers with only minor modifications (e.g., Canniff et al., 2018; Luro et al., 2018).

Since the time of some of the first egg rejection experiments ca. 100 years ago (e.g., Friedmann, 1929), multiple cues have been suggested to influence foreign-egg recognition. Accordingly, the host may rely on low intraclutch variation to facilitate the recognition of the distinct, outlier parasitic egg in the clutch (e.g., Davies and Brooke, 1989; Øien et al., 1995). In turn, according to the egg arrangement hypothesis, the host may examine disruptions to the arrangement of their eggs and use it to be alerted that their nest could be parasitized (Polačiková et al., 2013; but see Hanley et al., 2015b). Furthermore, placing a stuffed dummy of the adult parasite beside the host nest along with experimental parasitism may lead to the increased rejection of the parasitic egg suggesting that witnessing a parasitism event by the host may also narrow eggshell recognition thresholds and enable egg rejection (e.g., Bártol et al., 2002; Hanley et al., 2015c). Nest sanitation behavior, wherein the host removes debris from its nest, is also hypothesized to be responsible for recognition of differently shaped parasite eggs (e.g., Moskát et al., 2003; Guigueno and Sealy, 2012). Finally, the clutch size hypothesis predicts that psychophysically (e.g., according to Weber's law), it is easier to recognize the odd-egg-out in smaller vs. larger clutches (Akre and Johnsen, 2014).

However, egg recognition and rejection, according to perceivable differences in the phenotypes between the parasite and the host eggs (Manna et al., 2017), are by far the best studied aspects of anti-parasitic defense behaviors (reviewed in Honza and Cherry, 2017). Thousands of completed egg rejection experiments suggest that hosts can use distinct egg traits to recognize parasitic egg, such as color, maculation, shape, size, odor, or weight (Honza and Cherry, 2017). The majority of studies examined visual traits, particularly eggshell color and maculation, with a general conclusion that magnitude of difference between self and foreign eggs increases the probability of rejection (e.g., Avilés et al., 2010; Honza and Cherry, 2017; but see Hauber et al., 2020). Taken together, the experiments also imply that specific eggshell traits differ in their importance for the recognition and rejection processes (Honza and Cherry, 2017). However, there are still missing quantitative estimates on overall eggshell characteristics and their effects on host behaviors that prevent us from further exploring and discussing their significance for egg rejection as an antiparasitic strategy (Turner and Hauber, 2021). Knowledge of the latter will help us to better understand the cognitive processes involved in the brood parasite – host coevolution and also to design informative future experiments to fill in the missing gaps.

Brood parasitism research has suggested a variety of factors affecting the egg recognition process in hosts (Soler, 2017). Ongoing debates concluded that experimental egg type (natural or model egg stimuli) used in an experiment considerably affects not only the host's response but also the interpretation of results and a use of any stimulus type should be carefully justified (Hauber et al., 2015; Lahti, 2015; Stoddard et al., 2018). On the one hand, the use of natural stimuli allows researchers to observe biologically relevant reactions and these results can be generalized (e.g., Stevens et al., 2013). On the other hand, model (artificial) stimuli can be especially advantageous when planning a carefully designed experiment allowing exact alterations of the focal traits (Igic et al., 2015; Yang et al., 2019). Several review studies already examined the effect of egg type stimuli on their rejection probabilities (Honza and Cherry, 2017; Turner and Hauber, 2021) but we are still missing a comprehensive survey estimating such stimuli's effects in a standardized comparison.

The study of Langmore et al. (2005) suggested that open nesters reject eggs more often than species breeding in closed nests. However, they also showed that the effect of nest type (open vs. closed nest) disappears after controlling for nest light availability suggesting a crucial role of illumination within the nest (see also Honza et al., 2014). Regarding visually-relevant traits, such as shell color and maculation, they may have less important function in birds utilizing closed nests, which might rely more on tactile traits (Mason and Rothstein, 1986; Langmore et al., 2003; Tosi-Germán et al., 2020). Quantitatively, it still remains to be explored if open or closed nesters allot different importance on visual and non-visual egg traits during the decision process.

Finally, the two most studied avian host-brood parasite systems, the Old World cuckoos (*Cuculus* spp.) and New World cowbirds (*Molothrus* spp.), have been shown to considerably differ in their coevolutionary and ecological relationships with their hosts (Winfree, 1999). Unlike in cuckoos, there is little evidence that the cowbird lays mimetic eggs (Rutledge et al., 2021), suggesting that the evolutionary arms-race in this brood parasite system has not escalated relative to their Eurasian counterparts. It is, thus, a critical question if the differences between the parasitic systems and their co-evolutionary histories are also reflected in the hosts' emphasis on different egg traits when recognizing the foreign egg (Luro and Hauber, 2020).

In this study, we have built upon the previous review by Honza and Cherry (2017) with the aim to provide a formal meta-analysis through a quantitative measure of the magnitude of the experimental effect (effect sizes) for the egg characteristics involved in the recognition of parasitic egg in the host nest. Such a quantitative assessment across multiple host species and lineages of diverse parasitic species and lineages has not yet been conducted, although qualitative reviews of experiments on individual species' egg rejection cues have begun to appear in the published literature (e.g., Turner and Hauber, 2021).

Here, we used a multi-host and -parasite approach to examine effects of three extrinsic factors (variation in egg type stimuli, differences in nest architecture types, and different co-evolutionary histories with a parasite) that had been previously proposed to play an important role in hosts' egg-rejection responses and particularly relevant to parasitic egg traits. For this purpose, we employed recent meta-analytic statistical tools to provide unbiased quantitative estimates (Harrer et al., 2019a). The aim of this study is primarily exploratory, and thus we formulated predictions of major interests based on our overview above. We predicted that hosts use mainly visual traits (eggshell color and maculation characteristics) to recognize the foreign egg in their nest and this would be more pronounced in open nesting hosts. In line with the debate regarding artificial stimuli (e.g., Stoddard et al., 2018), we also assess the role of natural vs. model eggs' use in egg rejection experiments. Specifically, we predict that model eggs will be rejected at lower rates compared to natural ones because artificial eggs are difficult or impossible to pierce and remove *via* puncture ejection (Antonov et al., 2009). Finally, we predicted that hosts of Old World cuckoos better discriminate by color and maculation relative to other traits than cowbird hosts due to several million years longer coevolutionary experience with more mimetic parasite eggs in the former group of hosts (Caves et al., 2017; Krüger and Pauli, 2017). We make this prediction because we know from prior research that egg rejection belongs to a different class of recognition systems compared to other recognition tasks faced by nesting birds (e.g., nest hygiene: Hauber et al., 2021).

MATERIALS AND METHODS

We sought out published studies exploring eggshell traits affecting antiparasitic egg rejection behavior. We searched the Web of Science Core Collection for studies published up to 31 December 2020. We used search terms using Basic Search and All Fields option: (brood parasitism or egg rejection or egg characteristics or defense* or defence*) AND (cuckoo* or cowbird* or vidua* or honeyguide*). This resulted in 1,608 studies we exported into Microsoft Excel Worksheet. We also noticed nine relevant studies published between 1972 and 1999 but not included in the search's output and, thus, we manually entered these studies into analyses.

We screened all the studies identified and selected 62 studies fulfilling the following criteria for the analyses (see **Supplementary Figure 1** for selection procedure): (a) only *single* trait at a time was manipulated, (b) there was a *control* treatment (i.e., referential baseline rejection rate) conducted or available from the study population (for three studies we sourced control data from the same population but published in a different study), (c) the host species is known to have <100% rejection rate of foreign eggs, (d) the study reported at least the total sample size and the count or proportion of rejected eggs. We *a priori* decided to apply these four search criteria to ensure that we obtain credible effect size estimates. In the studies performing a valid egg experiment, using a control treatment was particularly limiting selection criterion and led to a notable reduction of the selected studies for final analyses. However, modifying or even excluding any of the four criteria would directly prevent obtaining a valid result. If a study manipulated more egg traits in more experimental treatments in one species (each experimental treatment still manipulated only *single* egg trait) or an egg trait was tested in more species than we included all these experimental treatments as a separate unique report for calculating the effect size for each. Thus, some studies may have been used to generate several effect sizes. We also attempted to identify studies experimentally manipulating *two* traits at a time, while also meeting the rest of criteria above, but only eggshell color with maculation traits yielded a reasonable sample size ($N = 12$ studies). Therefore, we reported the estimates only for the color-maculation summation trait effect.

We found that identifying the trait as being experimentally manipulated was challenging in some studies. For the eggshell's ground color trait, we excluded reports for which the authors did not state clearly the hue being used or the altered hue that was deemed as mimetic of host eggs. When using artificial eggs, the control eggs were painted to appear mimetic of the hosts' own eggs. For natural eggs, only highly mimetic conspecific eggs were used as a control group. The experimental treatments for the maculation trait included creating new spots on both immaculate or already maculated eggs or in three reports also removing spots in hosts with maculated eggs. Egg material (type) stimuli varied from real eggs to those created from clay, wood, plaster, plastic, or plasticine. Experimental treatment for the shape trait was performed by creating eggs slimmer or more spherical than the natural egg shape. Experimental treatment for the ultraviolet reflectance (hereafter: UV) trait involved only decreasing UV for all but one study. Further, some studies reported only egg ejections but not desertions (or egg burial) and vice versa, likely because egg desertion is not always an outcome of natural or experimental parasitism (Grim et al., 2011; Croston and Hauber, 2014; Soler et al., 2015). For the effect size calculations, we, thus, always used the rejection rates if both ejection and desertion (or egg burial) events were reported and the ejection rates if only ejection events were reported.

Statistical Analysis

All the analyses were performed in R 3.4.4 (R Core Team, 2020). We identified 10 different egg traits in 62 studies with 128 effect size reports (**Table 1**) but for statistical analyses we chose only egg traits with representative number of reports ($n \geq 5$), resulting in six egg traits from 56 studies.

We computed Cohen's h effect size for each report from difference in rejection rates between control and manipulated treatment and using sample sizes data provided in studies (Cohen, 1988). We then examined and corrected for the high between-study heterogeneity (Higgin's & Thompson's $I^2 > 90\%$ for all but one trait type) by detecting outlier reports, i.e., those in which the 95% confidence interval does not overlap with confidence interval of the pooled effect. We performed this test for each trait type separately using the function *find.outliers* implemented in R package dmetar (version 0.0.9000; Harrer et al., 2019b). After excluding studies identified as outliers, the between-study heterogeneity improved from substantial ($I^2 > 75\%$) to low or moderate ($I^2 < 75\%$; Higgins and Thompson, 2002) in four egg traits but remained substantial for egg color ($I^2 = 78\%$) and size ($I^2 = 82\%$). Thus, the effect size estimates for the two egg traits with the substantial between-study variability are under higher risk of producing biased overall estimates and should be interpreted with greater caution. To estimate the pooled confidence interval and each report's confidence interval, we employed the random effect model using the function *metagen* implemented in the R package meta (version 4.15-1; Balduzzi et al., 2019). After correcting for the heterogeneity, the final dataset included 46 studies with 81 effect size reports from 30 species.

To account for phylogenetic non-independence between the species, we used a phylogenetic tree of the host species generated from BirdTree.org[1] (Jetz et al., 2012). We applied a Bayesian

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[1] www.birdtree.org

TABLE 1 | Summary of 62 studies on egg trait manipulation to be considered for statistical analyses.

Egg trait type	Trait treatment	*N* studies	*N* species	Total reports	Final analyses	*N* exp.	Rejection rate (%)
Brightness	Baseline	1	1	1	No	20	0
	Altered	1	1	1	No	22	0.73
Color	Baseline	19	9	23	Yes	506	12.8
	Altered	19	9	23	Yes	603	56.1
Maculation	Baseline	26	19	33	Yes	1008	14.4
	Altered	26	19	33	Yes	596	48.2
Material	Baseline	13	13	17	Yes	355	47.0
	Altered	13	13	17	Yes	354	67.2
Odor	Baseline	2	2	3	No	114	22.8
	Altered	2	2	3	No	220	38.6
Pole	Baseline	3	3	3	No	91	28.6
	Altered	3	3	3	No	56	78.6
Shape	Baseline	4	5	6	Yes	81	38.3
	Altered	4	5	6	Yes	185	47.0
Size	Baseline	13	10	28	Yes	596	52.2
	Altered	13	10	28	Yes	456	60.1
UV	Baseline	8	10	13	Yes	282	19.5
	Altered	8	10	13	Yes	325	31.1
Weight	Baseline	1	1	1	No	13	69.2
	Altered	1	1	1	No	26	50.0

Some of studies reported more than one effect size and/or tested more species. Column "Final analyses" is "yes" if the egg trait was used in the final analyses. Column "Total reports" shows number of effect size estimates obtained for each egg trait type. Column "N exp." reports total number of individual egg experiments performed per the treatment.

random-effect model using the package brms (version 2.14.4; Bürkner, 2017) to calculate the pooled effect size for each egg trait. The identity of each effect size report was modeled as a random intercept effect. The covariance matrix of species relatedness was created using the package ape (version 5.4-1; Paradis and Schliep, 2019) and included as another random intercept effect. We set a weakly informative priors of Normal($\mu = 0, \sigma = 1$) for fixed predictors and Half-Cauchy($x_0 = 0.3, \gamma = 0.3$) for between-report heterogeneity (Williams et al., 2018; Harrer et al., 2019a). We ran 1×10^4 iterations with a burn-in phase of 1,000 to obtain >3,000 effective samples per parameter for posterior inference. The Potential Scale Reduction Factor ($\hat{R}$) was always 1.00 suggesting a good convergence of chains.

We performed four main analyses, (i) examining overall effect of egg trait, (ii) comparing egg trait effects between hosts parasitized by natural eggs and artificial model eggs (egg type stimuli), (iii) comparing egg trait effects between open-nesters and those breeding in enclosed nests (domed, holes, cavities) and (iv) comparing egg trait effects only in hosts of *Cuculus* cuckoo or *Molothrus* cowbird parasitic species. In the first analysis, we included only egg trait type (categorical with six levels; color, maculation, material, shape, size, UV) as a fixed effect and in other three analyses it was the interaction of egg trait type with experimental egg stimuli (categorical with two levels; natural, artificial), egg trait type with nest type (categorical with two levels; open, closed) and egg trait type with parasite (categorical with two levels; cuckoo, cowbird), respectively. We then calculated median with 89% credible interval for each effect using the package emmeans (version 1.4.8; Lenth, 2020) and prefer this interval because it has been shown to be more stable as 95% credible intervals if effective sample size for a parameter <10,000 (Makowski et al., 2019). However, re-calculation with 95% credible intervals led to the same conclusions (results not shown). Additionally, we performed a Bayesian equivalence test to formally examine difference of each trait type from the null value and differences between trait types themselves. We computed these tests using function *equivalence_test* from the package bayestestR (version 0.8.0; Makowski et al., 2019). Due to lack of theoretical knowledge, the null value was set as the region of practical equivalence at $\delta = \pm 0.1$, which corresponds to the effect size at half of Cohen's conventional definition for a small effect (Kruschke, 2018). Finally, we computed a Bayes factor using the package bayestest R and assumed that values of 3 and higher suggest an evidence for significant difference from the null value (e.g., Kruschke, 2018).

RESULTS

After correcting for the between-study heterogeneity (see section "Materials and Methods"), a total of 46 studies with 81 effect size reports were entered into our final analyses. We found significant overall effect on egg rejection for egg color (Cohen's h = 1.24, 89% credible intervals = 0.98–1.49), followed by maculation (h = 0.69 [0.45–0.94]) and UV (h = 0.43 [0.16–0.69]) (**Figure 1** and **Supplementary Table 1**). Effect sizes of egg material (natural vs. artificial model), shape, and size were small and each of their credible intervals overlapped with 0 (**Figure 1** and **Supplementary Table 1**). The same pattern of results was generated for eggshell trait types also when adding a fixed effect of

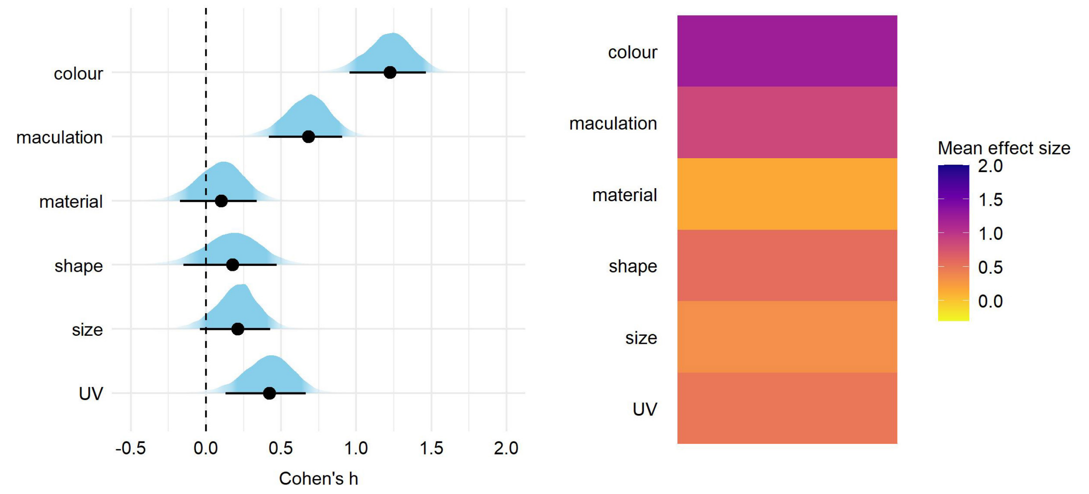

FIGURE 1 | Predicted overall effect size for egg trait types. On the left, for each trait type we show the parameter distribution with its median (black dot) and 89% credible interval (black line). On the right, the median effect sizes are plotted as a heatmap for each trait type.

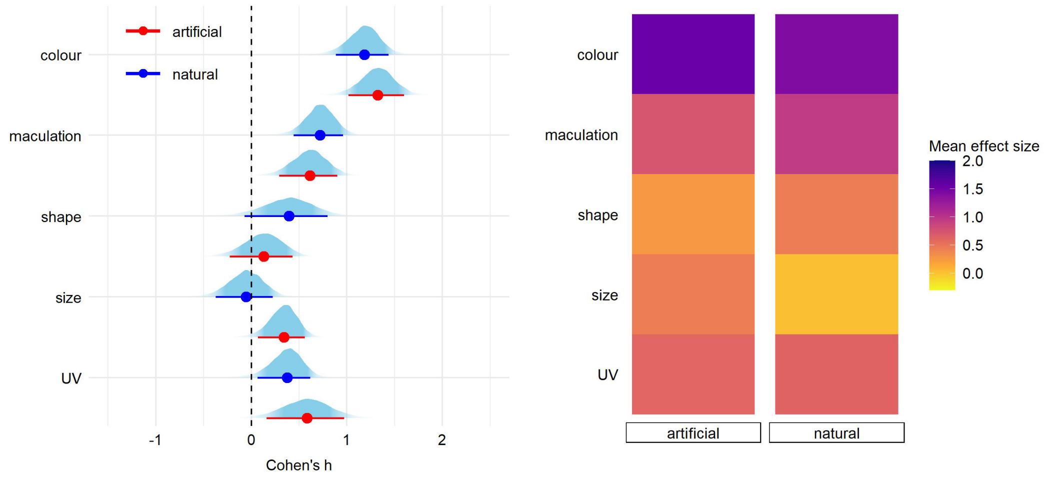

FIGURE 2 | Predicted effect size for egg trait types according to the experimental egg stimuli (natural or model egg). On the left, for each trait type we show the parameter distribution with its median (dot) and 89% credible interval (lines). On the right, the median effect sizes are plotted as a heatmap for each trait type separated by egg type stimuli.

stimulus type (natural vs. model), nest type (open vs. closed) or host-parasite system (*Cuculus* vs. *Molothrus* hosts; **Figures 2–4** and **Supplementary Tables 2–4**).

Pairwise comparisons within each egg trait type according to stimulus type (natural or model egg) were similar except of egg size trait (estimate = 0.39 [0.14–0.66]; **Figure 2** and **Supplementary Table 2**). This sole difference was driven by a high effect size found for smaller artificial eggs (Cohen's *h* = 0.59 [0.28–1.16], *N* = 10 reports) but not for other treatments (artificial larger = −0.14 [−0.55—0.27], *N* = 4; natural smaller = 0.03 [−0.29—0.43], *N* = 3; natural larger = -0.04 [−0.21—0.14], *N* = 4).

Finally, pairwise comparisons did not detect significant effect of nest type nor host-parasite system on any of egg trait (**Figures 3**, **4** and **Supplementary Tables 3,4**).

The only summation effect allowing us to estimate effect size was the simultaneous change in egg color and maculation (*N* = 12 reports). Even after excluding five outlier reports (see section "Statistical Analysis" for details), the between-study heterogeneity remained high (I^2 = 90%; 95% CI = 81.8–94.4) suggesting a caution for further interpretation of this overall effect size estimate. Bayesian random-effect model estimated high Cohen's *h* of 1.52 (89% credible intervals 0.86–2.05; *N* = 7 reports).

DISCUSSION

The results of this meta-analysis support earlier qualitative findings that visual traits play a dominant role in the recognition

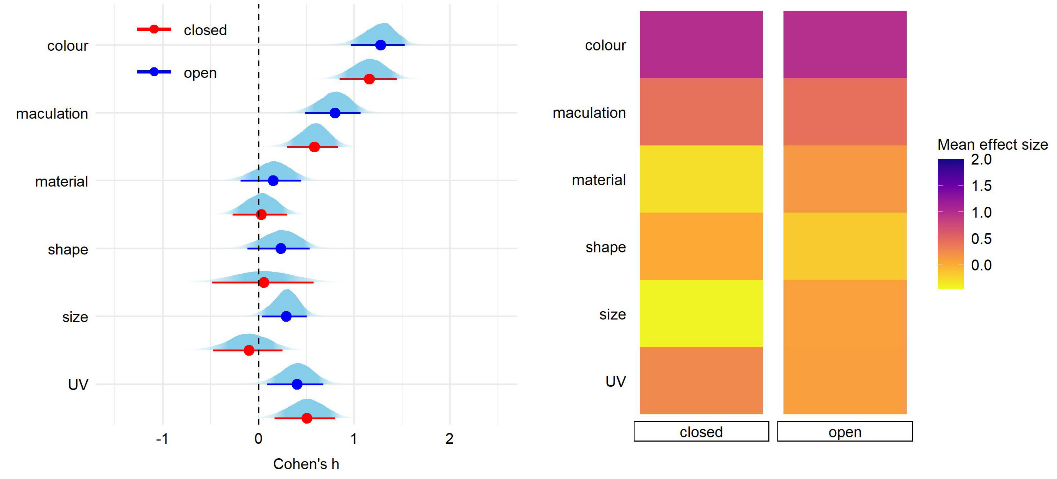

FIGURE 3 | Predicted effect size for egg trait types according to the breeding strategy (nest type: open or closed). On the left, for each trait type we show the parameter distribution with its median (dot) and 89% credible interval (lines). On the right, the median effect sizes are plotted as a heatmap for each trait type separated by host nest architecture.

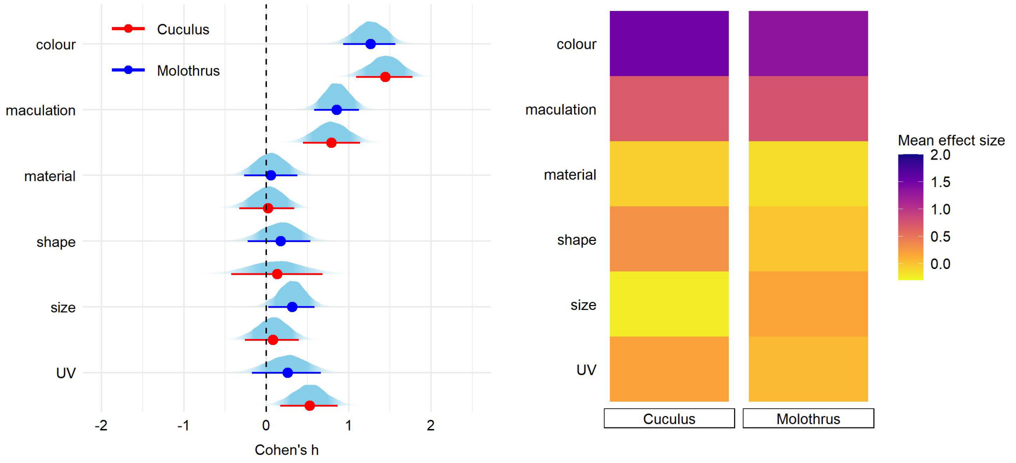

FIGURE 4 | Predicted effect size for egg trait types in hosts of *Cuculus* and *Molothrus* parasite species. On the left, for each trait type we show the parameter distribution with its median (dot) and 89% credible interval (lines). On the right, the median effect sizes are plotted as a heatmap for each trait type separated by parasitic lineage type.

of parasitic egg in the host nest (Honza and Cherry, 2017; Turner and Hauber, 2021). All the three visually-related eggshell traits, including color, maculation, and UV, showed no overlap with null effect sizes. The effect of the shell's ground coloration was particularly substantial and more important than either maculation or UV, and any other egg characteristic. However, we note that the visual egg traits are at the same time the most studied characteristic (55% of included reports; **Table 1**). Only partly vision-related traits such as egg shape and size showed small effects and the effect of other, also potentially partly tactile traits represented by natural vs. model materials, was negligible. Other hypothesized recognition cues, such as egg odor (e.g., Soler et al., 2014; Hauber, 2020) or weight (Ruiz-Raya et al., 2015) could not be statistically analyzed due to insufficient number of published reports (**Figure 5**). Therefore, our first recommendation is that more such studies address the potential roles of tactile-only or olfactory cued egg rejection behaviors in varied hosts of diverse avian brood parasites (also see Turner and Hauber, 2021). Finally, even in the studies performing a valid egg experiment, using a control treatment was particularly limiting selection criterion and led to a notable severe reduction of the selected studies for final analyses (**Supplementary Table 1**). However, modifying or even excluding any of the four selection criteria (see section "Materials and Methods") would have directly prevented obtaining a valid meta-analytical result.

Further, examining the three impactful extrinsic factors potentially affecting the relative importance within each egg trait showed that birds responded similarly regardless the bird is presented with artificial or natural experimental eggs, breeding in open or closed nest and parasitized by Old World cuckoos or New

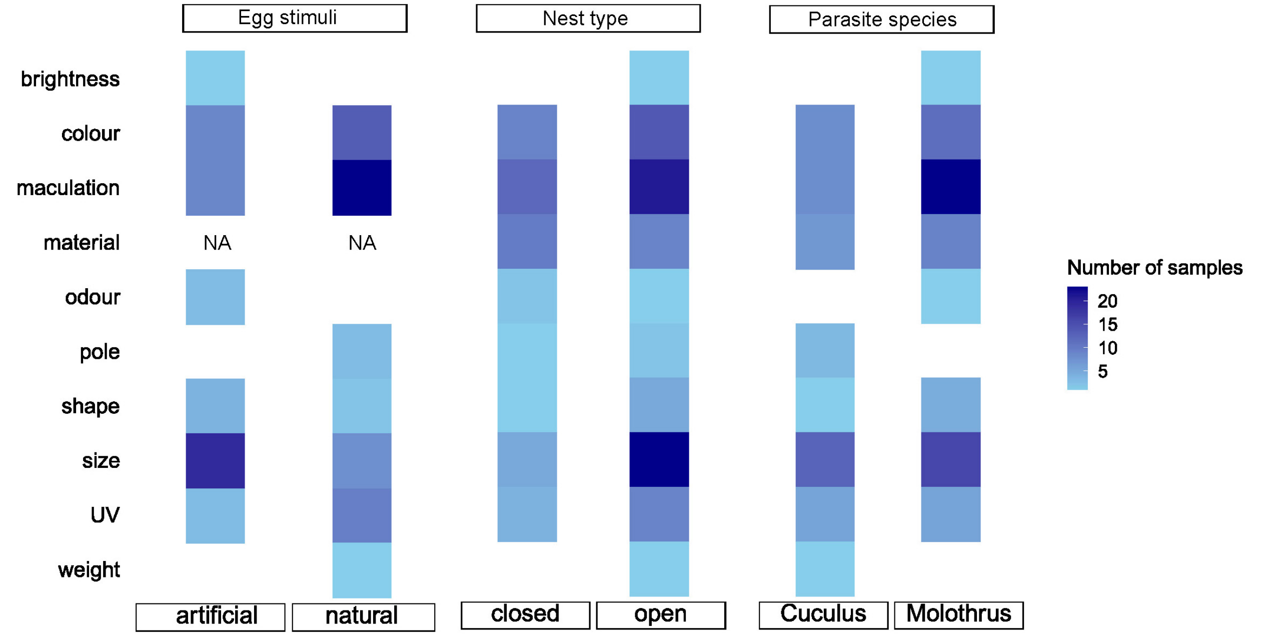

FIGURE 5 | A heat map showing number of samples (number of effect size reports) in our meta-analysis dataset. Blank squares represent no experiments available to be included in our data set for a particular treatment. NA, treatment does not exist in our dataset.

World cowbirds. The latter lack of difference between cuckoo vs. cowbird hosts may be due to the prior, naturally selected adaptations of the mostly insectivorous hosts of both parasite types, whereby visual discrimination of salient recognition cues, such as colors and patterns, may have been similarly preadapted to non-egg relevant traits, such as shared foraging contexts (e.g., Luro and Hauber, 2020).

In turn, the only within-egg trait difference was found for egg size, where model eggs with experimentally altered size were rejected more often than natural ones. More detailed exploration showed that this difference was caused by high rejection rates of artificial eggs. This was true particularly for experimental eggs smaller than the host egg. This treatment with smaller artificial eggs was also studied more often ($N = 10$ reports compared to smaller, $N \leq 4$ in other three treatments, i.e., larger artificial, smaller and larger natural egg) and 7 out of 10 reports were performed on *Turdus* spp. These thrush species are also known to reject smaller egg models at generally high rates (e.g., Grim et al., 2011; Samas et al., 2014; Hanley et al., 2017; Luro et al., 2018). This bias for testing strong rejecters in the treatment with artificial egg sizes might thus explain the higher effect size compared to published experiments using natural eggs in moderately rejecter host species.

The previous overview (Honza and Cherry, 2017) and our current meta-analysis both show a notable preponderance of studies to examine egg color and maculation characteristics (about a half of all studies in our data set, **Figure 5**). Yet, our second recommendation is that the potential for future studies on both of these visual traits still remains vast due to recent development of new analytical tools for data collection and approaches to statistical analyses (Stevens, 2011; Weinstein, 2018). For example, the eggshell color signal was recently assessed from two vision aspects, chromatic (hue) and achromatic contrasts (saturation; e.g., Avilés et al., 2010; Croston and Hauber, 2014; Hanley et al., 2017; Abolins-Abols et al., 2019; Manna et al., 2020). Similarly, egg patterning has been explored in a greater detail using advanced analytical tools (e.g., Schmitz Ornés et al., 2014; Stoddard et al., 2014). These studies provide important new insights into the sensory and cognitive processes of the hosts and show that the potential for future studies remains vast. Also, other recently emerged technologies, such as 3D printing (Igic et al., 2015), thermochromic coats (Hauber et al., 2019), or multispectral cameras (Attisano et al., 2018) open additional and novel research avenues to examine in greater detail various potential eggshell trait effects. Future meta-analyses can benefit from the more detailed studies by exploring each trait in greater depth. The direct quantification of the change in the manipulated egg trait will allow to include into analyses also the effect of magnitude of the manipulation. Such more detailed analysis was beyond the scope of this study here but we also note that the current level of methodological details and diversity in the methods would hinder these attempts. We recommend that the future studies should provide specific information, which allows to estimate the magnitude of egg trait manipulation whenever it is possible. For example, the magnitude of color contrast between control and manipulated eggs could be expressed in just noticeable difference units (JND; Vorobyev and Osorio, 1998) or as a simple proportion of change in quantities expressed with the International System of Units for some other traits.

Rothstein (1982) formally suggested that only one egg trait may play less important role on rejection than the summation of several egg characteristics. This "stimulus summation" hypothesis was supported by several other studies (Bártol et al., 2002; López-de-Hierro and Moreno-Rueda, 2010; de la Colina et al., 2012), including a biological replication of Rothstein's own study on American robins (*Turdus migratorius*) (Luro et al., 2018), while Underwood and Sealy (2006) concluded that in warbling vireos (*Vireo gilvus*) it was egg maculation itself that was a sufficient cue to recognize the cowbird egg. We found that Cohen's *h* of 1.52 (89% credible intervals 0.86–2.05) for the simultaneous change in egg color and maculation was somewhat higher, but still highly overlapping in its intervals with the effect size for the egg color trait only (Cohen's h = 1.23; 0.96–1.47). This single result does not provide a quantitative support for the "stimulus summation" hypothesis and more focally designed studies are clearly necessary before drawing any conclusions.

The greater importance of the color trait effect than any other eggshell traits (**Figure 1**) suggests the highest reliance of avian cognition processes on this particular visual parameter, irrespective of the nest's lighting milieu (**Figure 3**). At least from a human perspective, eggshell colors are diverse (Hauber, 2014; but see Hanley et al., 2015a), whereas other traits, including egg size and shape, are more limited in their variability (but see Stoddard et al., 2017). Also, all bird eggs have a ground coloration but not all of them are maculated, which might contribute to generally lower importance of maculation traits compared to the color as a reliable recognition cue. In turn, the effect of UV has been studied relatively often but it is rather assumed as a part of the color characteristic than a distinct trait (Cassey et al., 2008; Stoddard and Hauber, 2017). We classified the UV as a separate trait because this meta-analysis reflected the viewpoint and efforts in the field of brood parasitism research, whereby UV-sensitivity and -spectral reflectance are often treated as a critically avian-relevant perceptual cue (e.g., Honza et al., 2007; Croston and Hauber, 2014; Abernathy and Peer, 2015). Here we also examined the effect of egg material, which did not appear to generate reliably distinct effect sizes between model and natural egg stimuli (**Figure 2**). This conclusion is still important from a methodological point of view, because various materials are used to manufacture the artificial egg models. However, what is still missing from the experimental repertoire is a model egg stimulus that can be pierced by hosts whose beaks are too small for grasp rejection (e.g., Roncalli et al., 2017). Finally, we compared the eggshell traits' impact on egg rejection by hosts parasitized by Old World cuckoos vs. cowbirds and, contrary to expectations, found no statistical differences between these diverse set of hosts (**Figure 4**). This may be due to the use of artificial colors, rather than naturally mimetic cuckoo egg coloration, in studying the responses of hosts of both types of parasites, whereby even control treatments can be rejected by some hosts at unnaturally high rates (e.g., Abolins-Abols et al., 2019).

Vision is assumed to be the most important sense in birds (Martin, 2017). Accordingly, it is increasingly accepted that hosts recognize the foreign egg in their nest according to color and maculation (Honza and Cherry, 2017). Our results confirm quantitatively that visual components are essential during interactions with brood parasites during the egg stage. However, we must be reminded that egg characteristics that are not sensed visually have also attracted much lower research attention. Noticeably egg odor, weight, or surface texture remain unstudied (Turner and Hauber, 2021; **Figure 5**), and their relative impact on egg rejections remains mostly unknown and unquantifiable by us, too. Recent technological advances also open new ways to study in more depth any of the egg's visual characteristics and promise novel insights in the near future. We encourage continuing research efforts in this fascinating field of coevolutionary and ecological interactions.

DATA AVAILABILITY STATEMENT

The dataset generated for this meta-analysis is deposited in the Figshare digital repository (Dataset – Meta-analysis of avian egg traits; https://figshare.com/s/7320f0fb44c4f188c9eb).

AUTHOR CONTRIBUTIONS

MH conceived the study. MH and PS designed the study. PS analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.703208/full#supplementary-material

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Testing the Interspecific Function of Female Common Cuckoo “Bubbling” Call

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Female common cuckoo (*Cuculus canorous*) predator-like “bubbling” calls distract host parental attention and reduce the egg rejection rate. Such “bubbling” calls are also frequently used to attract males and deter territorial rivals in intraspecies contact, and these calls are an ancestral character in many cuckoo species. Although hosts have had sufficient time to become familiar with this call and evolve anti-parasitic strategies, why are the hosts fooled by this “bubbling” call? We propose two hypotheses. The first hypothesis proposes that call variation reduces the opportunity for host species to correctly assess cuckoo tricks. In contrast, the second hypothesis proposes that the cost of behavior may prevent the antiparasitic strategy from evolving. In the study, we tested the prerequisites of these hypotheses, by investigating whether cuckoo calls vary during the day and testing whether the predator-like calls suppress bird activities. Based on field recordings from three different areas, we found high overlap in the calls generated during different periods. Oriental great reed warblers (*Acrocephalus orientalis*), a host species, did not show different responses toward the playback of female common cuckoo calls generated before noon or afternoon. Based on bird count data, we found that predator-like call playback is insufficient for suppressing bird activities. Therefore, none of the prerequisites were supported by our field data. We discuss the potential reasons for our findings and hope to inspire more research examining female cuckoo vocalizations.

Keywords: acoustic signals, call variation, common cuckoo, female vocalization, playback

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INTRODUCTION

The arms race between the common cuckoo (*Cuculus canorous*) and its hosts is a classic example of coevolution (Poulin and Forbes, 2012; Moksnes et al., 2013). Theoretical models suggest that both participants are locked in an arms race, and the outcomes depend on a series of strategies shaped by coevolution (Takasu, 1998, 2003; Soler, 2014). Common cuckoos have evolved numerous strategies to increase parasitism success, such as laying mimetic eggs (Honza et al., 2014; Yang et al., 2016, 2017), adjusting the timing of egg laying (Seel, 1973; Johnsgard, 1997; Wang et al., 2020) and mimicking hawk morphology (Welbergen and Davies, 2011; Gluckman and Mundy, 2013). Host species have also evolved various strategies to reduce the risk of parasitism, including the ability to discriminate cuckoo eggs (Lang et al., 2014), mobbing behavior (Ma et al., 2018), and unique alarm calls when cuckoos are in the vicinity of host nests (Yu et al., 2017).

The "bubbling" call of female common cuckoos is also considered a parasitic strategy to mimic hawks. This predator-like call diverts the attention of reed warbler (*Acrocephalus scirpaceus*) parents and reduces the egg rejection rate (York and Davies, 2017). Playback of this call can effectively suppress the mobbing intensity of great reed warblers (*Acrocephalus arundinaceus*) (Marton et al., 2021). Even unsuitable hosts can be deceived. York and Davies (2017) found blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) increased their vigilance after hearing female "bubbling" calls, similar to how they respond to sparrowhawk (*Accipiter nisus*) calls; Jiang et al. (2021) found that both female common cuckoo and sparrowhawk calls elicited vigilance and escape responses from chickens (*Gallus gallus domesticus*). Although both female common cuckoo calls and sparrowhawk calls consist of rapidly repeated elements (York and Davies, 2017), there are clear differences between them. For example, the maximum frequency of female common cuckoo calls is always less than 2.5 kHz, and the minimum frequency of sparrowhawk calls can exceed 2.5 kHz (Deng et al., 2019b). As there are clear differences between female common cuckoo "bubbling" calls and sparrowhawk calls, why are the hosts fooled by this "bubbling" call?

One possible reason is that the hosts have not had sufficient time to evolve the ability to distinguish female common cuckoo calls from predator calls. However, this explanation is unlikely, given that predator-like female calls are a common trait in at least four cuckoo species (Kim et al., 2017; Yoo et al., 2020), indicating that they are not recently evolved strategies. Another possibility is that hosts have not had sufficient opportunities to become familiar with female common cuckoo calls and distinguish them from predator calls. This hypothesis would be supported if female common cuckoos rarely use this predator-like call (e.g., using this call after parasitizing a host's clutch) (York and Davies, 2017). However, female common cuckoos also use "bubbling" calls to attract males and deter territorial rivals when they fly or perch on branches (Deng et al., 2019b; Moskat and Hauber, 2019; Yoo et al., 2020). More than 90% of all calls occur during the morning rather than during the egg laying time in the afternoon (Gong et al., 2020). Thus, hosts have plenty of opportunities to become familiar with this mimetic call and discriminate it from the hawk calls.

In this study, we proposed two hypotheses concerning the "bubbling" call of female common cuckoos and tested the prerequisites of these hypotheses with field data. The first concerns call variation. Non-passeriform vocalizations are widely assumed to be simple and stereotyped and show little variation; for example, there is a high degree of consistency in the number of syllables (Møller et al., 2016, 2017) and call characteristics in individual male common cuckoo calls (Jung et al., 2014; Li et al., 2017; Zsebok et al., 2017). However, recent studies have revealed that individual male common cuckoo calls are more variable than previously thought (Deng et al., 2019a), and male common cuckoos can use these versatile vocalizations to encode different messages (Tryjanowski et al., 2018b; Xia et al., 2019; Moskat et al., 2021). Inspired by these observations, our first hypothesis is that female common cuckoos use different calls for interspecific and intraspecific communication. Specifically, characteristics differ between calls generated in the afternoon (i.e., when eggs are laid) and other periods. Consequently, host species may have little opportunity to become familiar with the female cuckoos' call used to mimic hawks and discriminate it from the hawk calls. We used field recordings from three areas to test whether there were consistent differences among calls generated at different times and conducted playback experiments to test whether a host species, oriental great reed warbler (*Acrocephalus orientalis*), showed different responses to female common cuckoo "bubbling" calls broadcasted at different times.

The second hypothesis concerns the benefit and cost of host antiparasitic behavior. When the cost exceeds the benefit, the behavior should be eliminated (or not evolve) (Szalai and Szamado, 2009; Higham, 2014); for example, the cost of misidentification prevents cuckoo fledglings from being identified by parents in many host species (Lotem, 1993). The benefit of distinguishing female common cuckoo calls from predator calls is clear: hosts can use female common cuckoo calls as a predictor of parasitism risk and increase antiparasitic behavior (e.g., mobbing behavior) to reduce the risk of parasitism. However, the cost of this behavior is also obvious: the hosts may be killed once they misidentify predator calls as a female common cuckoo call. If the cost exceeds the potential benefit, it is better to treat any calls similar to predator calls as a potential predatory threat (Ruxton et al., 2004). If this is the truth, we predicted other predator-like calls, besides cuckoos "bubbling" call, can also influence bird activities. So, we played calls from a neutral bird, the little grebe (*Tachybaptus ruficollis*), whose calls consist of rapidly repeated elements and had a similar structure as predator calls/female cuckoo calls. As prey birds escape or remain silent after hearing predator calls (Akçay et al., 2016; Santema et al., 2019), we predicted that fewer bird species should be observed after playback.

MATERIALS AND METHODS

Sound Recordings

To compare call characteristics, sound recordings were collected by passive acoustic recorder Songmeters (Wildlife Acoustics Inc., United States) from Liaohe Delta Nature Reserve (41.034°N, 121.725°E), Wild Duck Lake (40.417°N, 115.850°E) and Dagangzi National Forest (43.617°N, 126.133°E), China. Reed-bed habitat is the dominant habitat type at both the Liaohe Delta Nature Reserve and Wild Duck Lake; and Dagangzi National Forest consists of natural secondary forest. The common cuckoo predominantly parasitizes the oriental great reed warbler in both Liaohe Delta Nature Reserve (Li et al., 2016) and Wild Duck Lake, and parasitizes many forest birds in Dagangzi National Forest, such as Daurian redstarts (*Phoenicurus auroreus*) (Zhang et al., 2021). In Liaohe Delta Nature Reserve, 10 recorders were used from June 28th to July 29th, 2018; in Wild Duck Lake, 10 recorders were used from May 7th to July 8th, 2017; in Dagangzi National Forest, 8 recorders were used from May 17th to July 10th,

2018. Recorders were attached to trees or telegraph poles at a height of 3 m above ground and were set to record continuously at a sampling rate of 44.1 kHz and a sampling accuracy of 16 bits. The adjacent recorders were separated by a minimum distance of 200 m to avoid the same call from being recorded by two recorders. Recorders were checked approximately every 10 days to replace batteries and memory cards. A total of 7,200, 14,640, and 12,720 h of recordings were collected from Liaohe Delta Nature Reserve, Wild Duck Lake and Dagangzi National Forest, respectively.

Acoustic Measurements

Kaleidoscope software (Wildlife Acoustics Inc., United States) was used to automatically select female common cuckoo calls from the sound recordings. First, we entered the following acoustic features of our target sound (female calls) to create a recognizer: the frequency ranged from 600 to 2900 Hz, and the duration ranged from 1.6 to 4 s. These acoustic features were slightly larger than the actual parameters of female common cuckoo calls, but this was done to increase the detectability of calls by the software. We then manually checked all calls identified by the recognizer based on listening and visual inspection of the spectrograms. In total, we obtained 1,222, 1,431, and 124 female calls from Liaohe Delta Nature Reserve, Wild Duck Lake and Dagangzi National Forest, respectively (Gong et al., 2020).

All female common cuckoo call recordings were extracted and resampled at 22.05 kHz. Avisoft software (Avisoft Bioacoustics, Germany) was used to generate spectrograms with the following settings: fast Fourier transform length 256 points; Hamming window with a frame size of 100% and an overlap of 50%; frequency resolution 86 Hz; and time resolution of 5.8 ms. Female common cuckoo calls consist of a series of rapidly repeated "kwik-kwik-kwik" notes (York and Davies, 2017). Each "kwik" note represents a continuous trace on the spectrogram. For each call, the maximum frequency, minimum frequency, duration, and the number of notes were measured (**Figure 1A**). For each hour, 10 randomly selected calls, or all calls (if fewer than 10 calls within this hour), were measured. A total of 118, 113, and 84 calls were measured from Liaohe Delta Nature Reserve, Wild Duck Lake and Dagangzi National Forest, respectively. The data are shown in **Supplementary Appendix 1**.

Playback Experiments

Host Bird Responses During Playback

To check whether host bird oriental great reed warblers can discriminate female common cuckoo "bubbling" calls generated in the morning or afternoon, these experiments were conducted at Wild Duck Lake on July 15th and 21st, 2021. The common cuckoo predominantly parasitizes oriental great reed warblers in this reed-bed habitat. The experiments date was near the end of breeding season, however, both common cuckoos and oriental great reed warblers were still active, frequently uttering calls or continuously singing high-pitched song.

Nine "bubbling" calls generated before noon and nine "bubbling" calls generated after noon were randomly selected from the 113 calls that were measured and recorded in the same area to create playback sounds. Calls from common kestrels (*Falco tinnunculus*) (**Figure 1B**), a common predator species in the study area, and oriental turtle doves (*Streptopelia orientalis*) (**Figure 1C**), a harmless bird, were used as a positive control and negative control, respectively. For common kestrels and oriental turtle doves, recordings from 9 individuals in each species were downloaded from Xeno-Canto[1] (**Supplementary Appendix 2**), and one call in each individual was used to generate the playback sound. In the playback sounds, the rate was adjusted to 1 call per 10 s. A total of four 90-s playback sounds were generated, and two 15-s breaks were inserted after 30 and 60 s to generate 2-min playback sounds. The rhythm of these sounds was similar to the playback sounds used in a previous study (Marton et al., 2021).

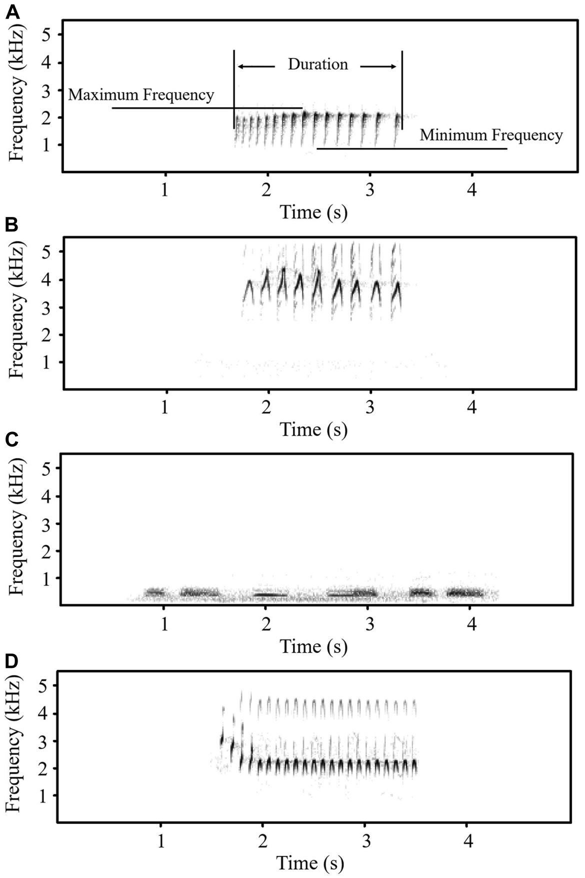

FIGURE 1 | Spectrogram of female common cuckoo "bubbling" call **(A)**, common kestrel call **(B)**, oriental turtle dove call **(C)**, and little grebe call **(D)**.

The sounds were played by a loudspeaker (E1; SMH Company, China), with the amplitude set to approximately 85 dB measured at 1 m with a sound level meter (NL-20; Rion Company, Japan). Playback experiments were conducted in the close vicinity of a singing oriental great reed warbler. The loudspeaker was positioned within 10 m from the target oriental great reed warbler, which was singing and always perched on the middle and upper parts of reeds. As target individuals were not banded, playback experiments were conducted at least 50 m

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[1] http://www.xeno-canto.org

apart to avoid repeated sampling from the same individual. There were 32 oriental great reed warblers involved in the playback experiments. These 32 individuals were randomly divided into four equal-sized groups, corresponding to the above four categories of acoustics used in the playback. For each individual, only one 2-min long sound was played. The observers with binoculars and stopwatches recorded whether and when the target individual stopped singing or flew away during the 2 min of playback. Some additional behavioral variables, such as scanning the surroundings, also reflect vigilance (York and Davies, 2017), but these behaviors were not recorded because of the difficulties in observing such behaviors in the dense vegetation used by the birds.

Bird Count Data After Playback

To test whether bird activities are suppressed by predator-like calls, these experiments were conducted at Xiaolongmen National Forest Park (40.017°N, 115.467°E), China, from May 24th to 28th, 2021. This area consists of secondary temperate deciduous broad-leaf forest. We played calls from female common cuckoos (**Figure 1A**), common kestrels (**Figure 1B**), oriental turtle doves (**Figure 1C**), and little grebes (**Figure 1D**). Oriental turtle doves are resident birds in the study area, and their call was used as a control. Both common kestrels and common cuckoos are breeding birds in the study area, and little grebes do not breed in this forest park. We used common kestrel calls rather than sparrowhawk calls as in previous studies (York and Davies, 2017; Xia et al., 2019; Jiang et al., 2021) because common kestrel is a more common predator than sparrowhawk in the study area based on our observations. The calls from common kestrels, female common cuckoos, and little grebes have a similar structure and consist of rapidly repeated elements (**Figure 1**).

As we did not collect enough recordings in the study area, we used acoustic files from Xeno-Canto (see footnote 1, **Supplementary Appendix 2**) to create playback sounds. For female common cuckoo calls, we did not distinguish the calls generated before or after noon because our aim in these experiments was to test whether bird activities were suppressed by any call types similar to predator calls rather than compare differences between calls generated at different periods. In addition, the acoustic characteristics highly overlapped in the calls generated before and after noon (seen in the Results). Recordings from 9 individuals were downloaded for each species, and one call from each individual was used. Similar to a previous study (Marton et al., 2021), the rate was adjusted to 1 call per 10 s, and then two 15-s breaks were inserted after 30 and 60 s, generating four 2-min long playback sounds.

The sounds were played by a loudspeaker (E1; SMH Company, China), with the amplitude set to approximately 85 dB measured at 1 m with a sound level meter (NL-20; Rion Company, Japan). Point counts were conducted at a total of 100 sites after playback. Sites were randomly divided into four equal-sized groups, corresponding to the above four categories of acoustics used in the playback. At each site, the loudspeaker was placed at approximately 1 m of a tree branch. The observers with binoculars were positioned approximately 10 m from the loudspeaker, and all bird species heard during a 3-min period within 30 m of the loudspeaker after playback were recorded. A 30-m radius was used because birds outside that distance were barely detected by the observers in the forest. As we had no prior information regarding an appropriate timeframe to make observations, our pragmatic solution was to choose a 3-min period for observation, not too long (exceeding efficient time) or too short (few birds were observed). Although birds were not individually ringed, the probability of counting an individual twice was very low, as two successive sites were separated by at least 200 m. Moreover, all point counts were conducted over a relatively short period during the breeding season to avoid the effect of season.

Data Analyses

Female common cuckoos generated calls from 3:00 to 20:00 in the study area, and the peak call output occurred during the morning (Gong et al., 2020). The calls were divided into two categories: calls generated before 12:00 and after 12:00. This division is based on the egg laying time by Common Cuckoos: 90% of egg laying occurred from 12:00 to 20:00 (Seel, 1973; Wang et al., 2020). As female Common Cuckoos give calls after parasitizing a host's clutch to divert host attention away from the clutch (York and Davies, 2017), we assumed that calls generated in the afternoon were mainly for interspecific communication (i.e., misdirect host defenses), and calls generated before the afternoon were for intraspecific communication (e.g., attract males and deter territorial rivals). We admit that this division is overly simplistic and somewhat arbitrary, as intraspecific calls could also occur in the afternoon, especially when cuckoos chase each other.

Multivariate analysis of variance (MANOVA) was used to assess the overall differences in the call characteristics between different periods (i.e., before or after noon) and among areas, followed by analysis of variance (ANOVA) to assess each individual call characteristic if there was a significant difference detected by MANOVA. Principal component analysis (PCA) with varimax rotation was used to compress the original variables into independent principal components, and discriminant function analysis (DFA) was used to determine whether calls generated before or after noon could be successfully split. The results from leave-one-out cross validation are reported as percentages of recordings correctly assigned in DFA.

For playback to oriental great reed warblers, birds that stopped singing or flew away during the 2 min of playback were scored as "response," and birds that continued to sing were scored as "no response." In two cases, the birds flew and approached the loudspeaker and then sang, and these two instances were also scored as "no response." Logistic regression was used to test whether "response" was affected by the time when the experiments were conducted. After confirming that there was no temporal effect, Fisher's exact test was used to compare the frequency of "response" among the four playback sounds (female common cuckoo calls generated before noon, female common cuckoo calls generated after noon, common kestrel calls, and oriental turtle dove calls).

For bird survey data, linear regression was used to test whether the number of species or number of individuals was affected by the time when experiments were conducted. After confirming that there was no temporal effect, both the number of species and the number of individuals were compared by ANOVA among the four groups (played calls from female common cuckoos, common kestrels, oriental turtle doves, and little grebes), followed by Tukey's test for *post hoc* pairwise multiple comparisons if there was a significant difference found in ANOVA.

All analyses were performed using SPSS 21.0 (IBM Corporation, United States). p values < 0.05 were considered statistically significant.

RESULTS

Our recordings revealed significant differences in call characteristics between periods (MANOVA: Pillai's Trace = 0.04, $F_{4,308} = 2.80$, $p = 0.026$) and among areas (MANOVA: Pillai's Trace = 0.36, $F_{8,618} = 16.91$, $p < 0.001$) (**Figure 2**). Specifically, calls generated before noon were longer in duration (ANOVA: $F_{1,311} = 8.10$, $p = 0.005$) than calls generated after noon, and there was no significant difference in other variables between different periods: maximum frequency (ANOVA: $F_{1,311} = 3.68$, $p = 0.056$), minimum frequency (ANOVA: $F_{1,311} = 0.09$, $p = 0.763$), and number of notes (ANOVA: $F_{1,311} = 1.87$, $p = 0.173$). For DFA, only 58.5, 60.2, and 61.9% of calls could be correctly classified into different periods in Liaohe Delta Nature Reserve, Wild Duck Lake and Dagangzi National Forest, respectively. There was high overlap in the calls between different periods based on the two principal components (with eigenvalues larger than 1) (**Figure 3**), and there was no clear timeline separating calls into different categories based on the measured acoustic variables (**Figure 4**).

Nearly all oriental great reed warblers kept singing without flying during the playback of female common cuckoo calls generated before or after noon (**Supplementary Appendix 3**). Only two individuals flew and approached the loudspeaker at 52 and 57 s, respectively. One of these two individuals quickly began to sing at the new location, and another stayed at the new location for 15 s without singing and then flew back to the original location and sang again. During the playback of the common kestrel call, 3 individuals flew away at 12, 35, and 37 s; 1 individual stopped singing at 46 s and then flew away at 68 s; 1 individual stopped singing at 62 s, and then generated alarm calls without flying; and another 3 individuals continued to sing. During the playback of oriental turtle dove calls, all 8 individuals continued to sing and did not fly. There was no temporal effect on the response of birds to playback (logistic regression: odds ratio = 2.00, $\chi^2_1 = 0.17$, $p = 0.678$). Common kestrel calls increased the response probability (stop singing or fly away) compared with the other three groups (Fisher's exact test: $p = 0.026$), and there were no differences observed among the other three groups (female common cuckoo calls generated before noon, female common cuckoo calls generated after noon, and oriental turtle dove calls).

A total of 33 species were heard after playback at all 100 sites (**Supplementary Appendix 4**). There were 3.10 ± 0.17 (mean ± standard error) species and 3.53 ± 0.20 individuals heard at each site. The number of species and the number of individuals were strongly positively correlated (Pearson correlation: $r = 0.93$, $p < 0.001$). There was no temporal effect on either the number of species (linear regression: coefficient = 0.46, $F_{1,98} = 0.13$, $p = 0.716$) or the number of individuals (linear regression: coefficient = −0.14, $F_{1,98} = 0.01$, $p = 0.923$). There was no significant difference in either the number of species (ANOVA: $F_{3,96} = 0.44$, $p = 0.728$) (**Figure 5A**) or the number of individuals (ANOVA: $F_{3,96} = 0.38$, $p = 0.769$) (**Figure 5B**) heard after the different playback sounds.

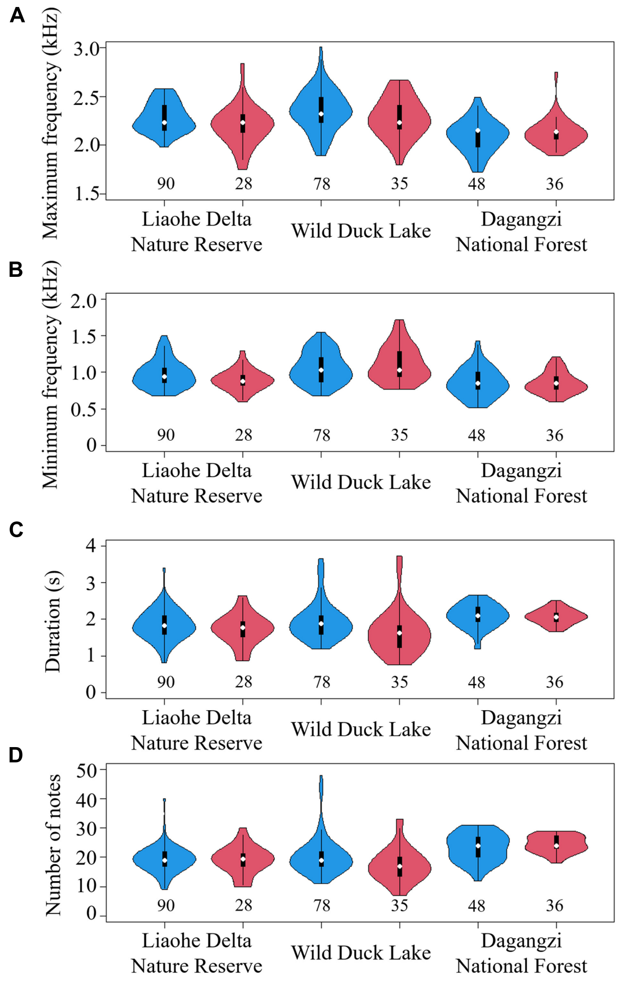

FIGURE 2 | Call characteristics between periods and among areas: maximum frequency **(A)**, minimum frequency **(B)**, duration **(C)**, and number of notes **(D)**. The blue violins indicate calls recorded before 12:00; the red violins indicate calls recorded after 12:00. Sample sizes are shown below each violin. In the middle of each density curve is a box plot, with black rectangle showing the ends of the first and third quartiles and white dot the median.

DISCUSSION

Common cuckoos and their hosts are an excellent system for studying coevolutionary arms races (Poulin and Forbes, 2012;

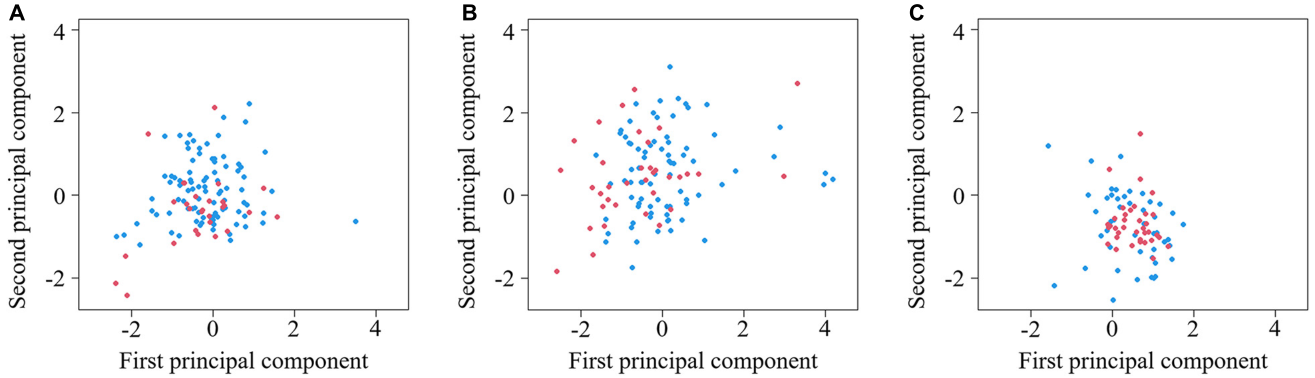

FIGURE 3 | Female common cuckoo "bubbling" calls highly overlapped between different periods in Liaohe Delta Nature Reserve **(A)**, Wild Duck Lake **(B)**, and Dagangzi National Forest **(C)**. The blue points indicate calls recorded before 12:00; the red points indicate calls recorded after 12:00.

Moksnes et al., 2013). Various strategies are adopted by cuckoos and their hosts. Recently, the "bubbling" call of female common cuckoos has been included in the long list of cuckoo tricks after the pioneering work by York and Davies (2017). This "bubbling" call type is frequently used when female common cuckoos fly or perch on branches (Deng et al., 2019b; Moskat and Hauber, 2019), and it is an ancestral character in many cuckoo species (Kim et al., 2017; Yoo et al., 2020). Thus, hosts have had sufficient opportunities to become familiar with this call and to evolve anti-parasitic strategies. Why are hosts still fooled by this "bubbling" call? We propose two hypotheses. The first concerns call variations in female common cuckoo and the second concerns the cost of antiparasitic behavior. In the study, we tested the prerequisites of these hypotheses, by investigating "bubbling" call variation and bird activities in predator-like calls playback. Based on the field recordings from three different areas, we found that there is a high degree of overlap in the calls generated between different periods. Oriental great reed warblers, a host species, did not vary in their responses to playback of female common cuckoo calls generated before noon or after noon. Based on the bird count data, we found that predator-like call playback is insufficient for suppressing bird activities. Therefore, none of these prerequisites are supported by our field data.

Although non-Passeriformes vocalizations are generally simple and stereotyped, many non-Passeriformes can use versatile vocalizations to encode different messages. For example, corncrakes (*Crex crex*) are known to express different levels of aggressive motivation through different call types (Rek and Osiejuk, 2011); African penguins (*Spheniscus demersus*) use four vocal categories under different circumstances (Favaro et al., 2014); and male ural owls (*Strix uralensis*) use different calls for territorial advertisement and for duetting with females (Zhou et al., 2020). For female common cuckoos, there are clear benefits, at least in theory, for separating call types corresponding to interspecific or intraspecific functions. The elaborate vocalizations may increase the probability that host species are fooled by cuckoos as well as the stimulation of sensory perception (Akre and Johnsen, 2014; Cui et al., 2016), which can reduce habituation in the distraction of host attention. However, our recordings do not support this idea. The acoustic characteristics largely overlapped between calls generated before or after noon, and DFA could not distinguish calls based on the measured characteristics. In this study, we split the calls based on time rather than interspecific or intraspecific functions. We admit that this division is overly simplistic, as many intraspecific calls can be mixed with interspecific calls into the afternoon group. However, we do not think the conclusions would be changed if other criteria were used to divide the calls. As the number of calls in the afternoon is quite low compared with the number of calls in the morning, we checked almost all afternoon calls by listening and visually inspecting the spectrograms when we measured the acoustic characteristics. We did not find any distinctive calls that are specially used for host species (e.g., after parasitizing a host's clutch).

For oriental great reed warblers, a host species in the study area, the induced behaviors during playback were not affected by whether female calls were generated before or after noon. Similar behavior indicates that there was no difference between calls generated in different periods, at least for oriental great reed warblers. This result is consistent with the acoustic analysis based on the call characteristics. Another interesting finding is that an interspecific function of female common cuckoo "bubbling" calls was not supported in the study, as the induced vigilance during cuckoo call playback was similar to that of the negative control (oriental turtle dove calls) and significantly lower than that of the positive control (common kestrel calls). This finding contradicts the findings of previous research (York and Davies, 2017; Jiang et al., 2021; Marton et al., 2021). The target species in previous studies always remained in an open area in the beginning of the experiments. For example, York and Davies (2017) presented playbacks to tits at experimental feeders; Jiang et al. (2021) presented playbacks to domestic chickens inhabiting open areas; Marton et al. (2021) started to broadcast playbacks after great reed warblers showed mobbing behavior to a cuckoo decoy. In this study, target individuals were perched on dense reeds. Thus, oriental great reed warblers might reduce their vigilance because of the shelter of dense reeds.

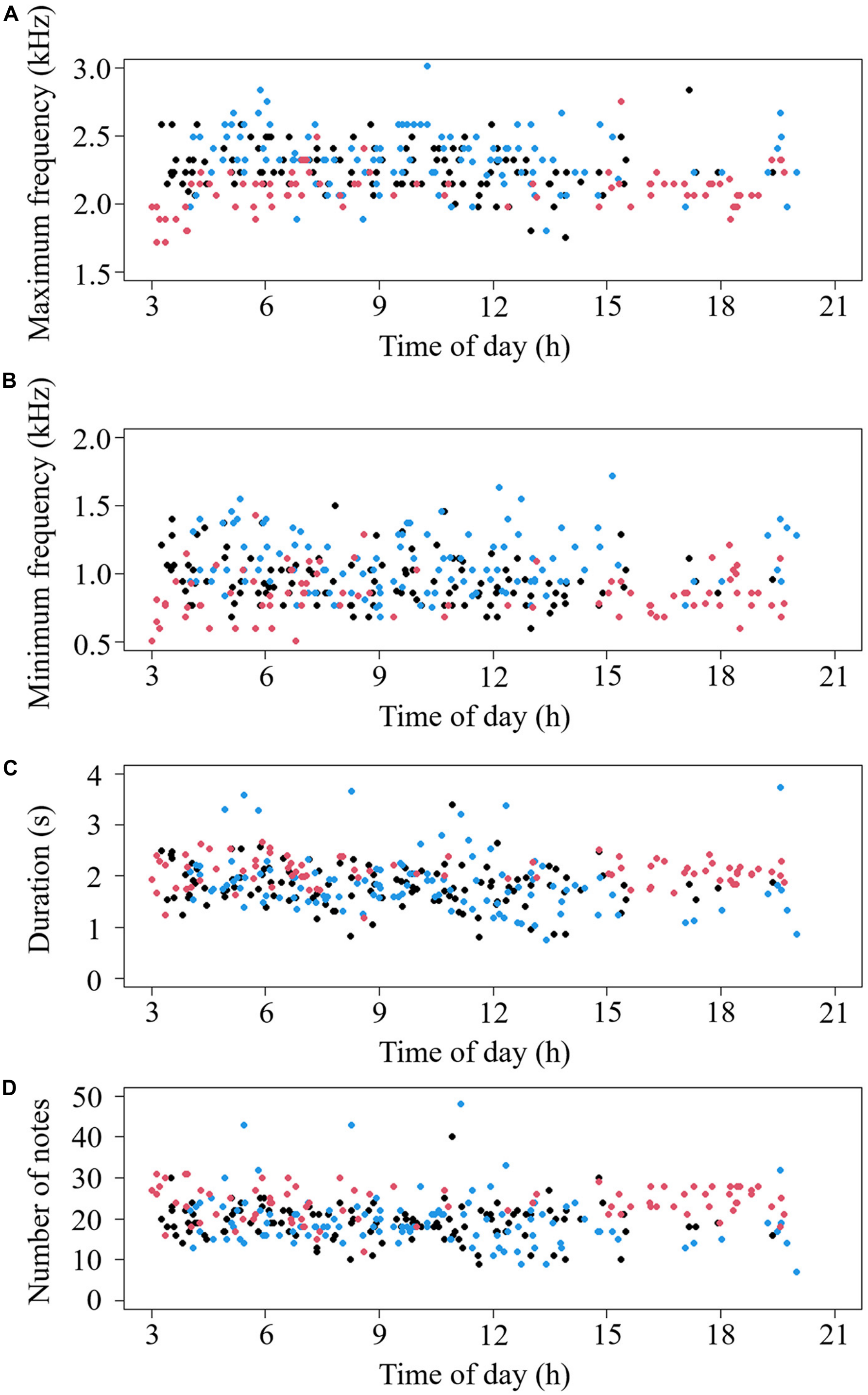

FIGURE 4 | Characteristics of female common cuckoos "bubbling" calls plotted against time of day: maximum frequency **(A)**, minimum frequency **(B)**, duration **(C)**, and number of notes **(D)**. The black points indicate calls from Liaohe Delta Nature Reserve; the blue points indicate calls from Wild Duck Lake; and the red points indicate the calls from Dagangzi National Forest.

Another possibility we could not rule out is that oriental great reed warblers respond to female cuckoo calls in some subtle ways, for example, through changes in posture and heart rate during playback.

The second hypothesis we proposed concerns the cost of host antiparasitic behavior. Theoretically, if costs exceed benefits, the behavior should be eliminated or not evolve at all (Szalai and Szamado, 2009; Higham, 2014). The cost of ignoring a predator

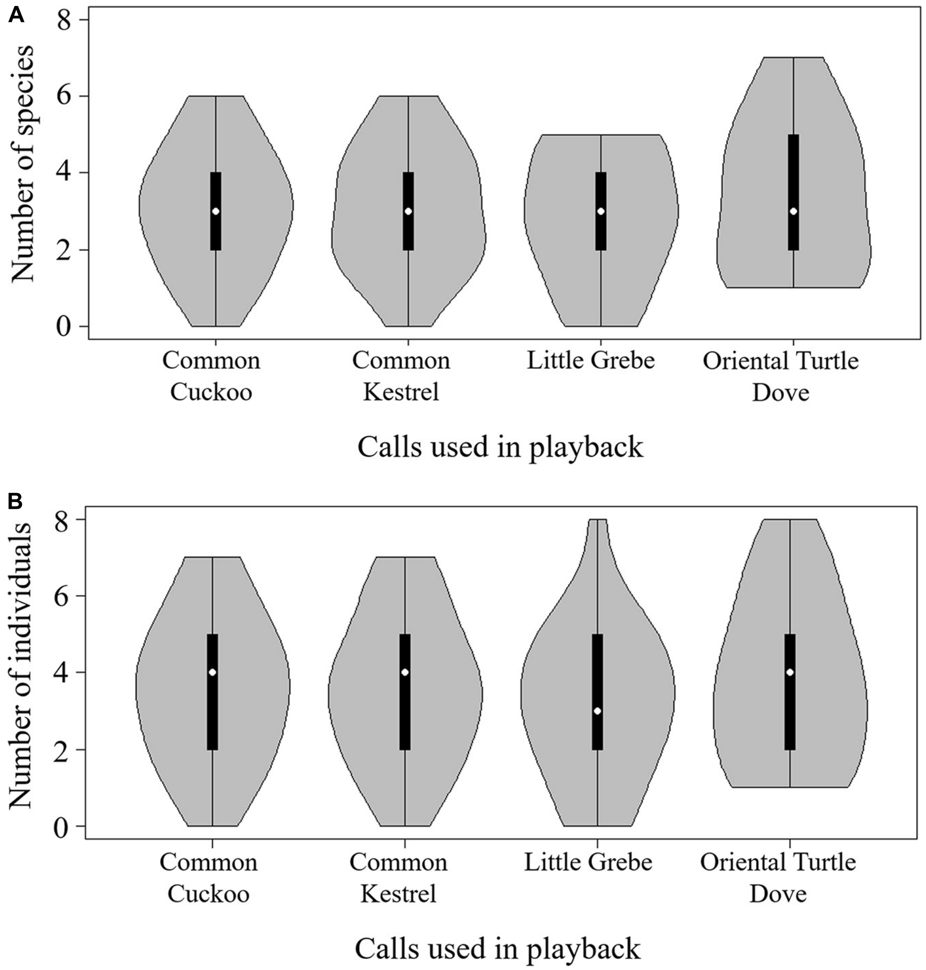

FIGURE 5 | Number of species **(A)** and individuals **(B)** heard after playback. Sample sizes for each group is 25. In the middle of each density curve is a box plot, with black rectangle showing the ends of the first and third quartiles and white dot the median.

can result in death (Creel and Christianson, 2008; Lima, 2009). Therefore, natural selection may favor prey species that can detect predatory cues (Ruxton et al., 2004). We counted bird species and the number of individuals after playback predator-like calls. We predicted that fewer birds would be observed after playback because bird activity would be suppressed (e.g., escaping, remaining silent) after hearing predator calls (Akçay et al., 2016; Santema et al., 2019). However, this prediction was not supported by our data: we did not find fewer species or number of individuals after playing back calls of female common cuckoos, common kestrels, and little grebes compared with the control (oriental turtle dove calls). These negative results may not stem from a lack of statistical power. If there was a medium effect of predator-like calls with a 0.3 effect size, as suggested by Cohen (1988), the power of ANOVA with 25 data points in each of the four groups could reach 0.69 (calculated by the "pwr" package in R software). This 0.69 statistical power is greater than the power of approximately 0.44 in most animal behavior studies (Jennions and Møller, 2002). Another possible reason for the negative result is that acoustic signals alone are insufficient for stimulating the prey response (Randler and Randler, 2020). Taxidermic models could be used in future studies to create a more realistic environment (Zachau and Freeberg, 2012; Tryjanowski et al., 2018a).

Acoustic signals play a key role in modifying bird behavior (Todt and Naguib, 2000; Slater, 2003). Recent research has shown that acoustic signals are involved in the arms race between common cuckoos and their hosts (York and Davies, 2017; Jiang et al., 2021; Marton et al., 2021). Female common cuckoos frequently use "bubbling" calls for both interspecific and intraspecific functions during the breeding season (Deng et al., 2019b; Moskat and Hauber, 2019; Yoo et al., 2020). The starting point of our study is the question: Why are the hosts fooled by this "bubbling" call? Two hypotheses based on call variations and the cost of antiparasitic behavior were proposed, and the prerequisites of these hypotheses were tested. None of these prerequisites are supported by our field recordings and playback experiments. More studies should be conducted to broaden our understanding of the vocalization of female cuckoos.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the research protocol was approved by the Animal Management Committee at the College of Life Sciences, Beijing Normal University, under license number CLS-EAW-2016-017.

AUTHOR CONTRIBUTIONS

CX conceived and designed the study. MT, JL, XL, and CX performed fieldwork. YW and CX analyzed the data. CX and AM wrote the manuscript with assistance from MT and YW. All authors reviewed and approved the final manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.725222/full#supplementary-material

Supplementary Appendix 1 | Call characteristics in female common cuckoos.

Supplementary Appendix 2 | The following recordings were downloaded from Xeno-Canto (http://www.xeno-canto.org) and used in the playback calls: : 138120, 181351, 466764, 479827, 53403, 550539, 557084, 557087, 605602 for female common cuckoos; 142344, 264925, 300296, 366079, 377557, 388806, 431372, 509603, 590630 for common kestrels; 120409, 19795, 285728, 286084, 286091, 409198, 426927, 473070, 491550 for oriental turtle doves; and 100940, 183440, 268729, 26929, 377058, 42554, 510716, 596757, 648836 for little grebes.

Supplementary Appendix 3 | Oriental great reed warbler responses during call playback.

Supplementary Appendix 4 | Bird count data after call playback.

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Eavesdropping on Referential Yellow Warbler Alarm Calls by Red-Winged Blackbirds Is Mediated by Brood Parasitism Risk

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Referential alarm calls that denote specific types of dangers are common across diverse vertebrate lineages. Different alarm calls can indicate a variety of threats, which often require specific actions to evade. Thus, to benefit from the call, listeners of referential alarm calls must be able to decode the signaled threat and respond to it in an appropriate manner. Yellow warblers (*Setophaga petechia*) produce referential "seet" calls that signal to conspecifics the presence of nearby obligate brood parasitic brown-headed cowbirds (*Molothrus ater*), which lay their eggs in the nests of other species, including yellow warblers. Our previous playback experiments have found that red-winged blackbirds (*Agelaius phoeniceus*), a species also parasitized by brown-headed cowbirds, eavesdrop upon and respond strongly to yellow warbler seet calls during the incubation stage of breeding with aggression similar to responses to both cowbird chatters and predator calls. To assess whether red-winged blackbird responses to seet calls vary with their own risk of brood parasitism, we presented the same playbacks during the nestling stage of breeding (when the risk of brood parasitism is lower than during incubation). As predicted, we found that blackbirds mediated their aggression toward both cowbird chatter calls and the warblers' anti-parasitic referential alarm calls in parallel with the low current risk of brood parasitism during the nestling stage. These results further support that red-winged blackbirds flexibly respond to yellow warbler antiparasitic referential calls as a frontline defense against brood parasitism at their own nests.

Keywords: brood parasitism, host-parasite interactions, heterospecific eavesdropping, playback presentations, referential alarm calling

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INTRODUCTION

Acoustic signals are used across diverse lineages to signal a variety of information, such as food sources or predatory threats (Bradbury and Vehrencamp, 2011). Some acoustic signals, known as functionally referential calls, denote to specific objects in the environment, and are often used to warn conspecifics of particular predator types (e.g., flying vs. ground), each requiring different behavioral responses to evade (Evans et al., 1993; Oda and Masataka, 1996; Evans, 1997; Rainey et al., 2004a,b; Zuberbühler, 2009; Suzuki, 2012). Listeners to referential calls must therefore be able to understand what is being referenced to determine the appropriate response based on the risk posed to them by the specific predatory threat denoted. Heterospecific eavesdropping upon referential calls is common across birds and mammals (Sherman, 1977; Magrath et al., 2015). Heterospecific eavesdroppers often demonstrate the same abilities as intended conspecific receivers do in decoding and responding appropriately to the information contained within the referential signals (e.g., Oda and Masataka, 1996; Rainey et al., 2004b; Suzuki, 2012; see Magrath et al., 2015, 2020 for reviews). For example, Verreaux's sifakas (*Propithecus verreauxi*) produce different referential alarm calls for aerial vs. terrestrial predators, which are heard by both intended conspecific receivers and by eavesdropping heterospecific black-casqued hornbills (*Ceratogymna atrata*) (Rainey et al., 2004a,b). Both types of listeners react to aerial alarm calls by hiding under cover, but, critically, the hornbills do not respond to the alarm calls signaling ground predators because they do not pose a threat to these birds.

Conspecific and heterospecific eavesdropping upon referential alarm calls occurs in songbirds within the context of improving nest defense or minimizing nest detection by predators (Gill and Sealy, 2003, 2004; Platzen and Magrath, 2005; Davies et al., 2006; Haff and Magrath, 2012; Suzuki, 2015; Yu et al., 2017). Avian nests can be threatened by at least two types of dangers: (1) nest predators that depredate eggs and nestlings, and (2) obligate brood parasites that solely lay their eggs in other species' nests (i.e., hosts), leaving the hosts to care for the costly brood parasitic young (Davies, 2010). Many host species exhibit strong frontline defenses against both threat types, responding aggressively toward both predatory and parasitic intruders on the territories to prevent their direct access to the nest (Welbergen and Davies, 2009; Kilner and Langmore, 2011; Feeney et al., 2012; Feeney and Langmore, 2015). There is some overlap between these nest threats, in that brood parasites may depredate eggs (e.g., mafia hypothesis, farming; Hauber, 2014; reviewed in Soler et al., 2017), and nest predators may also threaten adult survival (e.g., genus *Accipiter* hawks; Winkler et al., 2020). The main distinction between threats is that nest predators are of risk to hosts throughout the nesting cycle (laying, incubation, and nestling stages), whereas brood parasites pose the gravest risk when nests have eggs. Hosts are generally aggressive toward brood parasites during laying and incubation, when the nest is at highest risk of successful brood parasitism, and less aggressive (compared to other threats such as nest predators) during either the pre-nesting or the nestling stages when the risk of parasitism is low (Neudorf and Sealy, 1992; Gill and Sealy, 1996; Fasanella and Fernández, 2009; Lawson et al., 2021b; see Lawson et al., 2021a for a meta-analysis). Conversely, nest predation costs remain high (even increasing) as the brood ages due to its unchanging outcome (i.e., partial or total reproductive failure; Gill and Sealy, 1996; Fasanella and Fernández, 2009; Ruiz et al., 2018).

There is an adaptive benefit for hosts facing both nest threats to be able to discriminate brood parasites from nest predators and respond based on current risk. For potential hosts, anti-parasitic defense hinges on the early detection of brood parasites prior to the parasites' discovery of the host nests (Sealy et al., 1998). Thus, hosts of brood parasites should evolve to eavesdrop upon referential alarm calls that signal brood parasitism risk as an early warning system to maximize their frontline nest defenses. Yellow warblers (*Setophaga petechia*; hereafter "warblers") emit a referential alarm call to signal the presence of a generalist obligate brood-parasite, the brown-headed cowbird (*Molothrus ater*; hereafter: cowbird) (Gill and Sealy, 2004). Specifically, warblers produce "seet calls" to warn conspecifics of nearby cowbirds. After hearing seet calls or producing them, female warblers return to and sit upon their nest, which may prevent the cowbird from inspecting or laying an egg into the nest (Gill et al., 2008; Lawson et al., 2021c). Seet calls are primarily produced in response to the sight and/or sound of cowbirds themselves or seet calls emitted by conspecific warblers, and almost exclusively during laying and incubation stages, when the nest is at the highest risk of parasitism, and not during the pre-nesting or nestling stages (Sealy et al., 1998; Gill and Sealy, 2004; Gill et al., 2008; Lawson et al., 2021b).

Our previous research found evidence that red-winged blackbirds (*Agelaius phoeniceus*, hereafter "blackbirds"), another North American host of brown-headed cowbirds (Searcy and Yasukawa, 1995; Strausberger, 2001; Shaffer and Goldade, 2003), eavesdrop upon and respond to nearby yellow warbler seet calls during their own laying and incubation stages (Lawson et al., 2020). Blackbirds are phylogenetically and vocally distinct from yellow warblers, but often nest within the same wetlands as the warblers, with greater proximity to blackbirds linked to lower parasitism upon nearby yellow warbler nests (Clark and Robertson, 1979). Blackbirds are larger than cowbirds and yellow warblers, and frontload their anti-parasitic nest defenses, using both vocal and physical aggression toward cowbirds to prevent them from accessing and parasitizing the nest (Robertson and Norman, 1976, 1977; Ortega and Cruz, 1988; Neudorf and Sealy, 1992; Gill et al., 1997, 2008; Strausberger and Horning, 1997; Cruz, 1999; Yasukawa et al., 2016). Blackbirds are not known to have a referential alarm call system of their own, but they do eavesdrop upon the seet calls of yellow warbler neighbors: in Lawson et al. (2020) we found that during the incubation stage, blackbirds of both sexes responded more often to the warblers' seet calls relative to their generic "chip" alarm calls, and with similar urgency and vocal aggression toward playbacks of seet calls as to both cowbird chatters and nest predator calls. However, because there was equal response to both types of threats (brood parasite and nest predator), these findings implied that blackbirds do not perceive seet calls as a cowbird-specific referential signal *per se*, but rather as an alarm call for a nest threat.

Understanding how referential alarm calls are perceived by heterospecifics can be informed by testing under different conditions, such as varying levels of risk posed by the referent. For example, yellow warblers themselves respond less aggressively to referential seet calls during the nestling stage likely because there is little to no brood parasitism risk during this stage (Neudorf and Sealy, 1992; Gill and Sealy, 1996), and the same pattern can be seen across other hosts toward models of their respective brood parasites (Fasanella and Fernández, 2009; reviewed in Lawson et al., 2021a). Furthermore, blackbirds presented with taxidermy cowbird and nest predator models across nesting stages respond equally to both models during incubation, but more strongly to the nest predator during nestling stage (Neudorf and Sealy, 1992; also see Henger and Hauber, 2014). To determine whether blackbirds recognize seet calls as referential alarm calls denoting brood parasitism risk, we expanded on our previous playback study conducted during the incubation stage (Lawson et al., 2020), and presented playbacks of cowbird chatters, seet calls, nest predator calls, and a non-threatening control species to blackbird nests during the nestling stage, when the risk of brood parasitism is low. We predicted that if blackbirds respond to seet calls as a referent for brood parasites, aggressive responses toward cowbird chatter and seet calls should be lower than aggressive responses to nest predator calls, but comparable to each other, during the later stage of nesting.

MATERIALS AND METHODS

This playback experiment was conducted during April–July 2020 and used the same sites, playback files, and playback methodology as in Lawson et al. (2020), which tested blackbirds' responses to the same playbacks during the egg/incubation stages during the prior 2 years. The methodology is described briefly below; for more detailed methodology, see Lawson et al. (2020).

Sites and Study Species

Playbacks occurred at sites in Champaign (n = 3) and Vermilion counties (n = 3) in east central Illinois, United States, where blackbirds and yellow warblers both breed (Lawson et al., 2020). Both species are parasitized by cowbirds in Illinois (Rodewald, 2015; Merrill et al., 2017; pers. obs.). Blackbirds arrive as early as February but do not breed until late-April through late-July, with peak breeding season mid-May to mid-June (Lawson et al., 2020; Yasukawa and Searcy, 2020). Yellow warblers arrive on the breeding grounds in late-April with peak breeding mid-to-late May (overlapping with blackbirds; Kelly et al., 2019; Lawson et al., 2021b,c).

Playback Stimuli Construction

For our experiments, we used four of the playback treatments from Lawson et al. (2020): (1) female cowbird chatter (brood parasite), (2) yellow warbler seet calls [cowbird-specific anti-parasitic alarm call (Gill et al., 1997; Sealy et al., 1998; Gill and Sealy, 2003, 2004; Gill and Bierema, 2013)], (3) blue jay (*Cyanocitta cristata*, a nest predator commonly seen at our sites; Smith et al., 2020), calls and (4) wood thrush (*Hylocichla mustelina*, a non-threatening sympatric heterospecific control, Kelly et al., 2019) songs. Including a nest predator call along with a brood parasite was critical to determine whether the blackbirds' responses to the seet call are antiparasitic or general (Rothstein and Robinson, 1998). Audio file construction is described in detail in Lawson et al. (2020). Briefly, audio files were edited and filtered in Adobe Audition CC 2019 and included five exemplar files for each treatment sourced from different individuals, with one exemplar chosen randomly for each playback trial to avoid pseudoreplication (Kroodsma et al., 2001). Each exemplar contained vocalizations from at least three individuals.

Playback Experiment

We conducted playback trials at active blackbird nests that were ≥50 m apart, which is the mean territory size for blackbirds (Searcy and Yasukawa, 1995). Blackbirds are polygynous harem breeders, and are highly defensive of territory boundaries with little to no overlap with other males, with females being site-faithful to male territories (Searcy and Yasukawa, 1995). Therefore, only testing nests ≥50 m apart (i) reduced the likelihood that we tested the same parents twice at different nests, as the subjects were not banded, and (ii) allowed us to accurately record the stages of any additional nests on the male's territory. We searched sites 1–2 times weekly for active nests. Nest contents were checked every 3 days to ensure playback trials occurred during the nestling stage. We conducted playbacks at nests that only contained nestlings <9 days old to prevent forced fledging when inspecting the nest (blackbirds naturally fledge at 11–14 days old; Yasukawa and Searcy, 2020). Playbacks were conducted between 05:00 and 12:00 h local time with a FOXPRO NX4 game caller, placed ∼5 m from active nests. We placed the caller ∼1 m high in vegetation when possible and recorded data from >10 m away. Playback trials occurred for 10 min and were adjusted to broadcast at ∼90 dB at 1 m from the source (Lawson et al., 2020).

Blackbird nests received two of the four playback treatments, each on a separate day: cowbird chatter (n = 23), yellow warbler seet calls (n = 22), blue jay calls (n = 20), and wood thrush songs (n = 17), for a total of 82 playbacks. The time lapse in between the first and second playback at each territory ranged from 24 to 72 h later (mean = 30.4 h) to avoid habituation. Nests were randomly assigned treatments to minimize the potential for an effect of treatment order. Six nests were not retested as they were depredated between trials. Furthermore, the focal female did not appear within the playback range for two of the trials, and thus, these trials were dropped from the data analyses.

During the playback trial we recorded the following behavioral responses from both parents within 30 m of the speaker: (1) response latency (sec after the start of trial when a switch to behaviors signaling playback detection occurred: posturing, hopping, alarm calling, or attacking the speaker) (2) closest approach to the speaker (m); and (3) the number of alarm calls produced ("checks," "chits," "chonks" used interchangeably as nest defense alarm calls by both sexes, and "cheers" which are only produced by males, Beletsky et al., 1986; Knight and Temple, 1988; Yasukawa, 1989). We only recorded responses of the focal male and focal nesting female (determined by observing which female fed the nestlings), and not other females within the harem. The focal birds were visually tracked by an observer throughout the entire trial while another recorded the behaviors. The presence of additional nest(s) with eggs, as well as age of nestlings in the focal nest, were included as variables in our models (see section "Statistical Analyses").

These studies were approved by the Animal Ethics Committee (IACUC) of the University of Illinois (#17259), and by United States federal (MB08861A-3) and Illinois state agencies (W20.6394).

Statistical Analyses

We evaluated whether playback treatment affected the same three response variables of interest (latency, total alarm calls, and closest approach) using a separate generalized linear model for each. Models were also separated by sex, due to the polygynous nature of blackbirds possibly leading to sex differences in nest defensive behaviors (Yasukawa and Searcy, 2020). For all latency and alarm call models we used a negative binomial general linear model to account for the large number of non-responses (0 s latency, no alarm calls produced) that varied by treatment. For the closest approach variable, we log-transformed the data after adding a small constant to obtain a normal distribution, and ran a linear model. All models included the following fixed effects: playback treatment, date (ordinal days after start of season – April 1st), trial order (to account for repeated playbacks at the same site), and age of nestlings (to account for the variation in ages of nests at time of playback). For models on male data, we also included the presence of another nest with eggs as another fixed effect, because males may have multiple females at different stages of nesting on their territory, and if there were differences in response over nest stages, presence of eggs may have affected the males' responses. If the presence of eggs was significant, we ran the same generalized linear model with an interaction term (treatment × presence of eggs) to determine if responses to specific treatments were affected by presence of a nest with eggs on the male's territory. For all models with significance, we ran *post hoc* Tukey tests to multiple compare treatment pairs of least-square means. All statistical tests were conducted in the statistical program R 4.0.5 (packages lme4, nlme, multcomp, emmeans, and car), with $\alpha = 0.05$. Effect sizes were calculated in R for all significant and non-significant outcomes.

RESULTS

Latency

Average latencies to respond varied significantly by treatment for both males ($F_{3,81} = 8.95$, $p < 0.001$; **Figure 1**) and females ($F_{3,79} = 7.02$, $p < 0.001$; **Figure 2**). Based on *post hoc* pairwise comparisons of least-square means, males responded more quickly to playbacks of blue jay calls compared to cowbird chatters ($z = 4.44$, $p < 0.001$), seet calls ($z = 6.30$, $p < 0.001$), and control wood thrush songs ($z = 6.25$, $p < 0.001$). Female latencies showed the same pattern, where females responded more quickly to playbacks of blue jay calls compared to cowbird chatters ($z = 3.14$, $p < 0.01$), seet calls ($z = 3.06$, $p = 0.01$), and control wood thrush songs ($z = 2.92$, $p = 0.01$). There was no significant difference in latency to respond to cowbird chatters compared to seet calls for either sex (males: $z = -1.80$, $p = 0.27$; females: $z = 1.13$, $p = 0.99$), and both sexes responded to cowbird and seet calls with similar latency to the control wood thrush (males: cowbird-wood thrush $z = -2.02$, $p = 0.18$, seet-wood thrush $z = -3.99$, $p = 0.97$; females: cowbird-wood thrush $z = 0.005$, $p = 0.99$, seet-wood thrush $z = -0.11$, $p = 0.99$; see **Supplementary Table 1** for all *post hoc* comparisons). For both sexes, neither date of playback (males: $F_{3,81} = 0.37$, $p = 0.54$, estimate $= -0.009$; females: $F_{3,79} = 1.10$, $p = 0.29$, estimate <-0.001) nor trial order (males: $F_{3,81} = 0.33$, $p = 0.56$, estimate $= 0.26$; females: $F_{3,79} < 0.01$, $p = 0.93$, estimate $= -0.01$) affected latency responses. While age of nestlings did not significantly influence male latency ($F_{3,81} = 3.07$, $p = 0.08$, estimate $= 0.22$), females responded more quickly to playbacks with increasing age of nestlings ($F_{3,79} = 3.93$, $p = 0.05$, estimate $= 0.12$). For males, presence of a nest with eggs on the territory did not significantly affect latency responses ($F_{3,81} = 3.15$, $p = 0.07$, estimate $= -1.4$).

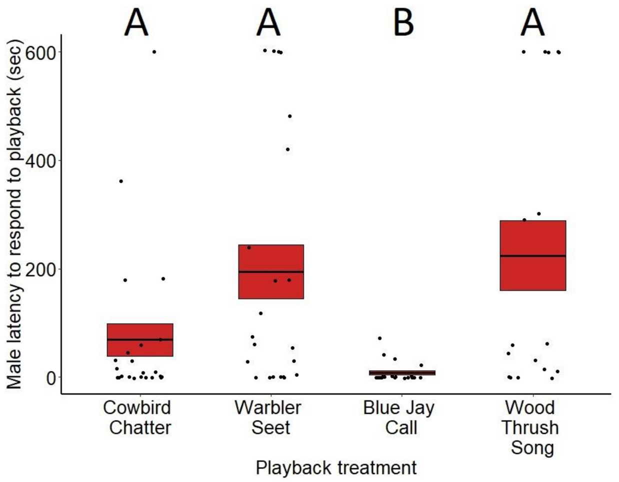

FIGURE 1 | Latency (in seconds) for male red-winged blackbirds to respond to the playback treatments at nests during nestling stage. Means are shown with the bold line, and shaded boxes represent standard errors. Boxes with different letters denote *post hoc* statistical differences between treatments. For the *p*-values of *post hoc* comparisons, please refer to **Supplementary Table 1**.

Closest Approach

Closest approach varied significantly by treatment for males ($F_{3,81} = 5.55$, $p < 0.01$; **Figure 3**) but not females ($F_{3,79} = 1.18$, $p = 0.32$; **Figure 4**). Based on *post hoc* comparisons, males approached playbacks of cowbird chatters more closely than playbacks of seet calls ($z = 2.73$, $p = 0.03$) and control wood thrush songs ($z = 3.81$, $p < 0.001$). Males also approached blue jay calls more closely than wood thrush songs ($z = 3.20$, $p = 0.05$). Closest approach did not differ between any of the other playback comparisons (cowbird-blue jay $z = -1.33$, $p = 0.54$, blue jay-seet $z = -1.34$, $p = 0.53$, seet-wood thrush $z = -1.35$, $p = 0.52$; see **Supplementary Table 2** for all *post hoc* comparisons). For both sexes, neither date of playback (males: $F_{3,81} = 0.30$, $p = 0.58$, estimate $= 0.004$; females: $F_{3,79} < 0.001$, $p = 0.98$, estimate <-0.001), trial order (males: $F_{3,81} = 0.40$, $p = 0.52$, estimate $= -0.15$; females: $F_{3,79} < 0.68$, $p = 0.40$, estimate $= -0.16$), nor age of nestlings (males: $F_{3,81} = 0.17$, $p = 0.68$, estimate $= -0.02$; females: $F_{3,79} = 0.87$, $p = 0.98$, estimate $= 0.04$) affected closest approach. For males, presence of a nest with eggs on the territory also did not significantly affect closest approach ($F_{1,81} = 3.20$, $p = 0.07$, estimate $= -0.60$).

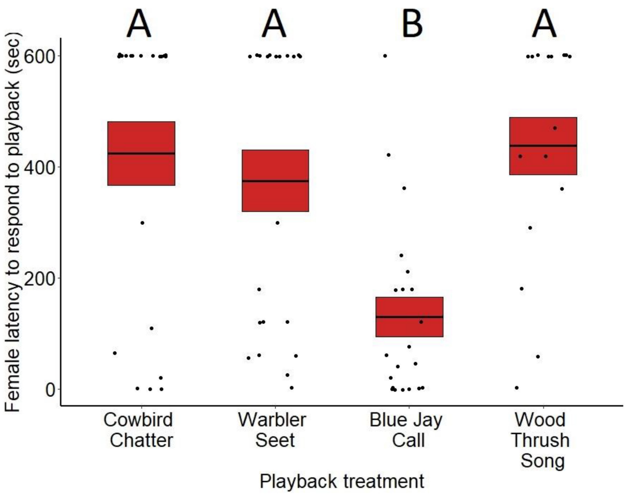

FIGURE 2 | Latency (in seconds) for female red-winged blackbirds to respond to the playback treatments at nests during nestling stage. Means are shown with the bold line, and shaded boxes represent standard errors. Boxes with different letters denote *post hoc* statistical differences between treatments. For the *p*-values of *post hoc* comparisons, please refer to **Supplementary Table 1**.

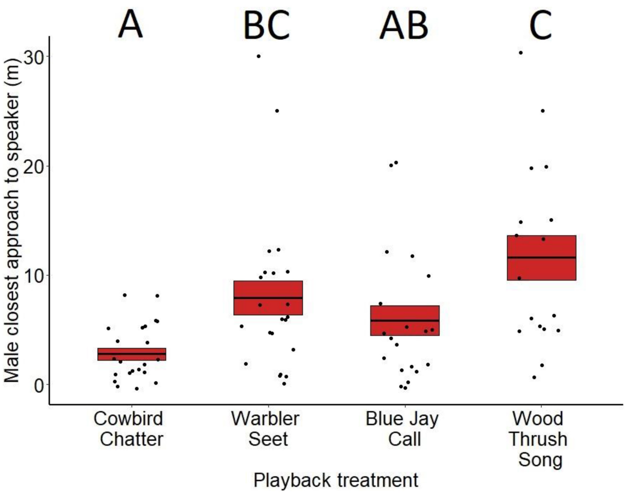

FIGURE 3 | Closest approach to the playback speaker (in meters) by male red-winged blackbirds for the different treatments at nests during nestling stage. Means are shown with the bold line, and shaded boxes represent standard errors. Boxes with different letters denote *post hoc* statistical differences between treatments. For the *p*-values of *post hoc* comparisons, please refer to **Supplementary Table 2**.

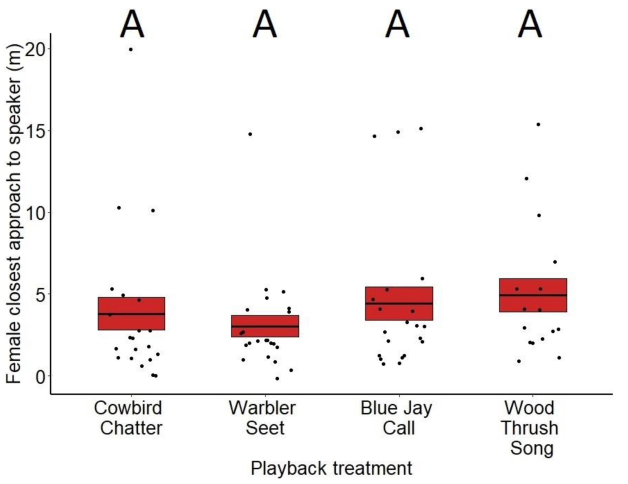

FIGURE 4 | Closest approach to the playback speaker (in meters) by female red-winged blackbirds for the different treatments at nests during nestling stage. Means are shown with the bold line, and shaded boxes represent standard errors. There were no significant pairwise differences; for the *p*-values of *post hoc* comparisons, please refer to **Supplementary Table 2**.

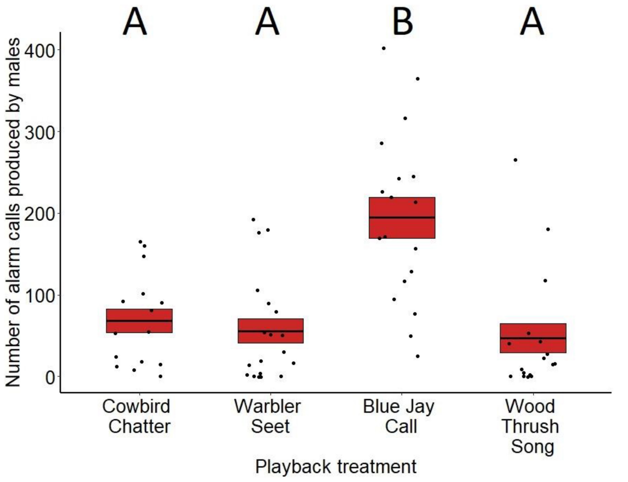

FIGURE 5 | Number of alarm calls produced by male red-winged blackbirds in response to playbacks at nests during nestling stage. Trials where males had an additional nest on territory with eggs are excluded. Means are shown with the bold line, and shaded boxes represent standard errors. Boxes with different letters denote *post hoc* statistical differences between treatments. For the *p*-values of *post hoc* comparisons, please refer to **Supplementary Table 3**.

Alarm Calling

Alarm calling varied significantly between treatments for both males ($F_{3,81} = 6.55, p < 0.001$; **Figure 5**) and females ($F_{3,79} = 8.92, p < 0.001$; **Figure 6**). Based on *post hoc* pairwise comparisons of least-squares means, males alarm called more toward playbacks of blue jay calls compared to cowbird chatters ($z = 2.53, p = 0.05$), seet calls ($z = 3.31, p < 0.01$), and control wood thrush songs ($z = 3.75, p < 0.001$). Female also alarm called more toward blue jay calls compared to cowbird chatters ($z = 5.84, p < 0.001$), seet calls ($z = 5.99, p < 0.001$), and control wood thrush songs ($z = 3.33, p < 0.01$). There was no significant difference in alarm calling responses toward cowbird chatters compared to seet calls for either sex (males: $z = 0.73, p = 0.88$; females: $z = 0.05, p = 0.99$), and both sexes alarm called similarly toward cowbird and seet calls compared to the control wood thrush (males: cowbird-wood thrush $z = 1.34$, $p = 0.53$, seet-wood thrush $z = 0.69$, $p = 0.90$; females: cowbird-wood thrush $z = -2.11$, $p = 0.14$,

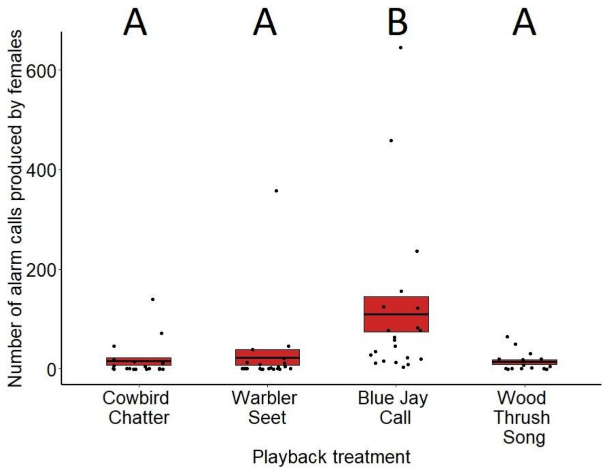

FIGURE 6 | Number of alarm calls produced by female red-winged blackbirds in response to playbacks at nests during nestling stage. Means are shown with the bold line, and shaded boxes represent standard errors. Boxes with different letters denote *post hoc* statistical differences between treatments. For the p-values of *post hoc* comparisons, please refer to **Supplementary Table 3**.

seet-wood thrush $z = 2.16$, $p = 0.13$; see **Supplementary Table 3** for all *post hoc* comparisons). For both sexes, date of playback had a significant effect on alarm calling (males: $F_{3,81} = 4.59$, $p = 0.03$, estimate = 0.01; females: $F_{3,79} = 8.28$, $p < 0.01$, estimate = 0.03), with blackbirds producing more alarm calls later in the season. Females also alarm called significantly more with increasing age of the nestlings in her nest ($F_{3,79} = 4.06$, $p = 0.04$, estimate = −0.22), and while males showed this same pattern, it was non-significant ($F_{3,81} = 3.65$, $p = 0.06$, estimate = 0.12). Trial order did not significantly affect alarm call responses (males: $F_{3,81} = 1.27$, $p = 0.26$, estimate = −0.27; females: $F_{3,79} = 2.56$, $p = 0.11$, estimate = −0.75).

For males, the presence of an additional nest with eggs on their territory significantly affected alarm calling responses ($F_{3,81} = 8.25$, $p < 0.01$, estimate = 0.95). When we ran the same generalized linear model with an interaction term we found a significant interaction between treatment and presence of a nest with eggs on alarm calling rates (treatment × eggs term: $F_{3,64} = 5.01$, $p < 0.01$). Specifically, males alarm called more toward playbacks of cowbird chatters (z ratio = 2.60, $p < 0.01$) and seet calls (z ratio = 2.63, $p < 0.01$) when there was an additional nest with eggs on their territory, while alarm calling toward blue jay playbacks was not significantly affected by presence of eggs vs. nestlings only (z ratio = −1.72, $p = 0.08$) (wood thrush playbacks were not included since no territories with the wood thrush playback had a blackbird nest with eggs). To determine how alarm calling between treatments varied without the influence of nests with eggs, we removed any trials with territories with eggs and reran the original general linear model. Male alarm calling still varied significantly by treatment ($F_{3,81} = 6.57$, $p < 0.001$), with *post hoc* comparisons showing the same patterns of higher alarm calling toward playbacks of blue jay calls compared to cowbird chatters ($z = 2.64$, $p = 0.04$), seet calls ($z = 3.40$, $p < 0.01$), and control wood thrush songs ($z = 3.71$, $p = 0.001$) as the model with no eggs. In addition, males showed no significant differences in responses between cowbird chatters and seet calls ($z = 0.52$, $p = 0.95$), between cowbird chatters and the control ($z = 0.93$, $p = 0.78$), and between seet calls and the control ($z = 0.43$, $p = 0.97$), similar to males with nestlings only.

DISCUSSION

Our current playback study found support for the hypothesis that both male and female red-winged blackbirds eavesdrop upon and respond to yellow warbler's seet calls specifically as a referent for "brood parasite" and not as a general nest-threat heterospecific alarm call. On the one hand, the risk of brood parasitism for hosts is highest when nests have eggs, and lower after the eggs hatch; on the other hand, the risk of predation remains high and often increases across nest stages, as the fitness outcome generally is the same – partial or total reproductive failure (Gill and Sealy, 1996; Fasanella and Fernández, 2009; Ruiz et al., 2018). In our own work, blackbirds demonstrated markedly different patterns of response toward brood parasitic vs. predatory threat playbacks depending on the risk posed by them across nest stages. During the nestling stage in the current study, blackbirds of both sexes responded equally and with low aggression toward calls signaling brood parasites (cowbird and seet calls) as to the control wood thrush songs, responding instead most aggressively toward nest-predatory blue jay calls. Contrastingly, when the same playbacks were presented during the incubation stage, blackbirds responded with equally strong aggression toward playbacks of cowbird chatters, seet calls, and blue jay calls (Lawson et al., 2020). Thus, blackbirds mediated in parallel their urgency to respond and aggression toward calls signaling brood parasitic danger, including referential seet calls of yellow warblers, depending on the level of the current threat of parasitism to their nest(s). Blackbirds mediated aggression depending on date in season and the age of their nestlings as well, showing increased aggression as the breeding season progressed and with increased age of their nestlings. Costs of renesting increase as the season progresses and reproductive value of offspring due to increased chance of survival increases (Montgomerie and Weatherhead, 1988; Gill and Sealy, 1996; Fasanella and Fernández, 2009; Ruiz et al., 2018) parents are thus expected to increase aggression toward threats toward their offspring with the greater age of their young. Similar patterns have been observed in other presentation studies with alarm-calling species (Regelmann and Curio, 1983; Montgomerie and Weatherhead, 1988; Campobello and Sealy, 2010; Lawson et al., 2021a,b).

A relevant distinction between male and female blackbirds is that in this polygynous mating system (Searcy and Yasukawa, 1995), males may have multiple nests at once on their territory, some even at different stages of development, while females only actively care for one nest. Males do not incubate eggs and provide limited paternal provisions for nestlings (e.g., Li and Hauber, 2021), but rather perform a sentinel role of protecting the territory from threats (Yasukawa and Searcy, 2020). However, our findings suggest that males actively monitor the progress of all nests within their harem, as those with additional nests with eggs still vulnerable to brood parasitism responded more strongly to cowbird chatters and seet calls compared to males with nests solely at the nestling stage. This statistical effect was not seen in blackbirds with eggs responding to blue jay calls, indicating a specifically anti-parasitic nest defense. This corresponds with previous work that showed male blackbirds alter provisioning rates based on age of nestlings, even after nests were swapped, supporting that male blackbirds actively monitor all the nests within their harem (Yasukawa et al., 1993). Blackbird males are also known to pay attention to social and vocal cues of females on their territories (Yasukawa, 1989), and this may also include cues from females regarding brood parasitism risk, leading to adjustments in the male's responses to cowbirds and cowbird-signaling calls.

Personal information vs. social information on risk likely affects host responses, as each has different reliability and cost. For example, we found that males approached cowbird and blue jay calls more closely than seet calls. The pattern of male closest approach was similar to that found by blackbirds during the incubation stage (Lawson et al., 2020), as well as to male yellow warblers during the incubation stage as well (Lawson et al., 2021b). Though both cowbird chatters and seet calls indicate brood parasitism risk, cowbird chatters directly indicate cowbird presence, whereas seet calls indirectly do so. Yellow warblers and blackbirds alike appear to more closely approach playbacks that directly signal threats (cowbird and blue jay calls) compared to social information of risk (seet calls) as acoustic presentations alone provide no visual target for responding subjects to direct physical aggression toward. Campobello and Sealy (2011a) found similar patterns in responses of yellow warblers presented with personal (cowbird model on nest, nest parasitized) or social information (conspecifics mobbing cowbird) on brood parasitism risk, where warblers responded more strongly to individually learned information. Conversely, reed warblers (*Acrocephalus scirpaceus*) in a similar experiment showed preference for social information on brood parasitism risk by common cuckoos compared to personal information (Campobello and Sealy, 2011b). Therefore, cost of acquiring personal information may also affect reliance on and responses to it, as common cuckoo nestlings eject all host eggs/nestlings from the nest unlike cowbirds (Campobello and Sealy, 2011b). Treatments did not influence female closest approach because females spent most of the time alarm calling near or on the nest during playbacks, resulting in an average of ∼5 m approaches across treatments, as this was the distance the speaker was placed from the nest.

Our combined set of blackbird playback studies brings to light new questions in the blackbird-warbler eavesdropping system that should be addressed in future studies. Yellow warblers nesting in close proximity to blackbirds experience lower rates of parasitism (Clark and Robertson, 1979), due to the blackbirds' aggressive frontline defenses toward cowbirds near their territories. Do blackbirds that nest near yellow warblers themselves experience a decrease in brood parasitism rate as well? Blackbirds that nest closer to yellow warblers show increased alarm calling responses to chatters and seet calls (Lawson et al., 2020), suggesting a "neighborhood watch effect" where blackbirds that have access to the yellow warblers' referential system are more primed to respond to their cowbird-signaling calls. Thus, the relationship between yellow warblers and blackbirds appears mutualistic, yet it is unknown whether blackbirds experience a similar decrease in parasitism of their nests when in proximity to yellow warblers. Our study also encourages future research into how blackbird males and many other host species mechanistically make the switch in behavior toward cowbirds as their nests transition from eggs to chicks. Yellow warblers of both sexes also demonstrate a shift in response toward cowbirds from incubation to nestling stage, but warblers of both sexes also interact with and care for the young, unlike most blackbird males at most nests (Li and Hauber, 2021). The mechanism underlying these shifts in behavior is unknown for either species, although endocrine factors, particularly testosterone and prolactin, play a strong role in parental (including paternal) care and different nest-attentive behaviors across the breeding stages in birds (Wingfield et al., 1990; Schoech et al., 1998; Van Roo et al., 2003; Ketterson et al., 2005; Møller et al., 2005; O'Neal et al., 2008).

Our set of playbacks conducted across nesting stages has led to firm support for heterospecific eavesdropping on a referential call signaling the presence of obligate brood parasites (also see Yu et al., 2019). Blackbirds appear to perceive the seet call as a warning specifically for brood parasitic danger, priming them for defensive responses to actual cowbirds. Moreover, blackbirds respond to warbler seets and cowbird chatters based on current risk of brood parasitism to their nests. Future research is needed to measure parasitism rates and fitness benefits of blackbirds nesting near yellow warblers; our study suggests that red-winged blackbirds may have a communicative and possibly mutualistic relationship with the warblers, whereby warblers provide the early warning system for cowbirds, and blackbirds keep cowbirds away from nearby nests.

Heterospecific eavesdropping on alarm calls signaling threats to fitness are seen across diverse taxa, including networks of co-existing species (e.g., tropical mixed-species bird flocks: Martínez et al., 2021). Eavesdropping in multi-species networks could improve threat detection in many biologically meaningful contexts (see Magrath et al., 2015 for review), including foraging (e.g., Batcheller, 2017), habitat selection (e.g., Mönkkönen and Forsman, 2002), and offspring defense (this study). It still remains to be seen, however, whether the symmetrical (whereby each interacting species recognizes the other's referential alarm call; Walton and Kershenbaum, 2019) or asymmetrical (whereby only one actor recognizes the other's call; this study) systems are more likely to evolve and be maintained by mutualistic selective forces. Both theoretical modeling and more empirical and meta-analytic work may be able to resolve these broader scale questions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Illinois Institutional Animal Care and Use Committee (IACUC) protocol #18040.

AUTHOR CONTRIBUTIONS

SL and MH conceived the study and drafted the manuscript. SL, SG, and MH participated in the design of the study and obtained funding. SL and JE carried out the field work and playbacks. SL conducted the data analysis. All authors critically edited and revised the manuscript, gave final approval for publication, and agreed to be held accountable for the work performed therein.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.706170/full#supplementary-material

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Analysis of Egg Variation and Foreign Egg Rejection in Rüppell's Weaver (*Ploceus galbula*)

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Egg appearance is notable for its variation and as a source of recognition cues in bird species that are subject to egg-mimicking brood parasitism. Here I analyze the egg appearance of an East African weaverbird species that has variable eggs and is a host of brood parasitism by an egg-mimicking cuckoo, in order to (1) compare population variation to variation within a clutch as a measure of the distinctiveness of eggs; (2) assess modularity versus correlation among egg appearance traits as an indication of the complexity of egg signatures; and (3) address whether the eggs are discretely polymorphic or continuously variable in appearance. I also compare three methods of assessing egg coloration: reduction of spectral data to orthogonal components, targeted spectral shape variables, and avian visual modeling. Then I report the results of egg replacement experiments that assess the relationship between egg rejection behavior and the difference in appearance between own and foreign eggs. Rüppell's weaver (*Ploceus galbula*) eggs are variable in appearance between individuals and consistent within a clutch, but vary widely in the distinctiveness of particular traits. Most aspects of color and spotting are decoupled from each other, including coloration likely to derive from different pigments. Egg ground color is bimodal, with a broad continuous class of off-white/UV eggs and another broad class of blue-green eggs. Variation in all other traits is unimodal and usually normal in distribution. Females reject foreign eggs on the basis of the difference in brightness of the ground color and spotting of foreign eggs relative to their own, and the difference in degree to which spots are aggregated at the broad end of the egg. This aggregation is among the most distinctive features of their eggs, but the brightness of the ground color and spotting brightness are not; the birds' use of brightness rather than the more distinctive chromatic variation to recognize eggs might reflect the salience of achromatic contrast in a dim enclosed nest.

Keywords: brood parasitism, self-recognition, trait variation, polymorphism, egg coloration, Ploceidae

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INTRODUCTION

Dramatic and stable trait variation between individuals of the same species, sex, and age living in the same environment has always been compelling to evolutionary biologists, as it requires a more complex analysis than a singular prediction from optimality (Poulton, 1884; Dobzhansky, 1951; Ford, 1965). Bird egg appearance is one of the traits that has consistently been considered in this context, with hypotheses for its variation including drift in the absence of function (Moreau, 1960), parental recognition of eggs when laying sites are crowded (Tschanz, 1959), a toss-up in the face of different selective agents (Kim et al., 1995), and previously unrecognized environmental variation (Blanco and Bertellotti, 2002). By far, however, the most frequently considered hypotheses for intraspecific bird egg variation arise in the context of brood parasitism, and these are also particularly intriguing scenarios because other organisms are evolving to nullify the function. If the victims of brood parasitism evolve the ability to recognize their own eggs and reject foreign ones, the brood parasites can engage in deceptive or aggressive mimicry of host eggs as a counteradaptation, and brood parasite egg polymorphism can result as a byproduct of differing host specialization between lineages within the same brood parasite species (Baker, 1913; Friedmann, 1928). Variation in the eggs of brood parasites might also be adaptive in itself, if they compete to parasitize the same nests and reject each others' eggs (Brooker and Brooker, 1990; Spottiswoode, 2013). As for the hosts, one anti-counterfeiting strategy is to evolve distinctive eggs to facilitate egg recognition (Bates, 1911) when the brood parasite exhibits egg mimicry. Egg distinctiveness can be achieved by decreasing intraclutch variation and increasing variation between individuals in egg appearance (Davies and Brooke, 1989). To the extent that this phenomenon is occurring, host egg variation has evolved to facilitate self-recognition, since egg appearance traits are an extended phenotype of the mother; although in practicality it can also be considered offspring recognition since the parental trait is physically attached to the offspring and functions in distinguishing them from others. This particular mechanism of egg appearance variation within a species—increasing distinctiveness as a counteradaptation to brood parasitism—has been invoked with evidence as an explanation for increased egg variation in a number of species, such as the great reed warbler (*Acrocephalus arundinaceus*) (Moskát et al., 2002), blackcap (*Sylvia atricapilla*) (Honza et al., 2004), village weaver (*Ploceus cucullatus*) (Lahti, 2005), white-plumed honeyeater (Landstrom et al., 2010), tawny-flanked prinia (*Prinia subflava*) (Spottiswoode and Stevens, 2012), red-billed leiothrix (*Leiothrix lutea*) (Yang et al., 2014), and *Paradoxornis* parrotbills (Yang et al., 2015). Here I introduce a new brood parasite - host system, where the host is known to have variable eggs between individuals.

Rüppell's weaver (*Ploceus galbula*) is a small passerine endemic to the Horn of Africa (Ash and Atkins, 2009) and the Arabian peninsula (Shirihai and Svensson, 2018; **Figure 1**). It is a member of the clade of true weavers (Ploceinae), named for the proficient nestbuilding of the males. The Rüppell's weaver male is yellow with a striped back, dark rusty mask, and red eyes. The female is a drab green typical of the genus, but lays eggs of any of a variety of pastel colors maculated with speckles and blotches ranging from tawny to nearly black. The species is common within its restricted range, but has received little research attention. Its literature so far consists, besides breeding records and species accounts, of brief treatments of its behavior (O'Grady and O'Grady, 1990; Al-Safadi, 1996; Lahti, 2013), the effect of light on eggshell color (Navarro and Lahti, 2014), and its range expansion in Arabia (Alshamlih et al., 2020). Rüppell's weaver can be considered an associative nester (Lahti, 2013), in the sense that where one is nesting usually one or more others will also be in close proximity, whether conspecifics, or congeners such as the lesser masked weaver (*P. intermedius*). A male builds the enclosed basket-like pendant nest, and the female lines it with greenery and soft material and then lays her eggs.

Like many other weavers, Rüppell's has been reported to be subject to brood parasitism by the diederik cuckoo (*Chrysococcyx caprius*) (Jennings, 2010; Erritzøe et al., 2012; Eriksen and Porter, 2017). This cuckoo is an obligate brood parasite, laying eggs in the nests primarily of weavers (Ploceidae) (Payne, 2005). A young diederik cuckoo upon hatching, usually before the host chicks do, ousts the weaver eggs or nestlings from the nest, and begs for food from its foster parent. Thus, successful parasitism by the cuckoo results in the total loss of a reproductive attempt by the weaver. Some weavers, such as the village weaver (*Ploceus cucullatus*), are known to use various egg appearance traits in connection with refined egg recognition as an anti-counterfeiting strategy, detecting foreign eggs, and removing them from their nests (Victoria, 1972; Lahti, 2006). The egg appearance traits that have been shown to be effective cues for discrimination in village weavers are egg ground color and spotting pattern (Lahti and Lahti, 2002). The refinement of egg appearance traits and egg recognition is especially necessary because the diederik cuckoo has evolved mimicry of weaver eggs (Payne, 1967), and diverged into apparent egg races, or gentes, that are specialized on different host egg morphs (Jensen and Vernon, 1970). In some weavers, individuals parasitize each other (Jackson, 1992a), which is not as detrimental as cuckoo parasitism as it merely adds an individual to the clutch; nevertheless it might still impose selection for egg recognition (Samaš et al., 2014; Lyon et al., 2015). At least in the village weaver, however, defensive traits function especially in response to interspecific (cuckoo) brood parasitism, as demonstrated by their decay in the absence of the cuckoo (Lahti, 2005, 2006), and the subsequent evolution of egg appearance in accordance with other agents of selection in populations freed from cuckoo parasitism (Lahti, 2008). Rüppell's weaver is not closely related to the other two weavers that have been studied intensively for egg variation and egg rejection (*P. cucullatus* and *P. taeniopterus*), but is in another of three main clades of African *Ploceus/Malimbus* weavers, each with nearly two dozen species (De Silva et al., 2019; Habig, Childers, and Lahti in prep). Close relatives of Rüppell's weaver vary widely in whether they have (or are known to have) variable eggs (Freeman, 1988; Urban et al., 2004).

A distinctive feature of egg appearance adaptation to cuckoo brood parasitism is that no particular trait value is expected to be consistently adaptive, because the parasites' eggs themselves evolve continuously in counteradaptation to host egg evolution (Davies and Brooke, 1988; Spottiswoode and Stevens, 2012). At a given time and place, cuckoo eggs can be expected to be most successful when they are similar (in whatever way leads to acceptance by the host) to the largest possible proportion of current local host eggs. This situation results in mutual frequency-dependent selection between host and parasite. The host's most adaptive strategy in terms of egg appearance features

FIGURE 1 | Rüppell's weaver (*Ploceus galbula*). *Top left*: a male at the nest; *top right*: a female inside the nest. *Bottom*: sample eggs, each laid by a different female.

is not a particular color or spotting pattern *per se*, but rather individual distinctiveness, consistency, and complexity, traits typically effective in defeating deceptive mimicry (Davies, 2011; Feeney et al., 2012; Caves et al., 2015). Any given weaver's eggs will be more distinguishable from foreign eggs (whether laid by another weaver or by a cuckoo that has evolved egg mimicry) if they are different in appearance from other prospective host eggs, if they are consistent in appearance with each other, and if egg appearance represents a combination of several components that are limited in their correlation with each other and so can develop (and evolve) independently. Each of these three characteristics would decrease the likelihood that a parasitic egg randomly laid in a weaver nest will resemble the host's eggs, and would thus increase the effectiveness of egg discrimination by the host. This study assesses egg appearance variation and egg rejection in the Rüppell's weaver, in order to test whether these predictions are met: whether their eggs are variable between individuals and consistent within a clutch, have multiple modular egg appearance features, and whether the birds attend especially to their eggs' most distinctive features when detecting a foreign egg.

I also address three auxiliary questions. First, bird egg appearance variation is often described as polymorphic when in fact it could simply be variable; avoiding presumption in this area and distinguishing discrete from continuous variation is a common challenge in trait evolution studies (Rankin et al., 2016; Davison et al., 2019). For weavers in particular, the hypothesis of distinct egg types with rare or absent intermediates is rooted in claims over a century old (Bates, 1911; Swynnerton, 1916), but researchers studying village weaver egg color variation have differed in their adherence to this view (Collias, 1984, 1993;

Din, 1992). Pairwise comparisons of eggshell spectra suggest that variation is continuous, except perhaps for white eggs (Lahti and Lahti, 2002; Lahti, 2005), although distributions of particular egg appearance traits were not specifically analyzed. Here I characterize the distributions of egg appearance features in Rüppell's weaver in order to determine their shape and modality. Second, two egg pigment classes are known to result in egg coloration, biliverdin and protoporphyrin, corresponding roughly to blue-green and reddish-brown coloration (Poole, 1965; Mikšík et al., 1996). Is variation in the production and deposition of these pigments decoupled in weavers, such that they could develop and evolve independently? If so, either no correlation will be found in blue-green and reddish-brown egg coloration between individuals, or a mild negative correlation if the presence of one partly obscures the other. Alternatively, a strong positive correlation between them would suggest a linked production or deposition mechanism; and a strong negative correlation would indicate a trade-off or zero-sum allocation of pigments. Third, three strategies of representing color are commonly used, all based on spectral reflectance, in assessing variation and egg recognition: reduction of all spectral data into objective orthogonal components, spectral shape variables that target spectral peaks or wavelength regions of interest, and avian visual modeling that transforms spectral data according to retinal cone sensitivity. Here I perform all three of these methods and compare the results, especially looking for pros and cons of the approaches. For instance, since birds are looking at their own eggs during egg recognition, does approximating the avian visual system improve the explanatory power of a model of egg rejection?

MATERIALS AND METHODS

Study Site and Species

Breeding Rüppell's weavers were studied at Awash National Park, Ethiopia (008°53'150"N, 040°02'147"E) in July–August 2010. Males of this species build the nests, to which they attract females, who line the nests, lay their eggs, and solely incubate them (Al-Safadi, 1996). In this study site, Rüppell's weaver is common, and flocks with other weaver species such as the chestnut weaver (*P. rubiginosus*), village weaver (*P. cucullatus abyssinicus*), and lesser masked weaver (*P. intermedius*). Rüppell's weavers found at this site resided in single male territories (containing 1–3 nests) or loose aggregations (e.g., 30 nests spread across an area of 25 × 25 m) (Lahti, 2013). Their nests are kidney-shaped and pendulous with an opening on the bottom, hung generally from *Acacia* trees between 2 and 5 m above the ground. Diederik cuckoos (*Chrysococcyx caprius*) were seen, and heard singing, throughout this study and in the vicinity of Rüppell's weaver nests, although no known instance of brood parasitism occurred in study nests over the short period (2–4 days) each was monitored. However, in one case an egg was found in a nest that was very different in appearance from the others, bringing the total number of eggs in the nest to 5, which was not otherwise observed in the sample. Clutch size was determined by repeated nest visits. Clutches were excluded from this assessment if nest or eggs disappeared (presumably to predation), if the nests were abandoned, or if nest visits did not continue at least 1 day following clutch completion.

Egg Size and Shape

Egg length (L) and widest width (W) were measured with digital calipers (±0.05 mm); their ratio is considered here as a measure of egg shape. Mass of freshly collected eggs (laid in the previous 1–4 days) was measured with a Pesola spring scale (±0.1 g). These measurements permitted a test of the standard equation for initial mass based on length and width developed by Hoyt (1979).

Egg Ground Color

Egg color was measured with an Ocean Optics JAZ modular UV-VIS spectrophotometer and a pulsed xenon light source. I held a 400-μm reflection probe at a 90° angle 5 mm from the sample. Measurements were standardized with a diffuse tile made of polytetrafluoroethylene that reflects >98% of light over all sampled wavelengths. I performed all measurements under an opaque cloth to avoid an effect of ambient light. Three measurements were taken per egg for ground color. Ground color was measured approximately halfway between the egg poles, in a spot as clear of maculation as possible. In each case the mean of the three measurements was used for analysis.

I assessed the standard error of measurement (SEM) for the spectrophotometric data, on which all ground color variables are based. Both human and instrumental precision in spectral measurements varies, and within-clutch variation can be very small in birds subject to egg-mimicking brood parasitism (Lahti, 2005), possibly approaching the margin of error. The SEM for spectrophotometric data includes imprecision of the measurer and of the spectrophotometric apparatus. SEM also includes any within-egg variation in color that might have been captured by the measurements. In this study, any measures of color variation and any statistically significant egg color morphs are considered potentially biologically relevant only at differences that are greater than the SEM. In this dataset, the global mean SEM for spectral measurements, based on three measurements per egg, was 2.58 (±1.34 across eggs). I also tested whether the relative SEM (mean SEM/mean% reflectance) varies such that it is consistently higher in some spectral regions than in others. The mean relative SEM across wavelengths was 0.048, which indicates that absolute SEM closely tracked% reflectance over the range of 320–700. Therefore the global mean SEM can be used: all measurements of ground color% reflectance should be considered ±2.58 due to random error.

I interpreted reflectance data for color analysis in three ways.

Reduction of Spectra Into Objective Uncorrelated Axes of Variation

I reduced spectral reflectance values to a few variables with principal components analysis (PCA). The axes in the default output of a PCA are determined so as to maximize the variance explained by the first factor, and then so on for subsequent factors. This often results in brightness (reflectance over all wavelengths) being a highly explanatory first component. I used rotations of the principal component axes, which are equivalent ways to parse the data into factors, to find additional components that were chromatic and had biologically interpretable loading patterns, especially given the range of spectral shapes observed in the population. The original PC1 was preserved for analysis, but the second and third factors were not easily interpretable. Three additional interpretable and explanatory variables were found in another rotation, the varimax. This rotation maximizes the variance of each factor's squared loadings. For spectral data, this translates into minimizing the overlap between factors in loading on wavelengths, so that a given region of the spectrum will inform only one factor to the greatest extent possible.

Measurements of Spectral Shape

I derived univariate measures from the spectral data in an attempt to quantify variation in the observed spectral shapes, using the software Avicol (Gomez, 2006). The spectral features that were distinguished based on visual inspection of the raw spectral data were a peak in the ultraviolet (UV), a peak in the blue-green, and a reddish-brown slope (see "Results" section and **Figure 2**, top panel). The following variables were derived to describe them, using the shapes (especially inflection points) characteristic of spectra found for this species in the present study (numbers are wavelengths in nm, R = % reflectance):

$$\text{Proportion UV } R = \int_{300}^{380} R(\lambda)\,\mathrm{d}\lambda \Big/ \int_{300}^{700} R(\lambda)\,\mathrm{d}\lambda.$$

The cutoff of 380 nm was chosen because it was approximately the wavelength of minimum reflectance immediately to the right of UV peaks in sample spectra of this species. Generally called "UV chroma," this term is misleading because the variable is strongly affected by reflectance in other areas of the spectrum besides the UV, including variation that has nothing to do with chromaticity. For instance, increasing reflectance at 700 nm will decrease the value of this variable, and a white egg will have a higher value than a brown egg even if the UV reflectance in both cases is identical and flat. Thus it is better considered simply as proportion UV, in the sense of UV reflectance as a proportion of total reflectance. A second variable focusing on the UV peak is:

$$\text{UV max} - \text{min} = \text{Abs}\left((R_{\text{uvmax}} - R_{\text{uvmin}}) \big/ R_{\text{uvavg}}\right),$$

where R_{uvmax} and R_{uvmin} are the maximum and minimum reflectance between 300 and 380 nm and $R_{\text{uvavg}} = \int_{300}^{380} R(\lambda)\,\mathrm{d}\lambda \Big/ 81$. This measure focuses on the UV peak without influence by reflectance in other areas of the spectrum. Two further variables focused on blue-green proportional reflectance and peak height:

$$\text{Proportion blue} - \text{green } R = \int_{400}^{650} R(\lambda)\,\mathrm{d}\lambda \Big/ \int_{300}^{700} R(\lambda)\,\mathrm{d}\lambda.$$

The cutoffs of 400 and 650 nm were chosen because they approximate the wavelengths of minimal reflectance on either side of the blue-green peak. As a measure of blue-green chroma this has the same issues as proportion UV R above.

$$\text{Blue} - \text{green chroma peak height } = (R_{400} + R_{650}) \big/ 2 - R_{500},$$

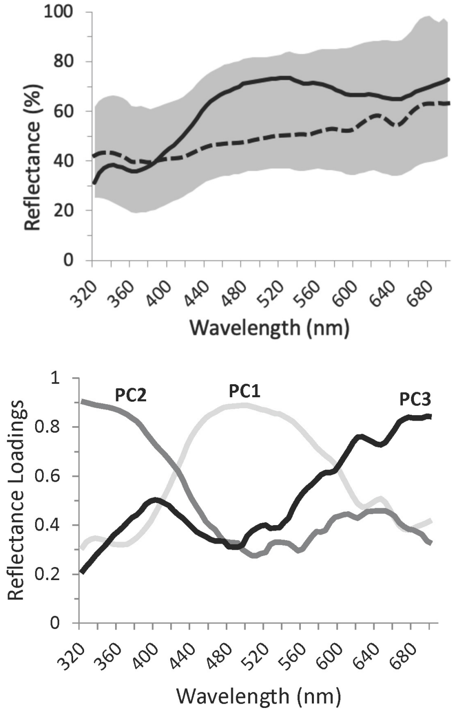

FIGURE 2 | Variation in Rüppell's weaver eggshell ground color (though influenced to some extent by maculation), in terms of spectral reflectance curves. Top: Range of reflectance in the sample, and two representative egg spectra. The shaded area defines the range of reflectance, across the bird-visible spectrum, of the ground color of 63 eggs in 26 clutches, three or fewer days after laying. The top and bottom edges of this shaded area represent extremes of brightness. To the extent that eggs exhibited chromatic variation in ground color, they were of a bluish hue, indicated by a reflectance hump between 400 and 650 nm, peaking around 500 nm. Extremes of blue chroma in the sample are exemplified by the solid curve (high chroma) and the dashed curve (low chroma). The tendency of the shaded area and the lines to increase in reflectance above about 600 indicates a small degree of reddish-brown in some of the eggs. In many cases this signal resulted from an inability to avoid fine spots when taking spectral measurements of ground color. Bottom: Factor loadings from a Principal Components Analysis of reflectance spectra of all study eggs, indicating three uncorrelated axes of variation: broadly speaking, in blue–green (PC1, explaining 30.8% of the variance), UV (PC2, explaining 23.0% of the variance), and reddish-brown (PC3, explaining 22.6% of the variance). The loading for a particular wavelength indicates the contribution of that wavelength to the respective PC. These curves represent a varimax rotation of the principal components, which distributes variation across the PCs and, in this case, highlights chromatic variation.

following Lahti (2008) where this was used as a measure of blue-green chroma in the eggs of another weaver species. This differs from proportion blue-green R in that it is robust to any variation in the blue-green curve except for peak height, and it is unaffected by variation in other areas of the spectrum.

$$\text{Proportion red} - \text{brown } R = \int_{650}^{700} R(\lambda)\,\mathrm{d}\lambda \Big/ \int_{300}^{700} R(\lambda)\,\mathrm{d}\lambda.$$

The cutoff of 650 nm was used because only reflectance above this wavelength can be unambiguously assigned to the red-brown slope rather than the blue-green peak. No more specific red-brown chroma variable was developed for ground color because this would be artifactual: most red-brown chroma was due to an inability to avoid fine dense maculation when gathering a ground color spectral reading. Finally, average reflectance was used to represent brightness:

$$\text{Brightness} = \left(\sum_{300}^{700} R\right) \Big/ 401.$$

Estimation of Photon Catches by the Bird Retina

I used the program TetraColorSpace (Stoddard and Prum, 2008) to convert spectral reflectance to estimated relative photon catches by each of the four cones of a typical UV-sensitive bird's eye (Goldsmith, 1990; Endler and Mielke, 2005). These cones correspond to maximum sensitivities at 372, 456, 544, and 609 nm, and are represented in TetraColorSpace output by wavelength-indicating variables u (UV), s (short), m (medium), and l (long), respectively (Rüppell's weaver peak sensitivities might vary from these values, but data are unavailable). These values are represented as proportions, and thus sum to 1. Any color as perceived by a bird can in this way be located in tetrahedral color space, where the value for each variable is maximized (has a value of 1, and all others 0) at one vertex of the tetrahedron, and where all four values are equal at 0.25 at the achromatic origin or centroid of the tetrahedron. TetraColorSpace then converts photon catches into spherical coordinates. From the achromatic center of this tetrahedron, the color vector's direction, or hue, is defined by two angles θ (the azimuth or horizontal angle away from a plane cutting perpendicular midway between the m and l vertices, ranging from $-\pi$ to $+\pi$) and φ (the elevation or vertical angle from the horizontal plane if the tetrahedron is arranged with the u vertex upward, ranging from $-\pi/2$ to $+\pi/2$). Since the outside surface of the tetrahedron represents the maximum distance from the achromatic center for a particular direction, the vector's magnitude r is considered saturation or chromaticity. Because different colors have different maxima, I used the standardized "achieved chroma" (r_A) that is scaled to its maximum for a given vector direction (Stoddard and Prum, 2008). I then applied these variables to an assessment of color variation using span analysis, volume analysis, and hue disparity analysis, all available in TetraColorSpace. Span analysis computes the average color contrast in terms of Euclidean distance between two points in the population. Volume analysis defines the three-dimensional size of the space occupied by the population by finding the minimum convex polygon containing all data points. Hue disparity analysis extracts hue (color vector angle) differences from differences in saturation (chromaticity, color vector magnitude), and represents contrast as a proportion, where 0 is the identical hue and 1 is its complementary, and thus maximally different, hue. All three of these measures ignore brilliance or brightness, however, so this feature was analyzed separately. Brilliance analysis in TetraColorSpace is equivalent to brightness analyses in Avicol, values being identical to three significant digits; only Avicol brightness data is presented here.

Egg Maculation

Four intuitive and quantitative measurements have been used previously to measure the variation in spotting of weaver eggs (Lahti and Lahti, 2002; Lahti, 2005): density, size, color, and aggregation toward the broad pole of the egg. However, in those studies the quantification of just a few eggs provided an index, and the bulk of the sample was assessed qualitatively according to the index. Here I use procedures in Adobe® Photoshop to further specify the method and fully quantify all spotting pattern characterizations. Eggs were photographed with a Nikon D200 digital SLR camera from three perspectives: lateral, and each pole of the egg. An 18% gray card and a ruler were placed in the background, and were used to standardize the image color (via "Curves") and size (via "Set Measurement Scale") after import. Spots were isolated in the image with the Magic Wand tool with anti-aliasing, by selecting contiguous ground (non-spot) area, after which spot measurements can be made automatically. In this species, each egg has two and sometimes three layers of spots separated by layers of ground color; these spots were considered separately, but all values are combined in analyses here. For spot density, size, and color, the lateral perspective of the egg was used. Density was calculated as a proportion: the ratio of the area occupied by spots to the total area of the egg in the image. Spot size was calculated as the ratio of the total area of spots to the number of spots counted, which yields the average spot area. Spot color was assessed in terms of brightness only, after spectrophotometry showed that this was the main axis of variation in spotting. Spectrophotometry was not ideal for measuring spots, however, as the area over which the reflection probe takes data is broader than most of the spots in this species. Instead, Photoshop was used to calculate the 8-bit gray value (range: 0–255, black to white) of the spots in the standardized photographs. The mean of this value was used for spot brightness. Spot aggregation toward the broad pole of the egg was assessed using the photographs of the two ends of the egg. The area of spots was calculated for each image, and the degree of aggregation was considered to be 1 − (area of spots at the narrow end/area of spots at the broad end). This yields a range between 0 and 1, where 0 is an even distribution of spots throughout the egg, and 1 is the aggregation of all spots at the broad end of the egg.

Polymorphism, Distribution Shapes, and Trait Interactions

Polymorphism in a strong sense would indicate discrete types with no intermediates. A weaker sense of polymorphism might be satisfied merely by a polymodal trait distribution. I tested for polymorphism in this weaker sense, of all egg color, maculation, size, and shape variables described above, using Hartigan's dip test for unimodality (Hartigan and Hartigan, 1985), along with visual inspection of histograms. Of particular interest was whether eggs that appear to be different colors (e.g., whitish vs. bluish) or have different color spots (light vs. dark) represent egg type morphs or continuous variation.

Distribution shape was examined by skewness and kurtosis; a significant departure from normality was concluded when the absolute value of the ratio of the statistic to its standard error (skewness/SES or kurtosis/SEK) was greater than 2. Significant skewness indicates asymmetry of the distribution around the mean, either to the right (positive values) or left (negative values). Significant kurtosis indicates either that the distribution has longer tails than normal (positive values) or is flatter than normal (negative values).

Trait interactions were investigated by conducting Pearson correlations among the variables, Bonferroni corrected for multiple comparisons. Certain comparisons were of particular interest: whether egg shape is conserved across egg sizes (whether length and width are correlated); whether variation in egg size is achieved primarily in one dimension or the other (whether length/width ratio is correlated with egg mass); whether color or maculation varied with egg size; whether different ways of measuring qualitatively similar color features were highly correlated; and whether any egg color features, such as different spectral peaks, are decoupled from each other.

Egg Appearance Distinctiveness

Based on the range and nature of population variation in egg appearance features, I calculated how much of this variation for each egg appearance trait was between individual (BI) vs. within-clutch (WC), using ANOVA to derive sums of squares, where WC = SS (error) and BI = SS (group, i.e., clutch). This permitted the production of a distinctiveness score $D = 1 - (\mathrm{WC}/\mathrm{BI})$ for each egg appearance variable. A score approaching 1 for a given feature indicates very little within-clutch variation compared to great between-individual variation, rendering the eggs within a particular clutch highly distinctive in the population with respect to that feature, and representing the ideal situation for egg discrimination. A score nearing 0 indicates equal within-clutch and between-individual variation, meaning that a random egg in a nest could be own or foreign with equal probability, rendering discrimination impossible on the basis of that feature. Ideally, both BI and WC would be calculated on the basis of the means of complete clutches; I performed this analysis first. This strategy is likely to be relatively accurate for WC, but might underestimate BI and thus distinctiveness in some cases because it restricts the number of clutches that are sampled. Therefore I also calculated D in a second way that is more accurate at assessing BI, via pairwise comparisons. Disparity measurements within a clutch were taken, allowing the inclusion of potentially unfinished clutches: two eggs for each WC comparison were chosen at random from clutches with more than two eggs, and one-egg clutches were excluded. This increased the sample of clutches, increasing the estimate of BI; however, WC was systematically underestimated, perhaps leading to an inflated estimate of egg distinctiveness in some cases. These two ways of estimating within-clutch and between-individual variation translate into D scores that are presented here as endpoints of a range. WC values cannot be considered to account fully for within-individual variation, however, as only one clutch was measured per female.

Egg Rejection Experiments

A single conspecific egg (hereafter, "foreign egg") was introduced into each experimental Rüppell's weaver nests ($N = 24$) to mimic parasitism by conspecifics or the diederik cuckoo. Two cases ultimately had to be discarded for missing egg appearance data. In accordance with the typical behavior of the diederick cuckoo (Friedmann, 1968), a host egg was removed from the nest at the same time, such that there was no change in the number of eggs in the nest as a result of the experimental procedure. The experimental protocol and relevant portions of the data analysis followed Lahti and Lahti (2002) and Lahti (2006) on four populations of another weaver species (the village weaver *Ploceus cucullatus*), so that results would be comparable between species. I wrote a number for identification on the side of each egg. In six control nests, the eggs were handled and numbered and replaced in the same nest. In five of these cases the female continued to incubate the eggs and did not reject any. In one nest both eggs were gone (the nest was empty) on the day after the manipulation; predation and the weaver's rejection of both of her own eggs cannot be distinguished in this case, although in no other case did a weaver reject her own eggs but no foreign egg. I checked each nest 24–28 h ($n = 13$) or 48–52 h ($n = 9$) after experimental parasitism and noted whether any eggs were damaged or missing, in which case they were considered rejected. A previous study on village weavers showed that all rejections happened within 24 h (Lahti and Lahti, 2002). All egg replacements were performed during or within 3 days after the laying period of the female. Experimental eggs were chosen in real time in order to achieve approximately a 50% rejection rate, as binary logistic regression is only as powerful as the less common of the two response states.

Egg appearance differences between host and foreign eggs were calculated for each variable separately, as the absolute value of the difference between the foreign egg and the host egg that was nearest to the foreign egg in that variable, whether that host egg was already in the nest at the time of the egg replacement or was laid afterward. Thus, different host eggs were compared to the foreign egg for different variables if the host clutch varied in which egg was minimally different from the foreign egg. The reason for comparing the closest egg in the clutch for each appearance variable rather than the mean of all host eggs, as is sometimes done, is to avoid confounding host-foreign clutch differences with host within-clutch variation (Lahti, 2006). For any particular mean value of a host egg appearance variable, a foreign egg is necessarily less distinctive in a host clutch with higher variation around that mean than with lower variation. Using the mean value would ignore this difference. Comparing a foreign egg to the nearest egg in the clutch for each variable is one way of circumventing this issue.

The calculated egg appearance differences included all the variables described above, including three ways of measuring color [PCA of spectra (three variables), reflectance peaks and proportions (five variables), and photon catches in TetraColorSpace (three variables)]; maculation (four variables); and size and shape (four variables). For spectral measures of color, differences were calculated between the reflectance values of the ground color of the two eggs between 320 and 700 nm. A (second) PCA was performed on these values. The spectral differences between host and foreign egg ground color were most interpretable without rotation of the principal components. In this analysis, three factors explained 73.2% of the variation, with no remaining component explaining more than 1.7%. PC1 loaded highly across 400–700 nm, and so represents differences in brightness excluding UV. PC2 loaded positively in UV (max at 355 nm) and negatively in blue (min at 490 nm); thus high and low PC2 values show differences in both blue and UV, but in opposite directions. PC3 loaded negatively on reddish-brown.

Factors potentially influencing egg rejection were analyzed by logistic regression (Hosmer and Lemeshow, 1989) with SYSTAT 10.0 software (SPSS Inc., Chicago IL), following Lahti (2006). The response variable was binary, namely whether or not the female rejected any eggs from her nest. In all cases of rejection, the female ejected at least the foreign egg, and sometimes damaged or ejected one of her own as well. In addition to all egg appearance difference variables, clutch size and whether the weaver laid a new egg after experimental parasitism were also included. First, variables were tested in univariate (single logistic regression) models, candidate variables being chosen on the basis of the log-likelihood of models at convergence; specifically, a likelihood-ratio (G) test was performed on each model, which results in a P-value of that model compared to a constant-only model. For each parameter in a significant model, a t-ratio compared the estimate to the standard error (SE) of the parameter. To correct for possible misspecification of models, covariance matrices were adjusted according to quasi-maximum likelihood (QML) to yield revised SE, t, and parameter-specific P-values (White, 1982). The most appropriate multivariate (multiple logistic regression) model was chosen by forward stepwise regression (with $P = 0.15$ as the threshold for variable inclusion) followed by comparisons (G tests) of the final model with all nested subsets. Significant models were assessed in two ways. First, McFadden's Rho-squared (ρ^2), sometimes called "pseudo-R^2", was used as an estimate of explained variation or goodness-of-fit; it is similar in interpretation to the R^2 of linear regression, but generally with lower values (Long, 1997). Second, sensitivity and specificity assessed the predictive capacity of the model. Sensitivity is the true rate of egg rejection when rejection is predicted by the model; and specificity is the true rate of egg acceptance when acceptance is predicted by the model. For continuous variables except for proportions, each significant model also yielded an odds ratio, the increase in the probability of egg rejection for one unit increase in the parameter. Also reported is the difference in each egg appearance variable in the best univariate and multivariate models to result in egg rejection 50% of the time. This is the LD50, the "median lethal dose" in toxicology; as rejection results in the death of the foreign egg, the concept is directly translatable as the median lethal difference (between the own and foreign eggs).

RESULTS

Clutch and Egg Measurements

Each Rüppell's Weaver clutch usually consisted of three eggs, less often two or four (mode 3, mean 2.74, SD 0.54, $N = 23$). Using Hoyt's (1979) equation for egg mass, this species' mass coefficient was calculated as $K_w = 0.499$, such that initial mass can be estimated with minimum error as $0.499 \times LW^2$, where L and W are egg length and width, respectively. The error of this estimate (its departure from known egg mass values) is $5.4 \pm 4.3\%$. Length, width, and mass were normally distributed. Egg shape was not conserved across egg sizes (length and width were not correlated). Variation in egg size was not achieved primarily in the length or width dimension (length/width ratio was not correlated with egg mass). Thus, an egg of any particular size was not significantly biased within the observed range of shapes, and an egg of any particular shape was not significantly biased within the range of sizes. Egg size and shape characteristics are listed in **Table 1**. Egg size and shape features were highly distinctive among clutches, the mean estimates for D scores exceeding 0.80 for length, width, mass, and shape. Egg shape (length/width ratio) was the most distinctive at $D = 0.90$. Neither egg ground color nor maculation correlated with egg size or shape.

Eggshell Ground Color

Representative Rüppell's weaver eggs are illustrated in **Figure 1** (bottom panel), and the range of reflectance of sample eggs' ground color over the bird-visible spectrum is illustrated in **Figure 2** (top panel). Such graphical representation, although showing variation in brightness (average reflectance) in the sample, obscures chromatic (spectral shape) variation. Examples of the two ends of the continuum of spectral shape in this sample are represented by the two curves in **Figure 2** (top panel).

Qualitative assessment of the shapes of the reflectance spectra yielded three main results. First, all eggs have a peak in the bird-visible portion of the ultraviolet (UV) region of the spectrum (320–400 nm), peaking toward the lower end of this range and reaching a minimum around 380 nm. Second, most eggs have another peak around 500 nm, broadly spanning from 400 to 650 nm, corresponding to blue-green (e.g., solid line in **Figure 2**, top panel). Eggs vary in terms of which of these two peaks is higher. Some eggs have no blue-green peak, resulting in a whitish apparent color, although they are in fact ultraviolet. Third, all eggs have maculation, and in some cases this influences the appearance of the ground color, resulting in a nearly linear (but irregular) inclining curve between 450 and 700 nm (e.g., dashed line in **Figure 2**, top panel).

Quantitative analysis of color was by reduction of spectral data into orthogonal components, identification of broad spectral shape features, and estimation of bird retinal photon catches. **Table 1** contains descriptive statistics, departures from normal distribution, and D scores.

Spectral Principal Components Analysis

As expected, PC1 in the unrotated principal components output loaded consistently and heavily (0.8–1) over the entire spectrum, and therefore can be considered brightness. This factor explained 67.5% of the variation in the data. Following varimax rotation, such that each wavelength contributes maximally to a single factor, the loading curves strikingly resembled the spectral shapes of particular color patterns observed in the raw data, facilitating biological interpretability and comparison with the other analyses: PC1 (which explained 30.8% of the variance) loaded in a broad peak centered at 500 nm, representing blue-green. PC2 (which explained 23.0% of the variance) rose in its loading as wavelength shortened, into a plateau in the UV. PC3

TABLE 1 | Statistics relating to appearance of Rüppell's weaver (*Ploceus galbula*) eggs and the structure of their variation[1].

		Population variation: mean ± SD (range)	**Distribution shape[2]**	***D*[3]**
Ground color: spectral principal components	Unrot PC1 (brightness)	n/a[4]		0.33–*0.49*
	Varimax PC1 (blue–green)	n/a[4]		0.07–*0.27*
	(Varimax PC2 (UV)	n/a[4]		*0.64*–0.71
	Varimax PC3 (red–brown)	n/a[4]		0.78–*0.80*
Ground color: retinal cone stimulation	UV (u)	0.19 ± 0.02 (0.15–0.23)		*0.66*–0.68
	Short (s)	0.26 ± 0.01 (0.24–0.29)	Weakly bimodal (P = 0.02); Flat (K = −2.1)	*0.79*–0.83
	Medium (m)	0.28 ± 0.01 (0.27–0.30)	Weakly bimodal (P = 0.02); Flat (K = −2.4)	*0.78*–0.79
	Long (l)	0.27 ± 0.02 (0.25–0.31)		0.77–*0.84*
Ground color: tetrahedral hue & chroma	θ	0.97 ± 0.95 (−0.16 to 2.51)	Weakly bimodal (P = 0.02); Flat (K = −2.5)	*0.76*–0.78
	ϕ	−1.26 ± 0.15 (−1.44 to −0.84)	Skewed right (S = 3.9)	0.70–*0.77*[5]
	Achieved *r*	0.26 ± 0.09 (0.06–0.40)		*0.65*–0.68
Ground color: spectral shape features	Prop. UV *R*	0.15 ± 0.02 (0.11–0.19)		*0.29*–0.54
	UV max–min	0.30 ± 0.10 (0.12–0.57)		−0.35 to *0.07*
	Prop. blue–green *R*	0.67 ± 0.02 (0.63–0.71)		*0.67*–0.75
	Blue–green peak height	8.2 ± 6.6 (−3.8 to 19.6)	Bimodal (P = 0.002)	*0.86*–0.87
	Prop. red–brown *R*	0.15 ± 0.01 (0.13–0.18)		*0.80*–0.85
	Brightness	54 ± 10 (32–77)		0.36–*0.52*
Maculation (spotting)	Density	0.19 ± 0.12 (0.05–0.43)	Flat (K = −2.1)	0.96–*0.98*
	Brightness	130 ± 17 (88–168)		0.72–*0.80*
	Size	0.18 ± 0.10 (0.07–0.79)	Skewed right (S = 11.4); Long tail (K = 29)	0.32–*0.40*[5]
	Aggregation	0.67 ± 0.21 (0.14–0.94)	Skewed right (S = 2.7)	0.81–*0.96*
Egg size and shape	Length	20.6 ± 1.1 (17.7–23.0)		0.87–*0.88*
	Width	13.8 ± 0.4 (12.9–15.0)		0.76–*0.92*
	Length/width ratio	1.49 ± 0.08 (1.31–1.66)		0.90 = *0.90*
	Mass	1.97 ± 0.24 (1.35–2.65)		0.72–*0.86*

Population variation yields $D = 1 - WC/BI$, a distinctiveness index comparing within-clutch variation to between-individual (population) variation; values closer to 1 indicate egg features that are more distinctive and so should better permit discrimination of foreign eggs in a clutch. See text for interpretation of egg appearance variables.

[1] *N = 63 eggs in 26 clutches (ground color); 66 eggs in 29 clutches (maculation); and 72 eggs in 31 clutches (egg size and shape).*

[2] *Blank values indicate that neither skewness, kurtosis, or Hartigan's dip tests indicated a significant departure from normality. S = abs (skewness statistic/standard error of skewness). K = abs (kurtosis statistic/standard error of kurtosis). Bimodality P-values derived from Hartigan's dip test for departure from unimodality.*

[3] *The range of values is between those derived from complete clutches (normal text), and two random eggs per clutch (italics).*

[4] *All PCs are devised to have a mean of 0 and a variance of 1.*

[5] *F-tests assume normally distributed data, so these values may be biased.*

(which explained 22.6% of the variance) had a loading pattern rising in the long wavelengths, resembling the reflectance of reddish-brown (**Figure 2**, bottom panel). None of the spectral variation deviated from normality. Distinctiveness of variables was highly variable, and roughly opposite to the amount of explained variation. Thus the two PC1s (brightness and blue-green) conferred very little distinctiveness, but PC2 (UV) was higher (D = 0.64–0.71), as was PC3 (red-brown) (D = 0.78–0.80; **Table 1**).

Spectral Shape Measurements

Spectral shape variables differed widely in their variation and contribution to distinctiveness. Proportion UV R and UV max-min both measured UV reflectance, but in different ways, the former representing it as a proportion of total reflectance, and the latter in terms of peak height in relation to average UV reflectance only. These variables were completely uncorrelated (r = 0.02). Proportion UV R varied less in terms of standard deviation relative to its mean (CV = 0.13), and conferred distinctiveness to eggs only weakly (D = 0.29–0.54); UV max-min, despite greater relative variation (CV = 0.33), was the least distinctive feature of all egg appearance variables between clutches, generally varying more within a clutch than between individuals (D = −0.35 to 0.07). Blue-green chroma was measured in two ways as well, one as a proportion of total reflectance (Proportion blue-green R) and one as the reflectance difference between the max and min in the short to medium VIS wavelengths (Blue-green chroma peak height). These values were highly correlated (r = 0.91), but differed greatly in their standard deviation relative to the mean, with proportional blue-green CV = 0.03, and the blue-green peak height CV = 0.81, second in magnitude only to the spherical coordinate θ among egg appearance variables. Both measures of blue-green contributed to egg distinctiveness between clutches: D = 0.67–0.75 for Proportion blue-green R, and D = 0.86–0.87 for

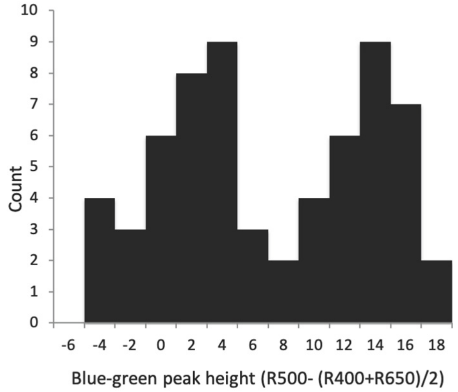

FIGURE 3 | Histogram of blue–green spectral reflectance peak height of Rüppell's weaver eggs (63 eggs in 26 clutches), suggesting a bimodal distribution of color.

Blue-green chroma peak height, the highest value among ground color variables. Blue-green chroma peak height also was strongly bimodal ($P = 0.002$), which is visually evident from the histogram (**Figure 3**). Red-brown (long wavelength) chroma had a low standard deviation around its mean (CV = 0.07) but nevertheless contributed substantially to distinctiveness (D = 0.80–0.85). Brightness was variable in terms of range (32–77% reflectance), but less so in terms of standard deviation around the mean (CV = 0.19), and conferred only weak to moderate distinctiveness to eggs (D = 0.36–0.52). No spectral shape features besides Blue-green peak height departed from unimodality or normality. Between these measures, proportional measures of blue-green and UV were negatively correlated ($r = -0.78$), as was the blue-green peak height with the proportional measure of red-brown ($r = -0.61$).

Retinal Cone Stimulation

Bird retinal cone stimulation values, as calculated by TetraColorSpace, yielded a quantitative assessment of color that takes into account estimated wavelength-specific photon catches by the four cones of a typical bird retina of the UV sort, i.e., one of whose cones' maximal sensitivity is in the UV. Mean color span (Euclidean distance in color space) was 0.036 (s^2 0.0003); volume (size of a minimum convex polygon enclosing the points in color space) was 0.00002; mean hue disparity (color contrast proportion) was 0.33 (s^2 0.052, max 1.0); average brilliance (mean proportional reflectance over 300–700) was 0.54, and average chroma (r) was 0.068 (s^2 0.0005). This value is the magnitude of the saturation vector starting at the achromatic origin at the centroid of the tetrahedron, uncorrected for variation in how far from that point a vector can go within a tetrahedron; correction yields an "achieved r" r_A of 0.26 (s^2 0.008), which is thus the proportion of maximum saturation.

The four wavelength-specific photon catch variables corresponding to retinal cone sensitivities (u = UV, s = short, m = medium, and l = long) are proportional and sum to 1. The three VIS cones had comparable mean stimulation, with the UV cone value being only 70% of these. The variation in all cone catches was remarkably low compared to other variables (CV < 0.1), indicating robustness of the relative stimulation of cones to the spectral variation in the sample. The correlations between these variables can be related to the typical colors observed in the sample: s and m were highly correlated ($r = 0.86$), reflecting their overlap at the peak of blue-green; l and s were negatively correlated ($r = -0.79$), as reddish-brown obscures the blue ground. The highest correlation was a negative relationship between m and u ($r = -0.93$). Despite the low variation in photon catch variables, the spherical coordinate θ varied widely (CV = 0.98). The mean egg hue in this dimension was a 1 (range −0.16 to 2.51) on a $-\pi$ to $+\pi$ scale where 0 is at the midpoint between m and l vertices. With respect to the other directional spherical coordinate, φ, which varies from $-\pi/2$ to $+\pi/2$, the mean egg hue was −1.26 (range −1.44 to −0.84). The chromaticity of the eggs was low, indicated by r_A values of 0.26 (range 0.06–0.40), where these values are proportions of maximum saturation. Both s and m, and the angular variable θ, were weakly bimodal ($P = 0.02$), and flatter than normal (K = −2.1 to −2.5). All of the photon catch variables and the three spherical coordinates were distinctive between clutches: all were in the D = 0.70–0.84 range except for u and the saturation variable r_A, which were less distinctive at D = 0.65–0.68 (**Table 1**). Visualization of photon catch results in tetrahedral space yields a distribution of points nearly in a plane, situated near the achromatic centroid, with variation primarily in two axes: between l and the midpoint of s and m vertices (i.e., between red and blue-green); and in the u (UV) axis (**Figure 4**). Also evident from visualization is an apparent bimodality, where some eggs are more UV and less blue–green, and others are less UV and more blue–green (**Figure 4**, right panel).

Methodological Comparisons

The three methods of ground color measurement, as determined by reflectance (based on spectrophotometry with an attempt to avoid spots), yielded largely concordant results, but with a few stark exceptions. For brightness (average reflectance), the unrotated PC1 correlated almost perfectly ($r = 0.97$) with a direct measure of average reflectance over the 300–700 nm wavelengths, and the latter perfectly agreed ($r = 1.00$) with automated TetraColorSpace assessment of brilliance. For UV, the varimax PC2, proportional UV reflectance, and the u photon catch in TetraColorSpace were all highly ($r > 0.9$) correlated with each other; the exception was UV max-min, the reflectance-adjusted UV peak height, which did not correlate. The tetrahedral directional (hue) coordinates were not correlated with UV, but the magnitude (saturation) variable R_A was perfectly ($r = -1.00$) negatively correlated with u. For blue-green, both s and m photon catches, proportional blue–green reflectance, and blue–green peak height measures were all highly correlated ($r > 0.8$), with blue–green peak height being most strongly correlated with the other measures ($r > 0.9$). The odd variable out was the varimax-rotated PC1, whose loading indicated blue–green reflectance, but whose values were not significantly correlated with any other variables aimed at blue–green chroma. For red–brown, all three measures (PC3, the proportional reflectance measure, and photon catch variable l) are all highly correlated ($r > 0.95$).

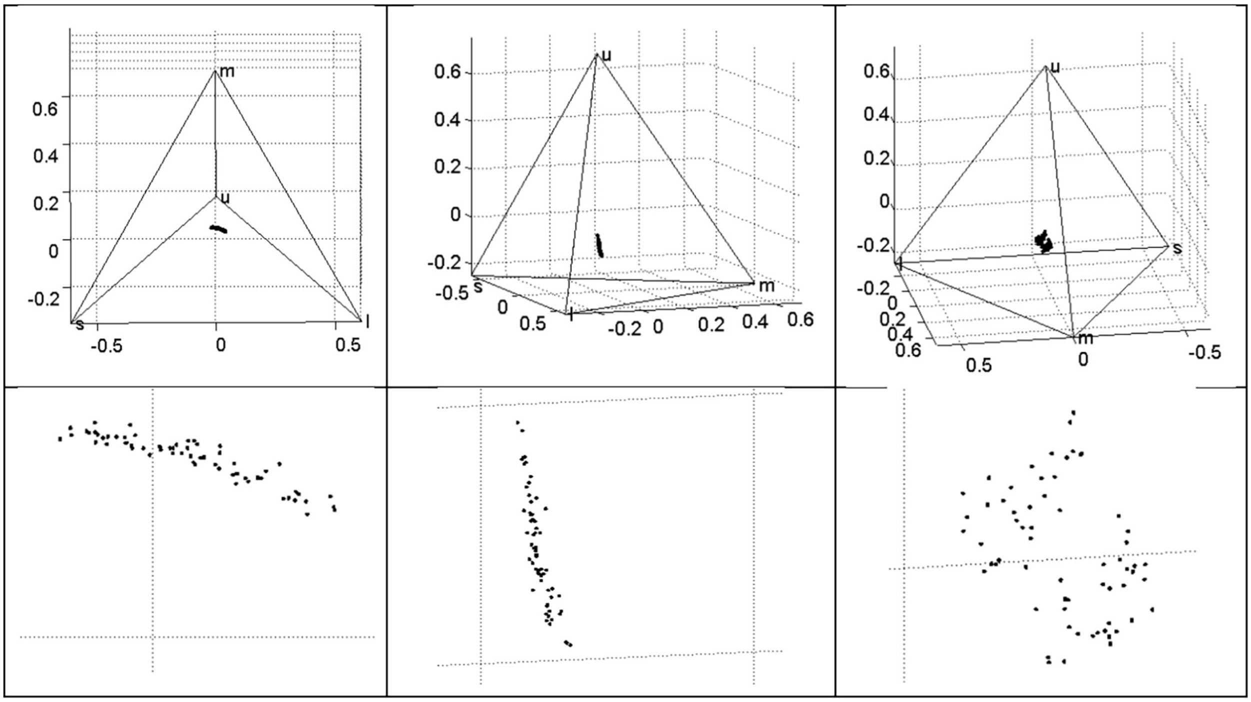

FIGURE 4 | Variation in Rüppell's weaver eggshell ground color (though influenced to some extent by maculation), in terms of photon catches for an average avian UV-type retina, plotted in tetrahedral space, where each vertex represents a cone type, and where proportional photon catch for a given cone type reaches unity at the vertex. Variation can be characterized as a plane near the achromatic origin (centroid) that varies mostly in the UV axis and the axis between long (red) and short-medium (blue–green) wavelengths. The variation is apparently bimodal (*right panel*).

The tetrahedral chromaticity vector r_A was again dramatic in its relation to particular colors, having no correlation ($r = 0$) with reddish-brown (l), but tracking variation in blue-green (m) closely ($r = 0.93$).

Variation in blue-green and reddish-brown was largely decoupled, all relevant correlations being moderately negative, including the proportional reflectance measures ($r = -0.34$) photon catch variables m and l ($r = -0.28$), and red-brown proportional reflectance versus blue-green peak height ($r = -0.61$).

Maculation

Maculation was in the form of spots and small blotches (**Figure 1**, bottom panel). Two layers were typically visible, as has been shown in many birds, being known as primary (darker) and secondary (lighter) spots (Baerends and Drent, 1982; Brown and Sherman, 1989) (Note that the naming of the spot layers was from the perspective of the viewer and opposite to the order of deposition). Although the spots in the different layers appeared to be of the same hue, they were separated by a layer of ground color and so the deeper secondary layer was distinctly fainter than the top primary layer. In some cases, three layers of spots seemed to be present.

Spot Density

The density of maculation ranged widely, covering 5–43% of the surface of the egg; it had the highest standard deviation relative to its mean of any spotting variable (CV = 0.63), and its distribution was significantly flatter (more even) than normal ($K = -2.1$). Spot density lent the highest distinctiveness to eggs of any egg appearance variable, at $D = 0.96$–0.98 (**Table 1**; raw variation shown in **Figure 5**, first panel).

Spot Brightness

To the eye, the brightness of spots had an extensive range of variation across eggs, from a light rust (e.g., **Figure 1** bottom row fifth egg) to almost black (e.g., **Figure 1** top row first egg). Although brightness in this study was averaged across primary and secondary spots, the mean for an egg still ranged widely, between 88 and 168 on a 256-level scale from black to white. Taking spot brightness variation within an egg into account, the range was much broader, especially in the darker direction (45–172). Nevertheless, brightness had the lowest standard deviation for its mean (CV = 18.5) of the spotting variables. Spot brightness lent only a moderate distinctiveness to eggs ($D = 0.72$–0.80) (**Table 1**; raw variation shown in **Figure 5**, second panel).

Spot Size

The size of spots averaged across an egg ranged widely, from 0.07 to 0.79 mm^2 in area, with a standard deviation around its mean second only to spot density for spotting variables (CV = 0.55). Contrasting examples of typical variation in spot size in this species are portrayed in **Figure 1**, bottom panel, top row, first and fifth eggs. The sample distribution deviated from normality

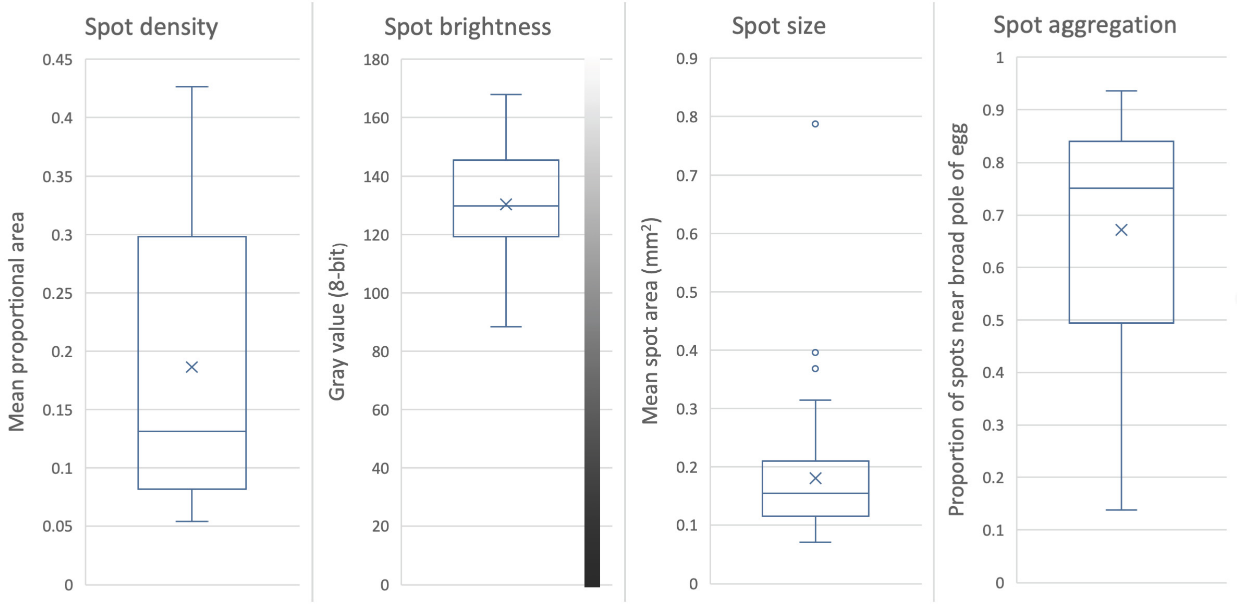

FIGURE 5 | Variation in maculation of Rüppell's weaver eggs (66 eggs in 29 clutches). Box and whiskers divide the data into quartiles, with the median value indicated by the midline, the mean by an X, and outliers by circles. Maculation on the eggs of this species consists of at least two layers of spots; values here are for all layers combined. Spot density is the mean proportional area of the eggshell covered with spots. Spot size is the mean area of an individual spot. Spot brightness is the mean gray value in 8-bit grayscale, range 0–255. Spot aggregation is the degree to which spots are clustered near the broad pole of the egg (range 0–1), as indicated by 1 – the ratio of the area of spots at the narrow end to the area of spots at the broad end.

far more than any other egg appearance variable, being skewed right, with a very long tail (*S* = 11.4, *K* = 29) (i.e., a small number of individuals laid eggs with many large spots). This variable consequently had the highest relative range (range/mean) of any egg trait. Part of this deviation was a result of one egg that had many large spots whereas most eggs only had a few or none of that size. Without that egg, the kurtosis is alleviated but the skewness remains. Spot size contributed only weakly to distinctiveness relative to other variables (*D* = 0.32–0.49), with or without the outlier egg (**Table 1**; raw variation shown in **Figure 5**, third panel).

Spot Aggregation

Most eggs had higher densities of spots near the broad pole of the egg compared to the narrow pole. The mean of this ratio was 0.67, but ranged from 0.14 to 0.94 with a skew to the right (*S* = 2.7), and a moderate standard deviation for the mean relative to other variables (CV = 0.31). The mean of the two estimates for distinctiveness conferred by spot aggregation was the second highest of any egg appearance variable after spot density (*D* = 0.81–0.96) (**Table 1**; raw variation shown in **Figure 5**, fourth panel).

Maculation Correlations

Spot aggregation was negatively correlated with brightness (*r* = −0.65) and even more strongly with density (*r* = −0.82). These effects result from the nature of spot aggregation—aggregated spots tend to overlap and become darker; and eggs with highly aggregated spots tend to cluster them very close to the broad pole of the egg, leaving much of the egg with a much lower spot density (e.g., **Figure 1**, bottom row fourth egg). Spot density was also correlated with brightness (*r* = 0.67) and size (*r* = 0.58). Lighter spots were denser than darker spots, as eggs with larger spots had denser spots, at least partly due to the means of measuring spot density, which is the proportion of eggshell area covered with spots. Spot density, brightness, and size all correlated moderately with the three measures of reddish-brown ground color (spectral PC3, proportion of reflectance from 650 to 700, and long-wavelength cone photon catch) (*r* = 0.48–0.69). Spot aggregation near the broad pole of the egg was also moderately negatively correlated with reddish-brown ground color (*r* = −0.40 to −0.49).

No egg spotting variables deviated from unimodality.

Across all egg traits, population variation was not related to distinctiveness (Pearson correlation of *D* and CV: *r* = −0.04). Within-clutch variation could not be statistically compared across traits or females, because in many cases the variance of individual clutches was too small to be reliable for comparison, or was within the known standard error of measurement (2.58 for spectral reflectance).

Egg Rejection Experiments

Rüppell's weavers practiced egg rejection within 28 h of experimental parasitism, by ejecting eggs from their nests. No act of egg rejection was directly observed, but broken eggs marked with the appropriate identification numbers were occasionally found beneath nests. As males were never observed entering nests during or after the laying period, the female

TABLE 2 | Logistic regression models of the rejection of foreign eggs by Rüppell's weaver.

Model	L–L	*P*	ρ^2	Sns	Spc	Variable	Est.	SE (raw)	*t* (raw)	*P* (raw)	SE (QML adj)	*t* (QML adj)	*P* (QML adj)	Odds ratio (95% CI)
Ground brightness	−10.4	0.002	0.32	0.69	0.69	Constant	−1.9	0.86	−2.2	0.030	0.83	−2.3	0.024	
						Ground brightness	0.26	0.11	2.4	0.016	0.10	2.6	0.009	1.29 (1.05–1.59)
Spot brightness	−10.1	0.001	0.34	0.71	0.71	Constant	−2.2	0.98	−2.3	0.024	1.14	−1.9	0.053	
						Spot brightness	0.14	0.06	2.6	0.010	0.06	2.5	0.012	1.15 (1.03–1.28)
Spot aggregation	−10.2	0.002	0.33	0.71	0.71	Constant	−1.9	0.92	−2.1	0.037	0.95	−2.0	0.044	
						Spot aggregation	20.4	9.2	2.2	0.028	13.0	1.6	0.12	n/a[1]
Combined	−3.0	<0.001	0.80	0.91	0.91	Constant	−8.6	4.6	−1.9	0.063	2.0	−4.4	< 0.001	
						Ground brightness	0.37	0.25	1.5	0.143	0.08	4.6	<0.001	1.45 (1.24–1.70)
						Spot brightness	0.15	0.09	1.7	0.087	0.05	2.8	0.005	1.16 (1.05–1.29)
						Spot aggregation	28.1	17.5	1.6	0.109	13.2	2.1	0.03	n/a[1]

Three univariate models and one multivariate model best predict egg rejection on the basis of the difference in appearance between the foreign egg and the nearest egg in the host clutch for each parameter. L–L is the log-likelihood of the model at convergence. P indicates the significance of the model relative to the respective constant-only model, as assessed by a likelihood-ratio (G) test. ρ^2 *is McFadden's Rho-squared or "pseudo-*R^2*". Sns is the model's sensitivity (rate of egg rejection when predicted), and Spc is specificity (rate of egg acceptance when predicted). t is the ratio of the estimate (Est.) to the standard error (SE) of the parameter. To correct for possible misspecification of models, covariance matrices were adjusted according to quasi-maximum likelihood (QML) to yield revised SE, t, and parameter-specific P values. Odds Ratio is the increase in the probability of egg rejection for one unit increase in the parameter.*

[1] *As spot aggregation is a ratio, one unit of change in the variable is impossible, so odds ratios are not interpretable.*

is assumed to be the egg ejector. Puncture ejection was occasionally implicated by the presence of yolk in the nest after rejection and by peck marks on eggs. The experimental egg was rejected in 11 of 22 cases (50% rejection rate), this level being deliberately targeted during the experiment by the choice of experimental eggs. In four cases, the weaver's own egg was rejected along with the foreign egg (36% rejection cost), although in two of these cases this resulted in an empty nest and so could also have been predation. In no case did the female reject her own egg but accept the experimental egg (0% recognition error).

Three single logistic regression models were significantly better than a constant-only model in predicting egg rejection. The variables in these models were ground color brightness, spot brightness, and the degree of aggregation of spots near the broad pole of the egg (**Table 2** and **Figure 6**). The effect of ground color brightness was the same whether measured as PC1 of spectral differences, Q_{avg} in Avicol, or brilliance in TetraColorSpace. Q_{avg} was used for subsequent analyses. Egg rejection was not associated with clutch size, whether the female laid another egg after experimental parasitism, egg size or shape, or any chromatic difference. The best multiple logistic regression model included the same three variables as the univariate models (**Table 2** and **Figure 7**); the equation of the curve is:

$$\ln\left(y/\left(1-y\right)\right) = 0.37Q_{\mathrm{GC}} + 0.15Q_{\mathrm{S}} + 28.2A_{\mathrm{S}} - 8.61,$$

where y is the probability of egg rejection; and Q_{GC}, Q_S, and A_S represent the differences in ground color brightness, spot brightness, and spot aggregation, respectively. G tests of nested models demonstrated the increased explanatory power of all three variables together. For instance, comparing this model to one with the two spotting variables alone yielded a G statistic of 5.2 with 1 df, for which $P = 0.02$ in favor of the three-variable model; lower P-values result from comparisons with other restricted models. No interactions among variables approached significance. The three-variable model yielded a ρ^2 of 0.80, a sensitivity of 0.91, and a specificity of 0.91. Odds ratios associated with this model indicated that for each difference of 1% in mean eggshell ground brightness across the spectrum between own and foreign eggs, the probability of rejection increased by 45% (95%CI: 24–70%); and for each unit of difference in spot brightness according to an 8-bit grayscale (256 levels), the probability of egg rejection increased by 16% (95%CI: 5–29%).

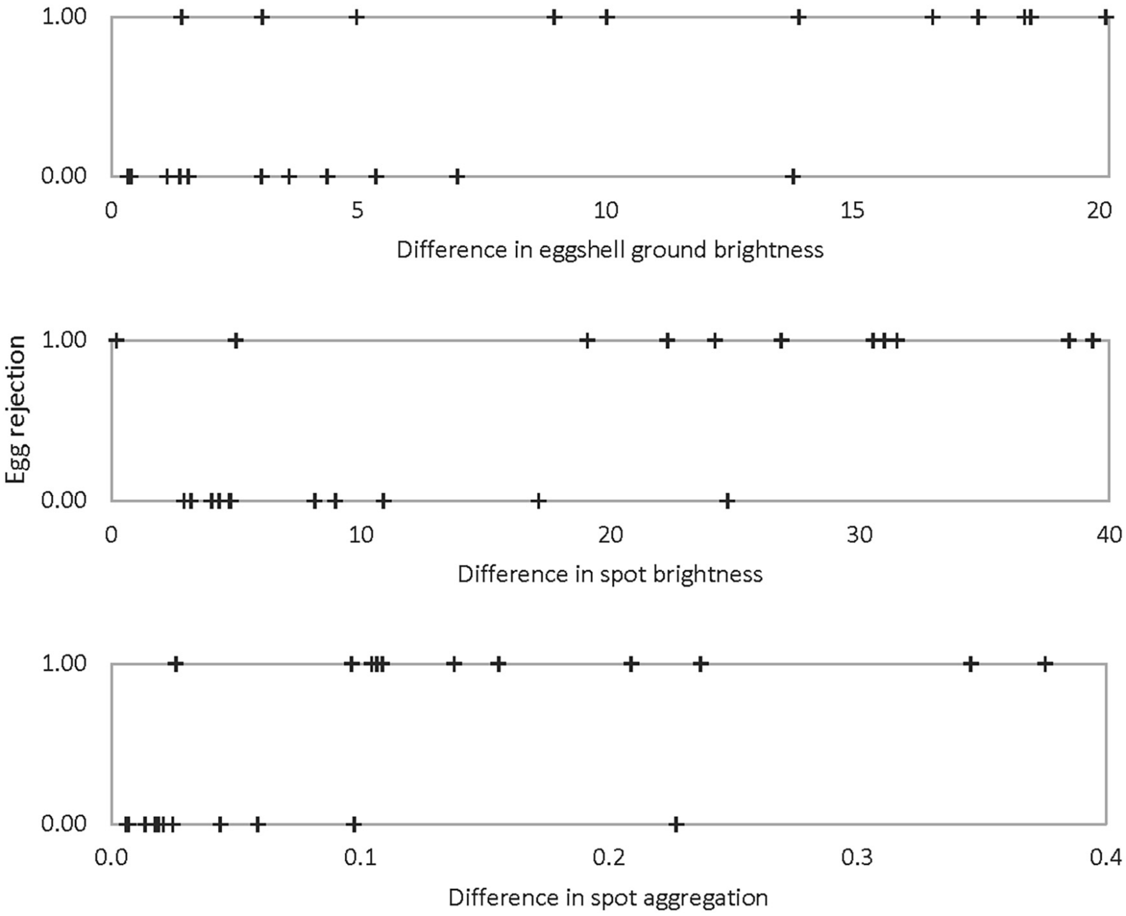

FIGURE 6 | Egg rejection by Rüppell's weaver in relation to the difference between own and foreign eggs in three variables: eggshell ground brightness (mean% reflectance across the UV-VIS spectrum), spot brightness (8-bit grayscale, 0–255), and degree of spot aggregation near the broad pole of the egg (0–1). Each of these three variables yielded significant single logistic regression models predicting egg rejection (ρ^2 = 0.32–0.33, P = 0.001–0.002).

The LD50 is the amount of difference that is predicted by the model to result in a 50% rejection rate. The LD50 is given by -c/x from a univariate logit model, where c is the constant and x is the estimate or coefficient. The single logit models yield an LD50 = 7.31 (in percent reflectance) for ground brightness, 15.7 (in 8-bit grayscale) for spot brightness, and 0.09 (a proportion) for spot aggregation. These models do not control for variation in other variables, however, and so other differences would account for some unknown proportion of egg rejection. Holding two of the variables constant at zero difference between host and foreign eggs in the three-variable multiple logit model yields an estimate of what difference in a single variable alone predicts 50% rejection: LD50 = 23.3 for ground brightness, 57.4 for spot brightness, and 0.31 for spot aggregation.

DISCUSSION

The eggs of the Rüppell's weaver (*Ploceus galbula*) in the Awash Valley of Ethiopia vary from off-white to light blue-green, sometimes with a faint cast of reddish brown. In terms of spectral reflectance curves, all eggs have a small peak in the ultraviolet, whether or not they have a blue-green peak or a reddish-brown slope. Reddish-brown and blue-green coloration varies nearly independently, but blue-green chroma is negatively correlated with ultraviolet. All eggs have spots varying from light rust to dark brown, of various sizes, densities, and degrees of clustering near the broad end of the egg; two layers of such maculation are usually evident. Size and shape of the eggs vary within narrow limits, with dimensions and size decoupled. Variation in all measured traits except for one is unimodal, indicating continuous variation rather than polymorphism. The exception is blue-green chroma, which is bimodal. Variation in nearly all traits is far higher between individuals than within a clutch; but egg appearance features vary widely in their distinctiveness, in terms of the proportion of population variation that is between individuals as opposed to within a clutch. Rüppell's weavers use some of these aspects of egg appearance to reject foreign eggs from their nests, which would function in defense against brood parasitism. Egg rejection appears to be accomplished by the female, by puncturing the egg and removing it from the nest. An individual is more likely to reject an egg the greater the

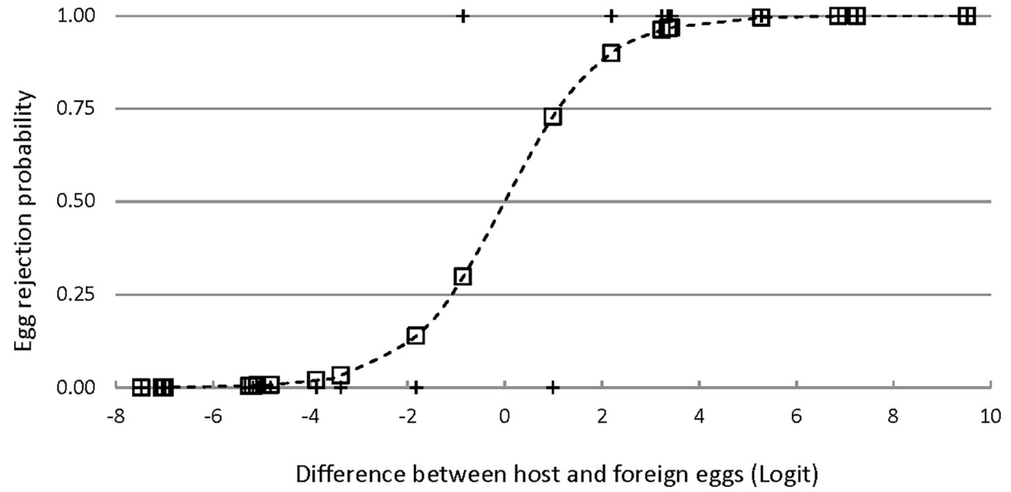

FIGURE 7 | Egg rejection by Rüppell's weaver in relation to the difference between own and foreign eggs in a multiple logistic regression model ($\rho^2 = 0.80$, $P < 0.001$) including three variables: eggshell ground brightness (mean% reflectance across the UV-VIS spectrum), spot brightness (8-bit grayscale, 0–255), and degree of spot aggregation near the broad pole of the egg (0–1). The logit values (X axis) integrate all three of these variables; the pluses indicate the observed responses (acceptance = 0, rejection = 1); and the squares indicate the probability of egg rejection for each instance of experimental parasitism as predicted by the model.

difference in appearance between her own eggs and a foreign egg, specifically in three features: the brightness of the ground color, the brightness of the spots, and the extent to which the spots are aggregated at the broad end of the egg. Although all three of these features are used to reject eggs and contribute to a similar extent, the population variation is sufficient for differences in any one of these traits alone to result in the rejection of conspecific or egg-mimicking heterospecific eggs.

Natural History

The clutch size reported here of 2.74 (mode 3, range 2–4) is consistent with estimates in the literature, which report three (Barnes, 1893; Archer and Godman, 1961; Gallagher and Woodcock, 1980) or two to four (Meinertzhagen, 1954; Mackworth-Praed and Grant, 1960). Al-Safadi (1996) is unusual in reporting a typical clutch size of two in Yemen, and a range from one to four.

The egg length (20.6 ± 1.1) and width (13.8 ± 0.4) reported here is likewise consistent with previous reports of 21.16 (range 19.3–23.6) × 13.96 (13.5–15.0) (Al-Safadi, 1996), and 20.0 (19.0–21.0) × 13.8 (13.5–14.5) (Archer and Godman, 1961). Al-Safadi's (1996) report of a mean freshly laid egg mass of 21.3 g must be a typographical error, and is likely supposed to be 2.13, which is in keeping with the present report of 1.97 ± 0.24 g.

Qualitatively, the appearances of Rüppell's weaver eggs found in this study are well described in general by Walters (2006) description of the eggs of this species as "blue or white, variably blotched or spotted brown". However, even the whitish eggs were of low reflectance compared to white-egged weaver species (e.g., the lesser masked weaver, or white eggs of the village weaver), and most eggs were usually tinted either with blue-green or light brown. Secondly, even the darkest blue eggs were very faint compared to medium blue-green egged weaver species (e.g., the Cape weaver *P. capensis*, or the darker blue eggs of the village weaver). Thirdly, the colors of the spots varied tremendously to the eye, from a light rust to nearly black.

Modularity vs. Correlation in Egg Appearance Traits

Signals of identity are predicted to be comprised of multiple highly variable traits with a limited degree of correlation with each other (Beecher, 1982; Dale et al., 2001). These features can be vital in the case of egg-mimicking brood parasitism where one organism is evolving to cause another to mistake identity, and when mistaken identity leads to the complete loss of a reproductive attempt. Low correlation among egg appearance traits have been found in the hosts of the cuckoo finch *Anomalospiza imberbis* and the common cuckoo *Cuculus canorus*, for instance (Spottiswoode and Stevens, 2010, 2011; Stoddard and Stevens, 2010). A comparative study has likewise shown that in two bird families, hosts of brood parasitism have unusually high entropy among different egg appearance traits (Caves et al., 2015). In the present study, the fact that a rotation of the principal components axes readily divided chromatic variation into three orthogonal components whose loading curves corresponded well to distinct colors (UV, blue-green, and reddish brown) indicates that these colors can vary independently of each other to a certain extent (**Figure 2**, bottom panel). The other methods of measuring color bore this out. Blue-green and reddish brown were weakly to moderately negatively correlated as predicted, whether measured as proportional reflectance in respective areas of the spectrum or in relative stimulation of visual cones. Although predicted here on the basis of separate pigments being responsible for the two colors, a weak to moderate negative correlation could also be a result of one color obscuring the other. This did occur: blue-green peak height above surrounding areas of the spectrum would decrease directly as a result of the reflectance slope characteristic of reddish-brown. Caution must be taken in interpreting pigment variation from color variation; although they sometimes clearly covary (Hauber et al., 2019), this is not always the case (Cassey et al., 2012).

Blue-green and ultraviolet coloration was strongly negatively correlated, as represented either by proportional reflectance or cone photon catches. This was somewhat surprising, as the same pigment (biliverdin) is responsible for both peaks (Lahti and Ardia, 2016). The relationship can also be seen in the particular shape of the color variation in tetrahedral color space. As shown in **Figure 4**, right panels, the eggs that are more UV are also further from the blue-green area between the short and medium wavelength vertices, whereas the eggs that are closer to that blue-green area are lower in UV. Since the long-wavelength light indicates reddish-brown, protoporphyrin might be modulating some of this negative correlation between blue-green and UV. A consideration of the spectra confirms that blue-green eggs have a rather low peak in the UV, which is overwhelmed by the broad blue-green peak in the central area of the bird-visible spectrum. More whitish eggs, on the other hand, have a peak in the UV which is small but proportionally significant without the interference of blue-green; in fact some eggs have no other chroma (no spectral slope or curvature) besides the UV peak.

Maculation variables were correlated moderately as expected given how they were measured, and also indicating three other points. First, lighter spots are denser; birds appear to be depositing similar amounts of pigment to each other, and so a higher area of coverage means less concentration in individual spots. Second, the fact that ground color and maculation variables are robust to variation in egg size and shape would increase the reliability of egg color and spotting for egg recognition across successive clutches and seasons. Third, correlations between spotting variables and reddish-brown ground color suggest an unintended influence of spots on ground color spectral measurements. Thus a slight reddish-brown tint to the ground color in this study cannot reliably be distinguished from the influence of nearby spots when maculation is highly dense (Akkaynak, 2014). The fact that spot aggregation and reddish-brown reflectance in the ground color are negatively correlated might best be explained by this artifactual effect; it means that when fewer spots are in the lateral area of the egg, the ground color is measured as less reddish-brown. Besides this effect, color and spotting variables were uncorrelated.

Distribution Shape and Modality in Egg Appearance Traits

Eggs of variable appearance within a species are usually referred to as polymorphic, which generally implies discrete variation with rare intermediates (Hoffman and Blouin, 2000). In the study of egg appearance, however, this is rarely distinguished from continuous variation, which can be substantial even if there are no discrete morphs or types. In the study of trait variation more generally, much theoretical and empirical work has focused on this distinction, and also on the subtler distinction between departures from continuous variation and departures from unimodality (Sacchi et al., 2013; Davison et al., 2019). For identity signals in particular, a multimodal distribution over continuous space is generally predicted (Tibbetts and Dale, 2007). Takasu's (2003) model of host egg variation under egg-mimicking brood parasitism begins with egg appearance varying continuously, but under a broad range of parameters ends consistently with discrete polymorphism. Spottiswoode and Stevens (2010) found continuous variation in the eggs of hosts of the cuckoo-finch. The two species of parrotbills or crow-tits (*Paradoxornis* spp.) appear to have eggs whose color is bimodal in distribution (Kim et al., 1995; Yang et al., 2010), although the variation at least in *P. alphonsianus* is continuous (Yang et al., 2010, Figure 1). These studies have related variation in egg appearance to brood parasitism. With respect to *Ploceus* weavers, Bates (1911) and Swynnerton (1916) both believed that egg appearance satisfied the condition for polymorphism, with discrete types and rare intermediates. Subsequent qualitative studies of egg color in the genus have tended to agree with or presume the existence of egg types as well (Bannerman, 1949), sometimes with indications that this might be a simplification (Victoria, 1972; Din, 1992). Collias (1984, 1993) treated egg color variation as continuous in brightness and chroma but discretely polymorphic in hue; but this may have been influenced by the fact that their particular color charts were organized in that way. Later more quantitative studies have treated all egg appearance traits as continuous in the northern masked weaver (Jackson, 1992a, 1998), village weaver (Lahti and Lahti, 2002; Lahti, 2005; 2006; 2008; Lahti and Ardia, 2016), and Rüppell's weaver (Navarro and Lahti, 2014). Collias (1984) concluded unimodality and continuous variation in egg shape and size in the village weaver from an examination of histograms. Otherwise, none of these studies conduct specific tests or visualizations to examine distribution shape or pattern of variation. All egg appearance traits can be assessed on continuous scales of measurement, but this does not mean that the variation itself is necessarily unimodal or even continuous.

Tests in the present study show continuous and unimodal variation in nearly all egg appearance traits, with one exception: ground color on the axis of more or less blue-green. This can be seen by visual inspection of histograms (**Figure 3**), the result of Hartigan's tests for departures from unimodality, and visual inspection of the scatterplot in tetrahedral color space (**Figure 4**, right panels). The variable that best indicates this is blue-green peak height, although it is also evident to a lesser extent in the photon catches of short and medium wavelength cones (which overlap in sensitivity at blue-green), and in the spherical coordinate θ. The tetrahedral plot indicates that this bimodality is not actually exclusively on the more or less blue-green axis, but is also evident on the more or less UV axis, as mentioned above regarding correlations. Thus the two classes of eggs are the more blue-green and less UV ones, versus the more UV and off-white ones. These cannot be called types, however, as there is substantial variation within a class. In fact, some eggs in one class are more similar in color to the eggs in the other class than they are to the most distant eggs in their own class. Whether this pattern of variation is discrete enough to be considered a dimorphism would thus depend on the precise definition chosen. Examples can be seen by comparing the top and bottom rows of eggs in **Figure 1**.

Other features of the distributions of egg appearance variation can be evident, and ecologically relevant, than just modality and the continuity of variation. A flatter than normal distribution, for instance, as indicated by negative kurtosis values, is a more even distribution. This minimizes the occupancy of any given place in the range, and thus can be predicted to be the ideal distribution for discriminability of recognition cues. The near-perfect distinctiveness of spot density in this study, for instance (see below), was made possible by the combination of a wide range of variation, and a significantly flatter distribution than normal (as determined by kurtosis). By contrast, spot brightness had a wide range as well, but a low CV, indicating a narrow normal distribution with two long tails; the histogram confirms this. This distribution shape, with most of the population clustering near the mean, renders this particular egg appearance variable less useful as a recognition cue. Narrow or highly skewed distributions that have most of their values clustered around a small area of the range provide an advantageous target for egg mimicry and thus can be predicted to be less adaptive than broader distributions.

Methodological Comparison

The three methods used to bridge the gap between spectral reflectance data and quantitative variables for analysis were largely concordant in their results, although each provided insights that were not necessarily revealed by both of the others. The variation in their contributions can largely be predicted by the ways in which they manipulate the data and divide it into separate variables (no method can reduce color variation to a single or even two dimensions). The two main advantages of PCA of spectral data, at least for certain applications, are that they are objective (do not assume anything about visual systems or important regions of the spectrum), and that the resulting variables are statistically independent of each other. The main drawback is that all comparisons must be made within the same analysis, prohibiting quantitative comparisons between studies (Doucet et al., 2006). In addition, interpretation of the axes can be challenging, and brightness can sometimes swamp chromatic variation (Burns et al., 2018); however, as shown in the present study, alternate rotations can relieve both of these constraints to some extent. In particular, the unrotated PCA yields a prominent initial component representing brightness, and largely uninterpretable subsequent components; but a varimax rotation, which is designed to maximally associate each variable (reflectance at a given wavelength) with a single component, yielded variation that broadly aligned with typical descriptions of color. Multiple rotations can be used in the same study (although it is rarely done), with the understanding that statistical independence applies only to components within a rotation. The varimax rotation did not completely remove the swamping effect of brightness, however, but instead distributed it among all the components; this may be why PCA was worse than the other two methods at identifying distinctiveness of egg features, with the amount of variance explained actually inversely proportional to the distinctiveness of the component. Blue-green coloration was a major axis of variation in this study, for instance, but the varimax PC1 included so much brightness as well that it had among the poorest distinctiveness of any egg appearance variable, and missed some of the most distinctive aspects of this color, such as its bimodality. This study also highlights another drawback to PCA, which is that the standardization of the variables (to a mean of 0 and a variance of 1) precludes easy comparisons of population variation among components.

Spectral shape variables that target spectral peaks or wavelength regions of interest can be helpful for particular questions where interpretability is at a premium, and *a priori* the variation of importance is known to be related to particular sorts of curvature or wavelength-specific reflectance level. The major drawback of this method follows from this advantage, however, which is the subjectivity or analytical uncertainty involved in identifying these features. The most typical variables are absolute or relative (proportional) reflectance within a certain wavelength range. Absolute reflectance has the major drawback of conflating chroma with brightness. Proportional reflectance overcomes this drawback, but at a cost of another: values are affected by irrelevant reflectance differences in other parts of the spectrum. Both sorts of variables can sometimes have the drawback of being associated with more than one color. For instance, in the present study, if the typical 625–700 nm bin were considered to be the variable that pinpoints reddish-brown, this would have been contaminated by the broad blue-green peak; thus 650 was a more precise cutoff, but at the cost of narrowing the range of wavelengths considered. For another example, if 300–400 nm were chosen as the bin for UV in this study, as is commonly done because of researcher bias arising from human vision, the reflectance between 380 and 400 nm will already often have passed its minimum and be rising again, leading to an underestimate of the bird-relevant UV peak. In this study, despite the stated drawbacks to relative reflectance, the correlations of those three variables (proportional UV, blue-green, and red-brown) with other measures were very high. Overall, this study sometimes succeeded and sometimes failed in the attempt to represent colors by spectral shape in a way that could be validated by other methods and could reveal the distinctiveness of egg appearance features. Since eggs that were blue-green varied little in hue, a blue-green peak height with fixed min (400 and 650 nm) and max (500 nm) wavelengths was one of the most effective of all egg appearance variables, most strongly indicating bimodality and with the highest distinctiveness score. On the other hand, UV max-min incorporated absolute UV reflectance and so was confounded with brightness in that area of the spectrum, making it the least effective variable in terms of distinctiveness and uncorrelated with other measures of UV.

Avian visual modeling that transforms spectral data according to retinal cone sensitivity provided additional insights and was very successful at separating chromatic variation from brightness. Surprisingly (and only evident by this method), egg variation was largely in a plane in tetrahedral color space (**Figure 4**), but was so oriented that variation was precisely divided in terms of cone photon catches, such that short, medium, and long wavelength cones equally participated in perception of the egg color; and with the UV cone taking a 70% share. The negative correlation between blue-green and UV coloration was most evident by this method ($r = -0.93$). A drawback to this method, however, is that the variables are fixed, in a manner irrespective of the color variation of the objects (eggs); whereas the construction of the PCA and spectral shape variables are governed by the variation in the objects. This is intentional, of course, as it is the central point of avian visual modeling. Nevertheless, it can hamper interpretation in some cases. In the present study, blue-green variation was the most dramatic color feature by all methods; however, in tetrahedral color space this variation does not line up with any particular cone, but lies midway between the short and medium wavelength cones, which divvy up the variation between them. Estimates of distinctiveness, chromatic variation, and modality were compromised by this sharing of the effect between two cones. The use of the spherical coordinates could potentially solve this problem, but they were less directly interpretable in terms of coloration. An advantage to tetrahedral color space is the diversity and specificity of the ways of measuring the range of coloration in a sample. In the present sample, since colors lay nearly on a plane, estimates of volume were close to 0, which of course is the volume of a plane of any size. A single point lying off the plane would have dramatically increased the volume measurement. This situation illustrates that the minimum convex polygon method of estimating color range is very sensitive to the overall shape of the scatter; if it lies only in two dimensions, volume should probably not be used. Span in this case, which considers Euclidean distance regardless of dimension, would be more effective if this data were to be compared, for instance, with another population or species.

Despite the great usefulness of avian visual modeling in general, one cautionary note is the possibility of differences between the birds that were used to parametrize the model, and the study species. Nevertheless, within passerines this has not often become a major issue regarding cone-specific photon catches (Burns et al., 2018). In general, the more that is assumed about a bird's perceptual and cognitive processing, the more the models should be parametrized by taxon-specific data. For instance, many studies of color discrimination in birds go one step further than cone-specific photon catches and estimate the ability of birds to detect color differences on the basis of photoreceptor noise (Vorobyev and Osorio, 1998). Thus a genus like *Ploceus*/*Malimbus* that has not been studied either for cone-specific photon catches or photoreceptor noise might compound error with each step deeper into the perceptual system. Moreover, referring to differences as "just noticeable" (JNDs) might imply a still deeper understanding of the perceptual processing than we have for any bird, which is not just a function of the photoreceptors but the brain (this contrasts with the typical uses of JNDs in psychology, which are rooted in subject responses as to what is noticeable). We still know too little about perceptual and cognitive processing and its variation between species to be too confident about the accuracy of what differences a bird can and does notice, despite the value of the photoreceptor noise models when they can be parametrized. In the meantime, some discrimination studies (like this one) might continue to use behavior alone (e.g., egg rejection) as a coarse indication of discriminability, although this strategy has its own drawbacks, such as the distinction between recognition and rejection (Ruiz-Raya and Soler, 2017).

One Component of a Predictive Theory of Egg Recognition: Distinctiveness

One of the most productive predictions in the study of brood parasitism is the expectation that a bird exhibiting egg recognition in counteradaptation to egg mimicry should lay eggs that are distinctive (Davies and Brooke, 1989), hence the relevance of the concept of "signature information" (Beecher, 1982; Caves et al., 2015). Bates (1909) first noted the variation between the eggs of different female weavers combined with uniformity within a clutch, and later proposed that it functioned in egg recognition (Bates, 1911). Much like human anti-counterfeiting strategies with respect to currency for instance, forgeries are more easily identified if the genuine articles are consistent in appearance with one another, different from other such objects elsewhere, and have a degree of complexity. Complexity has been dealt with above regarding multiple uncorrelated traits. With respect to the other two points, since they involve measurements of clutches nested within population samples, a simple ratio of sums of squares can provide a single integrated metric of distinctiveness. Specifically proposed here, $D = 1-\text{WC}/\text{BI}$, where BI is the sum of squares for the "treatment" (between clutches), and WC is the residual sum of squares (within clutches). For a species with variable eggs, some integrated metric of this sort is to be preferred over isolated measurements of population or intraclutch variation, as it is the relationship between these two features and not either in isolation that indicates the distinctiveness of the eggs in the average clutch within a population. In the data presented here, D ranged widely across traits, indicating major differences in signature information. The worst traits for discrimination were the PC for blue-green, the two UV spectral shape variables, brightness as determined either by PC1 or by average reflectance across the spectrum, and spot size. All of these variables had estimates of D that were <0.5. The PC for blue-green loaded so broadly that it was nearly brightness itself, explaining why other more focused measures of blue-green yielded higher distinctiveness. Perfect distinctiveness is 1, as that would indicate a near-zero numerator (within-clutch variation), relative to the magnitude of the denominator (between-individual variation), in the term to be subtracted from 1. In the present study, spot density made eggs almost perfectly distinctive, with 0.96–0.98 of the variation being between clutches. The other highly distinctive variables ($D \geq 0.85$) were the proportion of reddish-brown reflectance (which likewise represents spotting); blue-green peak height; the degree of spot aggregation at the broad pole of the egg; and egg shape, size, and mass. Although unreported above, the mean 8-bit grayscale value of primary spots in this sample was 75 ± 16 (range 45–107), and of secondary spots was 139 ± 13 (range 108–172), thus neatly partitioning the brightness space. Perhaps the distinctiveness that spot brightness actually confers to eggs is higher than suggested by my averaging these two layers of spots, if birds themselves can distinguish the differences in the two layers; this is not known for any bird.

With the appropriate data, *D* can be a useful measure, and comparable across traits measured at any scale, and across populations and studies. The sum of WC and BI, moreover, is the total sum of squares, which itself can be useful as a measure of total variation in a trait in the population. When comparing populations or species, however, one must be aware that smaller clutches will have less variation than larger clutches. More precisely, if eggs are laid in random order with respect to their appearance, there is a 0.67 probability that within-clutch variation will increase between 2 and 3 eggs, and 0.5 that it will increase again from 3 to 4 eggs, and so on with a proportion of $2/n$ where n is the clutch size with one additional egg. If final eggs are unusually different [as can be the case (Brant et al., 1950)], the bias is even greater. In many cases this is not a problem, because the true within-clutch variation is desired regardless of clutch size. However, if one needs to control for clutch size, for instance in comparing variation within a species across latitudes where clutch size varies, the solution is to subsample eggs randomly from the larger clutches when deriving *D*. This strategy can also be used if one has a small sample of known complete clutches, as in the present study. Here, the complete clutches were used for one estimate of *D*, and then the disparity between just two eggs from each clutch regardless of completion were used for another estimate of *D* (**Table 1**). The estimates were usually close together, as the true clutch size was not much over two. As the true clutch size increases, the inaccuracy of the subsampling strategy increases.

Among the limitations of *D* is that it does not indicate the magnitude of the differences. For instance, a value of 1 will be given for a trait that does not vary within a clutch no matter what the variation between clutches. Also, seemingly reasonable values can be calculated from variation that is not even perceptible by birds. These are standard limitations of any proportional variable, but are important to recognize in studies like this, for several reasons. For instance, every trait has some variation; and for egg appearance traits even in the absence of selection or function our null expectation is already that $BI > WC$ just from random genetic variation. Two further limitations relate not to *D per se* but to the practice of measuring variation more generally. One is that birds' eggs often vary very little within a clutch, and this is specifically predicted for hosts of egg-mimicking brood parasites. Within-clutch variation might therefore sometimes approach the error inherent in the equipment or the researcher. The standard error of measurement for the spectrophotometry in this study was 2.58, and remained relatively constant across wavelengths. Some clutches had a within-clutch variation in spectral reflectance at or under that value, and in many cases the difference between two eggs in a clutch in certain areas of the spectrum was less than that. For that reason, I could not run more focused analyses on differences in within-clutch variation. A second limitation of the present methods, as is typical of studies calculating within-clutch variation, is that we do not know how much variation an individual would exhibit from clutch to clutch, or from year to year. This is a "black box" of variation between the within-clutch and between-individual levels, which has important implications for interpreting distinctiveness for egg rejection (discussed below). One final, and again typical, limitation is that this study, and *D* in particular, presume that any parasitic egg will be within this study's sample range for each trait. This would indeed be expected if we were talking solely about intraspecific brood parasitism. It would also be expected if all diederik cuckoos that parasitize this particular weaver species lay eggs that are within the range of this host for all egg appearance variables. The accuracy of such an assumption depends on the particular extent and sort of coevolutionary relationship between the diederik and its hosts (Friedmann, 1968), which we still do not know. To the extent that any parasitic eggs laid in Rüppell's nests fall outside their range for any variable, *D* will underestimate the ability of weavers to discriminate against them.

Egg Rejection and Implications for Brood Parasitism

Rüppell's weavers rejected foreign eggs in their nest according to the difference between the foreign egg and their own eggs in the achromatic brightness of both ground color and maculation, and the degree to which spots were aggregated near the broad pole of the egg. More specifically, for each egg appearance variable the disparity was calculated between the foreign egg and every egg in the host's clutch, whether it was present at the time of the experiment or not. The minimum disparity was considered to be the difference between host and foreign eggs, so as not to confound this difference with intraclutch variation. The use of color and spotting but not size and shape is consistent with studies of egg rejection in several other species (Baker, 1913; Honza and Cherry, 2017), including two other *Ploceus* weavers (Jackson, 1998; Lahti and Lahti, 2002).

On the basis of distinctiveness alone, as described above, the prediction is that individuals should reject eggs based on spot density, reddish-brown reflectance (which amounts mainly to the same thing), blue-green peak height, spot aggregation, and egg shape and size. The prediction was therefore only met with respect to spot aggregation.

Distinctiveness of egg appearance traits was not related to their population variation. Since distinctiveness does not indicate the magnitude of variation but only the relative amounts within-clutch vs between-individual, an alternative hypothesis is that hosts might require signature traits to have high variation period, and that in the Rüppell's weaver even some of the more distinctive traits do not vary enough to be useful. The least variable traits measured, at $CV < 0.1$, were the four cone-specific photon catches, the proportional reflectances of blue-green and reddish-brown, and egg size and shape. The most variable traits, at $CV > 0.6$, were, in descending order, the spherical coordinate θ, blue-green peak height, and spot density. Thus, although there was no correlation overall, blue-green peak height and spot density were both among the most distinctive and the most variable egg appearance traits in the population; but neither they nor θ were used in rejecting eggs.

Since the eggs were whitish to pale blue-green, and the spots contrasted strikingly with this ground color in all individuals, differences in the aggregation of spots did indeed make for a ready basis for distinguishing clutches—the ratio of eggs at the broad pole to the middle ranged from 0.14 to 0.94, with a skewed distribution such that the mean was 0.67. Thus, in addition to being highly distinctive, spot aggregation was a qualitatively salient feature. The importance of this trait for egg recognition has been found in village weavers as well (Lahti and Lahti, 2002), and several other species (Polačiková and Grim, 2010; Polačiková et al., 2011).

Why, however, did birds use the brightness of ground color and spotting, instead of the more variable and distinctive chromatic variation, for egg recognition? This wording might beg the question, however, as just because a trait is more variable in terms of variation around the mean does not mean the bird sees it as more variable. Perhaps weavers rely more, in their dim enclosed nests, on achromatic contrasts via their double cones, than on the subtle differences in single cone photon catches. Although spectral shape features varied highly, the variation of all four single cone photon catches was very small (CV < 0.1 in all cases). Thus, although ground color brightness was only weak to moderate in its distinctiveness ($D = 0.36$–0.52), and only moderate in its standard deviation around the mean (CV = 0.19), it may have been a qualitatively salient feature in its achromaticity. Moreover, the range of ground color brightness variation was very high whether for bird or human eyes, ranging from 32 to 77% average reflectance over all wavelengths. Brightness of spots was more distinctive ($D = 0.72$–0.80), but had the lowest standard deviation for its mean among spotting variables (CV = 0.19). However, again, its achromatic contrast with egg ground color might have been salient, and the range of spot colors in the population on the 256-level grayscale was enormous (45–172 when both primary and secondary spots are considered, and still 88–168 if only the mean brightness across all spots on an egg is considered). These results highlight the importance not only of objective measures of variation and distinctiveness, but of salience to birds, a feature that is arguably much more difficult to assess and quantify.

Avian visual modeling might have been predicted to yield the variables that were most important in egg recognition, since the entire purpose is to approximate what the bird is seeing rather than relying on objective orthogonal variation or spectral curvature. However, birds did not use chromatic variation to reject eggs, and since nearly all of the visual modeling was in service to color rather than brightness or patterning, its failure to explain the basis for egg recognition is not surprising. The values for brightness in TetraColorSpace are equivalent to the direct measurement of average reflectance across the spectrum. One benefit of TetraColorSpace, however, as mentioned above, was the demonstration that the chromatic variation in photon catches was actually much lower than the objective variation of the spectral reflectance proportions or peak heights. In the low light of an enclosed nest (light environment was not considered in this study), the realized chromatic variation would be even lower.

Why do Rüppell's weavers have highly variable and distinctive egg features that they do not use to reject eggs? In particular, blue-green peak height and spot density were highly variable and extremely distinctive, but were not used to reject eggs. Assuming these features evolved for a function rather than by neutral evolutionary processes, there are at least four possible explanations. (1) The features they used in this study are only those they found salient for conspecific eggs; possibly the way cuckoo eggs differ from theirs might elicit some use of other egg appearance features. Rejection costs (rate of rejection of a host egg along with a foreign egg) might interact with this factor as well, limiting the kind or extent of variation used to reject eggs. The rejection cost observed here of 36% is comparable to the 41% observed with a higher sample size in the village weaver in The Gambia, but higher than the 12% found in South African spotted-backed weavers (thought to be the same species as the village weaver) (Lahti, 2006). A high rejection cost might mean that similar eggs, or eggs that are similar in certain features, are more likely to be accepted; but eggs of a greater difference from host eggs, and thus more likely to be parasitic, might be rejected on the basis of features different from those in the current study. (2) Rejection level might have been lower at the time and place they were tested here than they sometimes are. Environmental variables indicating probability of parasitism can influence egg rejection (Davies et al., 1996; Brooke et al., 1998). (3) Perhaps the weavers are using higher-level (feature-detection) pattern features to recognize eggs rather than the basic maculation variables tested here, but those pattern features are nevertheless dependent on the variation and distinctiveness of the lower-level features. In the tawny-flanked prinia, for example, although basic spotting variables explained rejection, the addition of a feature-detection algorithm for maculation pattern increased the explanatory power of the model (Stoddard et al., 2019). Finally, (4) certain features that are variable and distinctive in one clutch might not be in another clutch of the same female; egg shape and size, for instance, were highly distinctive in this sample, but were not used in egg recognition; in fact they rarely are so used (Honza and Cherry, 2017). The reason for this might be that they are variable between breeding attempts, depending for instance on resource availability, and thus are unreliable as signatures.

Finally, why do Rüppell's weavers reject eggs at all? The discussion here has focused mainly on the diederik cuckoo, and indeed they were singing and visible in the vicinity of the weaver nests throughout the course of this study, although no instance of brood parasitism was detected (unsurprisingly, given my restricted methods). Circumstantial evidence must currently suffice more generally. The diederik cuckoo apparently so depends on Rüppell's weaver in Arabia that sightings of the former are always within the limited and disjunct distribution of the latter (Jennings, 2010). In Yemen and Oman, the Rüppell's weaver is the only species that is a known host for the diederik cuckoo, and yet the cuckoo is a frequent breeding visitor in both countries (Eriksen and Porter, 2017). Some have proposed the alternative hypothesis, that intraspecific brood parasitism is the reason why the northern masked weaver (*Ploceus taeniopterus*) rejects eggs (Freeman, 1988; Jackson, 1992b); this hypothesis could apply to the Rüppell's weaver as well, in the absence of evidence. The agent of selection cannot be determined by the mere existence of egg rejection, as selection from intraspecific brood parasitism can lead to rejection of heterospecific eggs

(Lyon et al., 2015), and vice versa (Lahti, 2006). The evidence that intraspecific brood parasitism alone can promote the evolution of egg rejection is fairly uncontroversial (Lyon and Eadie, 2004). However, I am not aware of any species where intraspecific brood parasitism has led to the evolution of egg appearance variation, and there is reason to doubt that it would. Any given female in this case could be either a host or a parasite; to the extent she is a host, she benefits from having eggs that differ from those of conspecifics, but to the extent she is a parasite, she benefits from having eggs that match those of conspecifics. Whether these roles are typically played by different individuals or the same individual, they are in the same gene pool, which hobbles any evolutionary prediction.

Profitable next steps for research in this brood parasite – host system would be (1) to conduct tests that further investigate the features of eggs that are salient to the hosts in order to test the possibilities above as to why they had features they did not use; (2) to investigate individual differences in egg traits that are not seen within a single clutch, including environmental and developmental variation; (3) take a macroevolutionary perspective, looking into why some weavers have more advanced antiparasite defenses than others on the same trajectory, and why some even seem to have different trajectories. For instance, the lesser masked weaver nested alongside Rüppell's weaver in the study site, is known to be parasitized by the same cuckoo, but has nearly opaque nests, white invariable eggs, and a long narrow entrance tube (pers. obs.). Finally, and perhaps most important in terms of understanding brood parasitism, would be (4) to find out what the diederik cuckoos are doing.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC Queens College CUNY.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Variation in the Non-mimetic Vocalizations of Brood-Parasitic Indigobirds and Their Potential Role in Speciation

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Indigobirds (*Vidua* spp.) are obligate brood parasites in which imprinting on heterospecific hosts shapes adult vocal behavior and mating preferences. Adult male indigobirds mimic the songs and other vocalizations of their respective hosts, which signals their own host environment to prospective mates and has important implications for speciation. In this study, we examined variation within and among indigobird species in the non-mimetic components of their vocal behavior, including both chatter calls and their impressive repertoires of intricate non-mimicry songs. We test whether indigobird species in Tanzania (*V. chalybeata*, *V. codringtoni*, *V. funerea*, and *V. purpurascens*) differ consistently in general features of their non-mimetic vocalizations, and we test whether local ecological conditions influence vocal behavior. Indigobird non-mimetic song repertories are learned from and shared with other males of the same species. We find that local dialect "neighborhoods" are variable in size among species and regions, depending on habitat continuity and the distribution of male territories. Despite the complete turnover of the specific songs comprising non-mimicry song repertoires from one local dialect to the next, we find significant species effects for more general measures of non-mimicry songs such as repertoire size and diversity, frequency, song length, and pace. For some traits, we also found significant regional differences, which may be mediated by significant relationships between elevation and morphometrics. Chatter calls were broadly similar across both species and localities, but we found significant species and region effects for frequency and to a lesser extent pace. We discuss the possibility that learning and mimicking the vocalizations of different hosts might influence the production of non-mimetic vocalizations and explain many of the species differences we detected. Whether these species differences are purely due to phenotypic plasticity or also reflect genetic divergence in traits influencing sound production and/or female preferences, they may contribute to reproductive isolation among nascent and recently evolved indigobird species.

Keywords: *Vidua*, indigobirds, vocalization, brood parasitism, speciation

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INTRODUCTION

Songbirds (suborder Passeri) comprise a radiation of ca. 4,000 species with a remarkable diversity of morphological, ecological, and behavioral traits. As their name implies, songbirds are best known for their impressive diversity of songs, which are acquired through imitation during development (age-limited learners) or throughout their lifespan (open-ended learners) (Brenowitz and Beecher, 2005). Songbirds vary widely in both the size of their vocal repertoires and in the frequency and temporal features of song elements. Sexual selection is often hypothesized as an important driver of repertoire size (Eens et al., 1991; MacDougall-Shackleton, 1997; but see Byers and Kroodsma, 2009), whereas song traits (e.g., length and number of notes, frequency, modulation) can be shaped by numerous, non-mutually exclusive factors, including selection for optimal transmission through different physical environments (Morton, 1975; Nottebohm, 1985; Badyaev and Leaf, 1997), diversifying selection to enhance species recognition (Miller, 1982; Seddon, 2005), and as a by-product of selection on morphological traits (Podos, 2001; Huber and Podos, 2006). Adding to the overall diversity of songbird vocalizations, many species display geographic variation. If there are relatively sharp boundaries among conspecific populations with different song characteristics, these populations are recognized as having different dialects (Marler and Tamura, 1962). The formation, evolution, and maintenance of dialects can be shaped by selection on the songs themselves, the indirect effects of other evolutionary processes, and/or cultural evolution and drift, which depend on both the song learning and dispersal characteristics of a given species (Slabbekoorn and Smith, 2002; Podos and Warren, 2007).

Indigobirds (*Vidua* spp.) are obligate brood parasites that acquire part of their vocal repertoire through imprinting on their respective host species. Most of the ten recognized indigobird species are associated with one of the *Lagonosticta* firefinches, which have small repertoires of ca. 5–10 songs, alarm calls, and begging calls (Payne, 1973), but a few indigobird species are associated with more than one host and/or a host in another estrildid finch genus (e.g., Payne et al., 2005). Indigobirds imprint on host songs and calls during development, and adult males incorporate mimicry of host vocalizations into their singing (Nicolai, 1964; Payne, 1973; Payne et al., 1998), thereby advertising their success in having been reared by a particular host species. This serves as an important mate choice cue for females, in which imprinting on host vocalizations appears to guide both mate choice and the selection of nests to parasitize (Payne et al., 2000). These behaviors result in pre-mating reproductive isolation among indigobirds associated with different host species, with important implications for speciation and host-specific adaptation (Payne, 1973; Sorenson et al., 2003; Jamie et al., 2020).

While learning and mimicry of host songs has long been viewed as key to the establishment of behavioral/cultural reproductive isolation and therefore speciation in indigobirds, the non-mimetic components of indigobird vocal behavior have received less attention. All indigobird species produce similar "chatter" calls comprising rapid sequences of broadband notes (Payne, 1973). Chatters are most often associated with "comfort" behaviors like preening and bill wiping, but are also heard at the beginning of singing bouts, during flight, and when chasing other males (Payne, 1979). Qualitatively similar across species, chatter calls may serve as a general "password" (*sensu* Hauber et al., 2001) for the recognition of other male indigobirds (i.e., to discriminate indigobirds from non-indigobirds), but this has not been tested experimentally.

The vocal repertoires of male indigobirds also include ca. 10–20 distinctly different non-mimicry (NM) songs (Payne, 1973), each comprising a complex series of notes delivered in a highly consistent manner. Males advertising their territories alternate between NM songs and host song mimicry (Payne, 1973, 1979). There is clear evidence that indigobird males learn these complex NM songs from each other (Payne, 1985; Payne et al., 1998). As a consequence, neighboring males of the same indigobird species share broadly overlapping repertoires of NM songs, but the extent of overlap between conspecific males is negatively correlated with the geographic distance between their call sites, resulting in a complete turnover of NM song dialect "neighborhoods" across the landscape (Payne, 1973, 1985, 1987). However, the size of dialect neighborhoods, and possible interspecific variation in neighborhood size, has not been well studied. Indigobirds are also open-ended song learners, such that their song repertoires can change over time. Adult males have been observed copying the songs of neighbors that are frequently visited by females, or acquiring a completely new repertoire after dispersing outside of their original dialect neighborhood (Payne, 1985). Thus, the songs within a dialect neighborhood evolve over time, with new songs introduced by errors and/or innovation spreading via cultural transmission (Payne, 1973, 1985). Crucially, sympatric indigobird species have entirely non-overlapping repertoires of NM songs, indicating that males learn only from other males associated with the same host. Thus, dispersing males acquire songs only from conspecific males and can subsequently attract conspecific mates. This suggests that juvenile males discriminate among adult males based on their mimicry of different hosts and choose as tutors older male indigobirds that mimic the same host species that raised them.

The multifaceted vocal repertoire of each male indigobird thus contains three elements — chatter, mimicry of host songs and other vocalizations, and a complex repertoire of NM songs — that respectively convey its identity as an indigobird, its host association, and its membership in a local indigobird dialect neighborhood. It is important to note that indigobird species are also distinguished by evolved differences in adult male plumage and soft parts colors, and in the mimetic mouth markings of nestlings (Payne, 1973, 2005; Sorenson et al., 2003; Jamie et al., 2020), traits that clearly have a genetic basis. Thus, indigobird species may have evolved consistent differences in certain general features of their chatter calls and/or NM songs (e.g., frequency and/or temporal traits) even though the specific sequences of notes characterizing unique NM songs are not shared among allopatric populations of the same species. A consistent species difference in frequency, for example, might reflect the indirect effects of ecological selection on bill and body size or direct selection on effective signal transmission in different environments. Alternatively, differences between species may be attributable to males being raised in different host nest environments.

To date, there has been limited analysis of variation in chatter calls or in the general characteristics of NM songs among indigobird species. Payne (1973) measured the overall length of chatter calls and the number of chatter syllables per second in five species (1–8 localities per species), and found that all species occupy the same range of variation. Payne (1973) also measured the length, number of syllables, maximum frequency, and minimum frequency of NM songs in three species (3 localities per species), and similarly concluded that indigobird species are broadly similar in these measures. This result was not evaluated statistically, however, and a more rigorous analysis could provide greater insight into potential divergence in vocal behavior.

To further investigate the evolution of indigobird non-mimetic vocalizations and their potential role in speciation, we used population-level sampling and appropriate statistical methods to analyze intra- and interspecific variation in the chatter calls and NM songs of four indigobird species in Tanzania. Our sampling allowed more robust measurements of NM song dialect neighborhood size than in previous studies, and allowed us to test whether variation in chatter and NM song traits is better explained by species identity (and therefore host association) or sampling locality. If species identity better explains variation in these traits then there may be evolved differences in vocal behavior among species, or vocalizations may be shaped by plastic responses to different developmental environments (i.e., being reared by different host species). Conversely, if locality is the best predictor of vocal characteristics, then local ecological adaptation and/or phenotypic plasticity in response to local environmental conditions may affect the different species in a given region similarly. Our results find support for both effects in different aspects of vocal behavior.

MATERIALS AND METHODS

Study Sites and Sampling

Fieldwork was conducted in the United Republic of Tanzania during April and May of 2008 and 2009. Four species of indigobirds occur in Tanzania (*V. chalybeata, V. codringtoni, V. funerea*, and *V. purpurascens*), with two morphologically distinct subspecies of *V. chalybeata* distributed in the interior central plateau (*V. c. centralis*) and "coastal" lowlands (*V. c. amauropteryx*) (Payne et al., 1992). Singing male indigobirds (n = 114) were recorded for ca. 20 min and then captured using song playback at sites within three political regions: Iringa, Morogoro, and Ruvuma (**Figure 1** and **Table 1**). Since local ecology can affect body size (Ashton, 2002), and thus vocal attributes (see citations below), **Table 2** summarizes the average elevation of call sites as well as temperature and precipitation during the breeding season (April–June) for each of these regions. Standard morphological measurements (see below) were taken for each male. *Vidua funerea* and *V. purpurascens* cannot be reliably discriminated based on morphology (i.e., plumage and

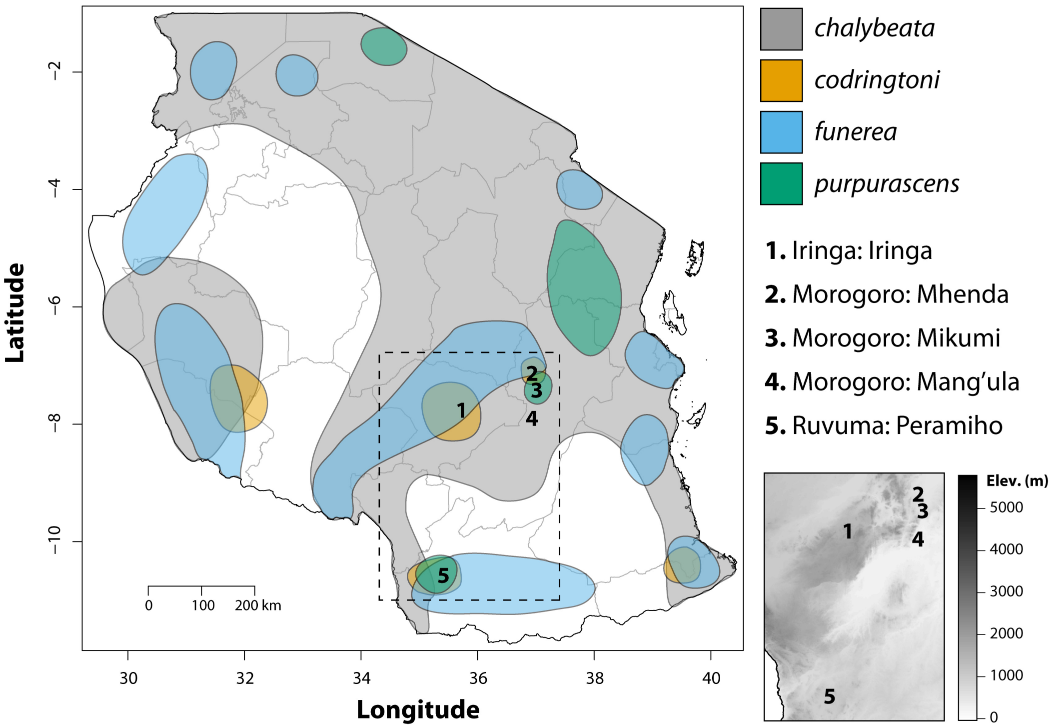

FIGURE 1 | Map showing the geographic distributions of the four indigobird study species in Tanzania adapted from Payne (2010). Numbers indicate sampling localities, interior gray lines show the boundaries of political regions, and the dashed box shows the location of the inset elevation map.

TABLE 1 | Locality and sampling information (*N* = number of recorded males).

Species	Host	Political region	Locality	Coordinates	*N*
Vidua chalybeata	*Lagonosticta senegala*	Iringa	Iringa	7.78° S, 35.70° E	18
		Morogoro	Mang'ula	7.87° S, 36.90° E	16
V. codringtoni	*Hypargos niveoguttatus*	Morogoro	Mhenda	7.20° S, 36.93° E	4
		Ruvuma	Peramiho	10.60°S, 35.35° E	17
V. funerea	*L. rubricata*	Iringa	Iringa	7.78°S, 35.70°E	14
		Ruvuma	Peramiho	10.60°S, 35.35° E	19
V. pupurascens	*L. rhodopareia*	Morogoro	Mikumi	7.39° S, 36.98° E	9
		Ruvuma	Peramiho	10.60°S, 35.35° E	17

TABLE 2 | Elevation, temperature, and precipitation data for sampled regions during the indigobird breeding season.

		Ave. high temp (°C)			Ave low temp (°C)			Ave precip (mm)		
Region	**Ave. elevation (m)**	**April**	**May**	**June**	**April**	**May**	**June**	**April**	**May**	**June**
Iringa	1588	24	24	23	15	14	12	57	10	1
Morogoro	431	30	28	27	20	19	16	198	79	19
Ruvuma	974	26	25	24	18	14	12	115	14	1

Climate data from www.climatedata.eu for the cities of Iringa, Morogoro, and Songea representing the Iringa, Morogoro and Ruvuma regions, respectively.

soft parts colors and morphometrics are all similar) in this part of East Africa, but can be differentiated by their mimicry of the unique songs of their respective hosts. Therefore, for the analyses presented here, individual males were assigned to species based on their host mimicry (i.e., host association).

Sharing of Non-mimicry Song Repertoires

The negative correlation between the extent of overlap in NM song repertoires between conspecific male indigobirds and geographic distance between their call sites has been quantified only for *V. chalybeata* (Payne, 1973, 1985), We tested the generality of this relationship by correlating geographic distance and repertoire sharing between all pairs of conspecifics within each political region. The latitude-longitude of each call site was recorded with a Garmin eTrex global positioning system (GPS), and the distances among call sites were calculated using the earth.dist function (fossil package) in R v4[1]. Song recordings for all individuals were visualized using Raven Pro v1.3 (Charif et al., 2008), and the first 100 NM songs were assigned to a song type (song type 1, 2, and 3, etc.) based on the different order and composition of syllables. NM songs are strikingly different from the clear whistles and calls of host species vocalizations that are well cataloged (see Payne, 1996), and each NM song is repeated in essentially identical form over the course of months not only by an individual male but also neighboring males that share the same songs (Payne, 1973). A sample of 100 songs is adequate to detect most or all of the distinct songs in each individual's repertoire (Payne, 1973; DaCosta, personal observation). Songs of nearly identical composition but with minor differences (e.g., a different number of introductory chatters or omission of a terminal note) were assigned to the same song type. Within each region, the similarity of NM song repertoires for each pair of conspecifics was quantified using the Jaccard index (Jaccard, 1901):

$$J_{ij} = \frac{S_{ij}}{R_i + R_j - S_{ij}},$$

where, the index J_{ij} varies from 0 to 1, S_{ij} is the number of shared song types, and R_i and R_j is the repertoire size of individuals i and j, respectively.

Body Size, Bill Size, and Call Site Elevation Measurements

The size and shape of a bird's body and bill affect its production of sound, and the evolution of these morphological traits can impose constraints on the frequency and pace of notes (Ryan and Brenowitz, 1985; Podos, 2001; Bertelli and Tubaro, 2002; Huber and Podos, 2006; Gillooly and Ophir, 2010). Body size may also be a sexually selected trait that indicates individual quality (Andersson and Iwasa, 1996), and in some birds it is positively correlated with repertoire size (Kipper et al., 2006; Hesler et al., 2012). We therefore measured body and bill size attributes of each individual and tested whether morphology significantly explains variation in NM song characteristics and repertoire size (see below). For each individual, JMD measured mass, wing length, tail length, tarsus length, bill length, bill width, and bill depth. Since many of these variables were correlated they were collapsed using a principal component analysis (PCA) in R (prcomp function).

Song characteristics may also vary with habitat if signals are optimized for transmission in the local acoustic environment (Morton, 1975; Nottebohm, 1985; Badyaev and Leaf, 1997). Indigobird males perch and sing at or near the tops of trees in relatively open habitats, so we did not measure structural aspects of the vegetation at each territory, but did record elevation above sea level for each call site using a GPS. Elevation, which is correlated with temperature and precipitation in Tanzania (**Table 2**), was used as a proxy for ecological differences among regions.

Measurements for Non-mimicry Songs and Chatter Calls

Non-mimicry songs were visualized and measured in Raven Pro. The first 100 NM songs recorded from each individual were assigned to a song type based on syllable composition (see above). The repertoire size of each male was calculated as the number of distinct song types observed in this sample (see **Supplementary Figure 1** for an example). The repertoire diversity of each male was quantified using the Shannon-Wiener index (Shannon, 1948; Wiener, 1948):

$$H^{'} = -\sum_{i=1}^{s} p_i ln(p_i),$$

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[1] www.r-project.org

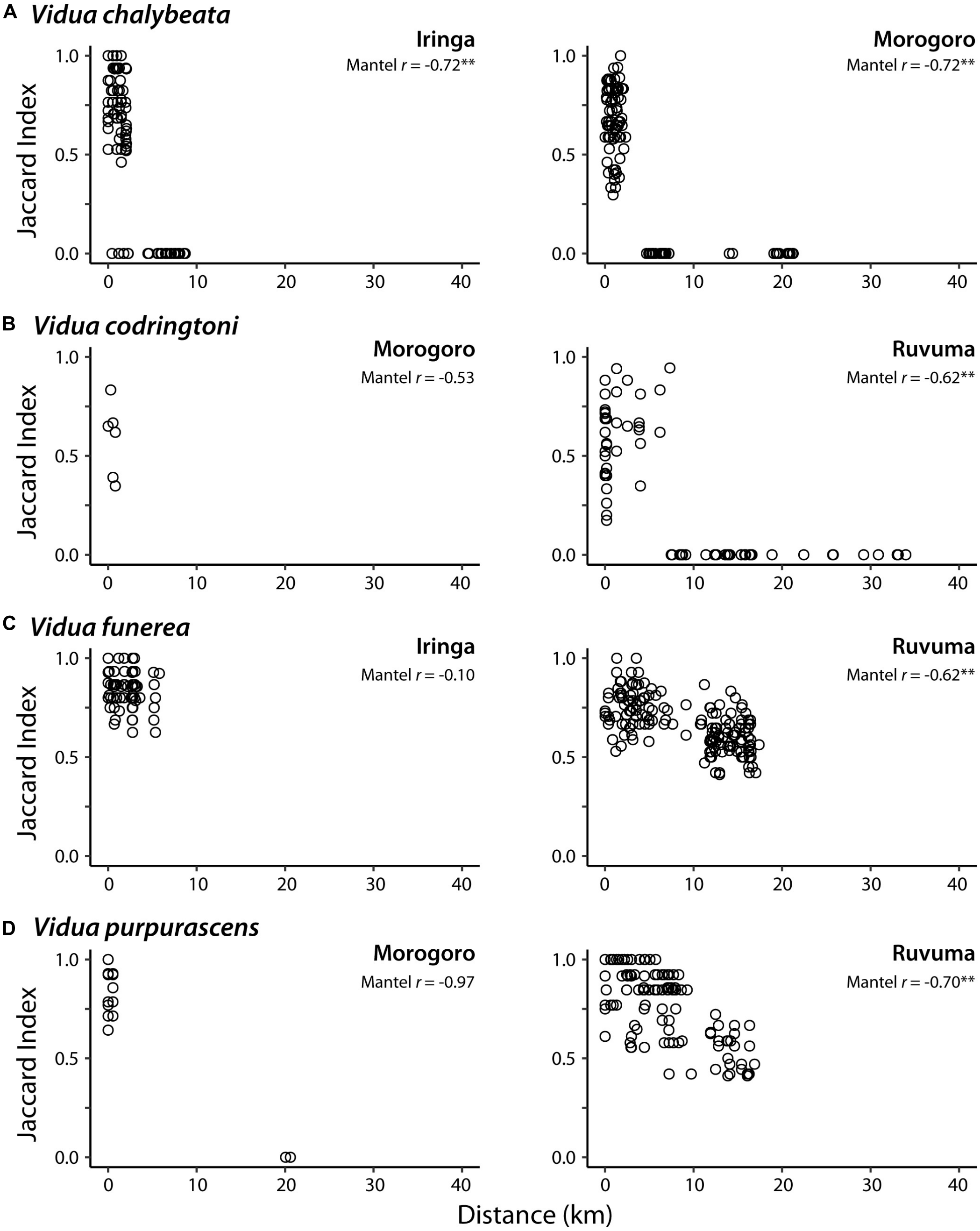

FIGURE 2 | Relationship between pairwise distance between call sites and non-mimicry song sharing (Jaccard index) for indigobird males in eight populations. Each species **(A–D)** was sampled from two of three regions: Iringa, Morogoro, and Ruvuma. Mantel r = Mantel coefficient; **Q-value < 0.01.

where, s is the number of distinct song types and p_i is the proportion of the ith song type in the sample. We subsampled 25 NM songs (every fourth song) for analyses of song characteristics. Introductory chatter notes often precede NM songs, and were included in song measurements only if they were found in every occurrence of a particular song type. For each song in the subsample, we applied a filter to remove low and high frequency background noise (below 500 and above 12,000 kHz, respectively) and then measured center frequency (the frequency that divides the song into two intervals of equal energy), maximum frequency (the frequency of highest energy), overall song length (in seconds), number of syllables, and pace (number of syllables per second). For each variable, measurements from the 25 songs were averaged to generate a single measurement of these traits for each individual. Some of these variables were highly correlated, so we summarized the data using a PCA.

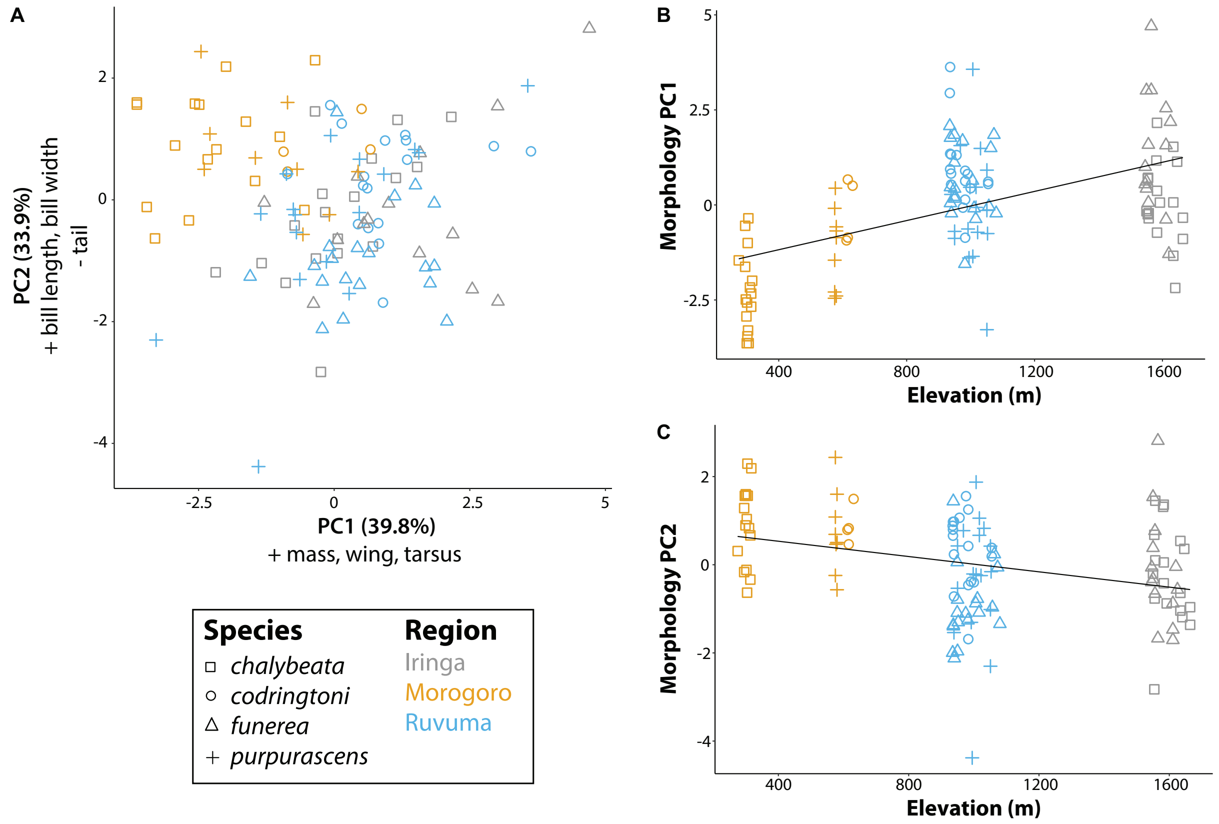

FIGURE 3 | Morphological variation of male indigobirds relative to elevation of their call sites. **(A)** The first two principal components of a PCA including measurements of mass, wing length, tail length, tarsus length, bill length, bill width, and bill depth. Variables with loadings > 0.4 (+) or < −0.4 (−) for each component are reported in the corresponding axis label. **(B,C)** Linear regressions for each of the first two morphology principal components with elevation as the explanatory variable. Lines show the least square regression line. Symbols and colors are used to denote different species and regions, respectively.

Chatter calls were also measured using Raven Pro with the same filter to remove background noise. The following characteristics from the first five chatter calls of each male were measured and averaged: center frequency, maximum frequency, overall length of the chatter, number of notes, and pace. These variables were also summarized with a PCA.

Statistical Analyses

All statistical tests were conducted in R v4. Within each species and geographic region, we tested for a correlation between pairwise measurements of geographic distance between call sites and song sharing (Jaccard index) by fitting a linear regression model in R (lm function). The significance of these regressions was quantified using a Mantel test (Mantel, 1967) with 1,000 permutations of the data in R (mantel function). PCAs to condense correlated song and morphometric variables were run with scaled variances (scale = TRUE) in R (prcomp function). For each of the first three principal components from the NM song PCA, we ran linear models (lm function) in R with the following factors: species identity, sampling region, morphology PC1, morphology PC2, and elevation. The anova and etaSquared functions were used to generate *P*-values and η^2 values for each factor. Similar linear models were run for the chatter principal components. For each linear model in which species or region was a significant factor, we used a Tukey-Kramer *post hoc* test (TukeyHSD function; Tukey, 1953; Kramer, 1956) to assess the significance of pairwise comparisons of species/regions. Across all Mantel, linear model, and linear regression analyses, we controlled the false discovery rate associated with multiple hypothesis testing by adjusting *P*-values into *Q*-values using the method of Benjamini and Hochberg (1995) in R (p.adjust function).

RESULTS

In pairwise comparisons of conspecifics within each region, sharing of NM songs was negatively correlated with the geographic distance between call sites, albeit with somewhat variable patterns among different species and localities (**Figure 2**). In Iringa and Morogoro, conspecific *V. chalybeata* males > 5 km apart had completely different repertoires (i.e., Jaccard index = 0) and were thus members of different dialect neighborhoods, whereas males within ∼2.5 km of each other typically shared 50–100% of their repertoires (**Figure 2A**). A similar pattern was observed for *V. codringtoni* males in

TABLE 3 | Linear model results for different measures of variation in the general characteristics of indigobird non-mimicry songs.

Response variable	Effect	η^2	*F*-value	*P*-value	*Q*-value
Repertoire size	Species	0.26	18.54	**<0.0001**	**<0.0001**
	Region	< 0.01	0.32	0.73	0.88
	Morphology PC1	< 0.01	0.16	0.69	0.87
	Morphology PC2	< 0.01	0.43	0.52	0.73
	Elevation	< 0.01	0.003	0.96	0.96
Repertoire diversity	Species	0.18	8.34	**<0.0001**	**0.0003**
	Region	0.01	2.51	0.09	0.18
	Morphology PC1	< 0.01	0.009	0.93	0.96
	Morphology PC2	< 0.01	1.90	0.17	0.29
	Elevation	0.02	2.72	0.10	0.20
PC1 (+song length, +no. syllables, –max frequency, and –center frequency)	Species	0.21	15.34	**<0.0001**	**<0.0001**
	Region	0.02	8.43	**0.0004**	**0.002**
	Morphology PC1	< 0.01	0.09	0.77	0.88
	Morphology PC2	< 0.01	0.07	0.79	0.88
	Elevation	0.04	6.22	**0.01**	**0.04**
PC2 (+center frequency, +no. syllables, +max frequency, and +song length)	Species	0.11	13.47	**<0.0001**	**<0.0001**
	Region	0.06	2.84	0.06	0.14
	Morphology PC1	< 0.01	0.24	0.63	0.84
	Morphology PC2	< 0.01	1.64	0.20	0.33
	Elevation	0.02	3.31	0.07	0.16
PC3 (+pace)	Species	0.19	32.96	**<0.0001**	**<0.0001**
	Region	0.06	18.59	**<0.0001**	**<0.0001**
	Morphology PC1	< 0.01	2.15	0.15	0.27
	Morphology PC2	< 0.01	0.004	0.95	0.96
	Elevation	< 0.01	0.11	0.74	0.88

Traits that contributed most (i.e., loadings > 0.4 or < –0.4) to each PC axis are indicated in parentheses below the respective PC. Positive and negative loadings mean that larger PC values are associated with larger or smaller trait values, respectively. P- and Q-values < 0.05 are shown in bold. F-value numerator degrees of freedom for each test: species = 3, region = 2, morphology PC1 = 1, morphology PC2 = 1, elevation = 1. Denominator degrees of freedom equals 105 for each test.

Ruvuma, with males separated by over 7 km not sharing any songs (**Figure 2B**). In contrast, both *V. funerea* and *V. purpurascens* had large dialect neighborhoods in Ruvuma, with conspecific males 15–20 km apart often sharing over 50% of their NM song repertoires (**Figures 2C,D**). Limited sampling and/or dispersion of individuals resulted in non-significant trends in the remaining comparisons. A single *V. chalybeata* male at Iringa was the only individual we sampled that did not share any songs with nearby males, resulting in Jaccard indices of zero for several pairwise comparisons with nearby males (**Figure 2A**).

Our analysis of morphological traits revealed significant effects of elevation (**Figure 3**). Overall body size, as captured by PC1 (39.8% of variation explained and positive loadings on all five morphometric traits), increased with elevation ($P < 0.001$; **Figure 3B**). PC2 (33.9% of variance explained), with positive loadings for bill length (loading = 0.51) and bill width (0.50), and a negative loading for tail length (−0.48), captured variation in bill size and tail length relative to overall body size and was also significantly correlated with elevation (P < 0.001). Thus, indigobirds from the lower elevation Morogoro region are smaller on average, but have relatively large bills and short tails. We used PC1 and PC2 scores for morphology as factors in linear models exploring variation in NM song and chatter attributes (see below).

Across all analyses of variation in NM songs, species identity was the most, and often only, significant predictor of variation (**Table 3**). *Vidua purpurascens* had significantly smaller and less diverse repertoires as compared to the other three species, whereas *V. chalybeata* and *V. codringtoni* had the largest and most diverse repertoires (**Figure 4**). We summarized other measures of variation in NM songs using PCA (**Figure 5A**). PC1 reflected differences in song length, number of syllables, maximum frequency, and center frequency with respective positive and negative loadings as follows : 0.52, 0.50, −0.51, and −0.47. Variation in PC1 scores was best explained by species identity ($F_{3,105} = 15.34$, *Q*-value < 0.0001), followed by sampling region ($F_{2,105} = 8.43$, *Q*-value $= 0.002$) and elevation ($F_{1,105} = 6.22$, *Q*-value $= 0.04$) (**Table 3**). Differences among species in PC1 scores (**Figure 5B**) indicate that *V. purpurascens* has longer songs with more syllables, which are sung at lower frequencies, whereas *V. codringtoni* has shorter songs delivered at higher frequencies. A smaller but significant regional effect was due to differences between Morogoro and Iringa (**Figure 5B**). For PC2 (31.9% of the variance explained with positive loadings on center frequency, number of syllables, maximum frequency, and song length), species identity was the only significant factor in the linear model (**Table 3**). As with PC1, *V. purpurascens* had the highest PC2 scores, whereas *V. chalybeata* had the lowest (**Figure 5C**). Because the same variables contribute to both PC1 and PC2, these axes are difficult to understand intuitively; results for the individual variables are shown in **Supplementary Figure 2**. Both species identity and sampling region were strongly significant predictors of variation in PC3 scores (**Table 3**), which captured pace (positive loading of 0.93). Among species, *V. chalybeata* and *V. codringtoni* sang the fastest and slowest songs, respectively, and songs were faster in Morogoro as compared to other regions (**Figure 5D**).

A comparable analysis of chatter calls revealed no species or regional differences in overall length or number of syllables, the variables contributing most to PC1 (39.8% of variance explained), but a few differences were detected for other measures of chatter call variation (**Table 4** and **Figure 6**). As captured by PC2 (33.9% of variance explained), chatter calls in *V. funerea* were significantly lower in frequency than in the other three species, whereas chatter calls in the Morogoro region were of significantly higher frequency (**Figure 6C**). Finally, *V. codringtoni* produced chatters with a significantly slower pace than in *V. chalybeata* and *V. purpurascens* (**Figure 6D**).

DISCUSSION

The complexity of indigobird vocal behavior is both a consequence and important catalyst of their evolutionary diversification as host-specific obligate brood parasites. As shown in earlier work (Payne et al., 1998, 2000), imprinting on host

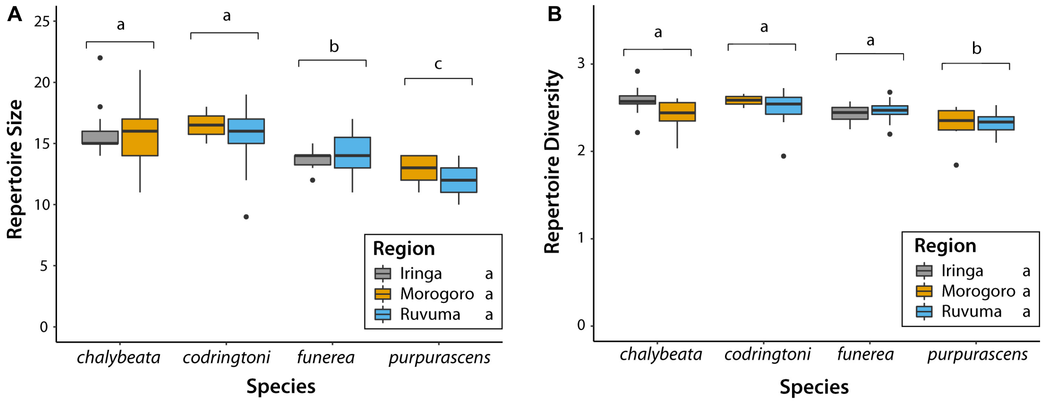

FIGURE 4 | Variation in **(A)** size and **(B)** diversity of non-mimicry song repertoires among indigobird species and sampled regions. Populations of the same species are grouped on the X-axis; boxplots are color-coded by sampling region. Species labeled with different lowercase letters (above brackets) differed significantly (adjusted $P < 0.05$) in *post hoc* tests. There were no significant differences among regions in these two measures.

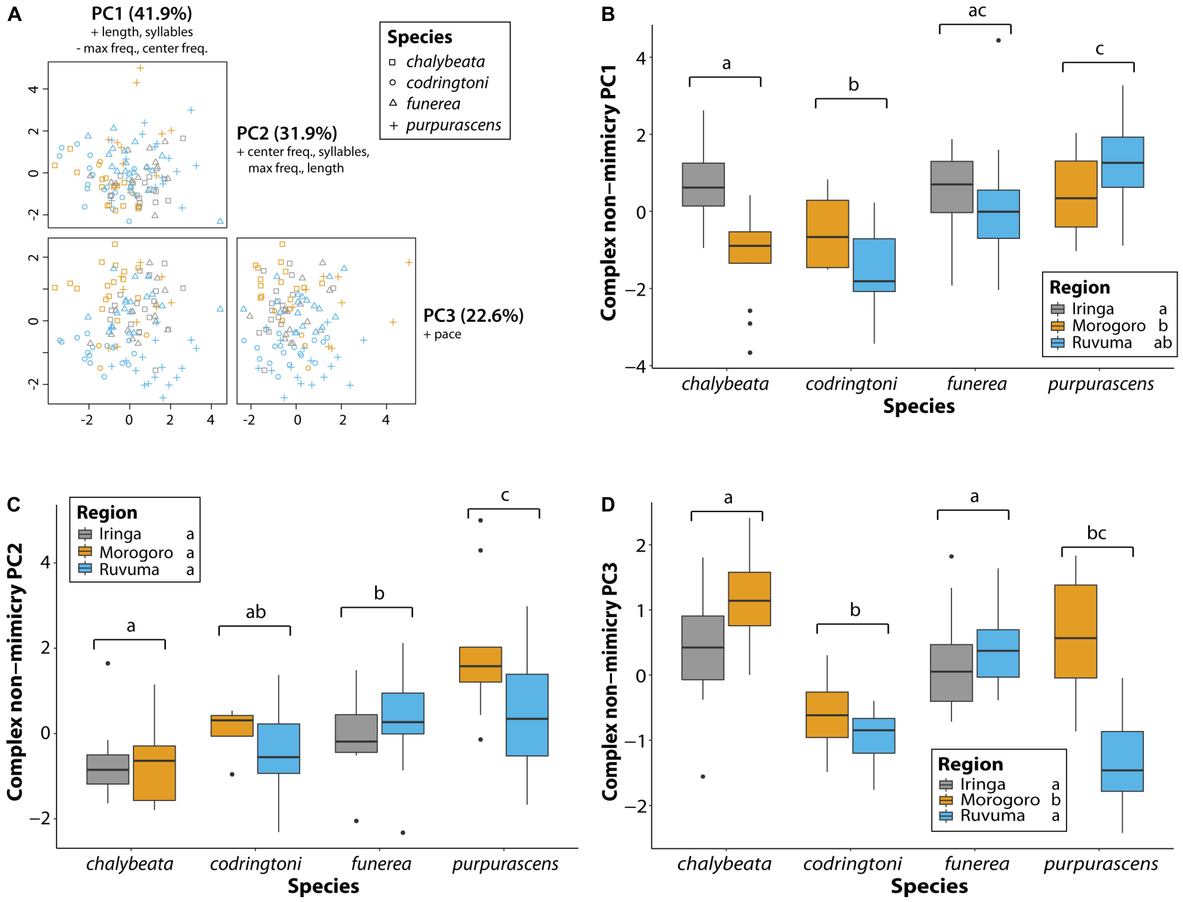

FIGURE 5 | Variation in general characteristics of non-mimicry songs between indigobird species and sampled regions. Song length, number of syllables, pace, center frequency, and maximum frequency measurements were collapsed via a principal component analysis **(A)**. Variables with loadings > 0.4 (+) or < -0.4 (–) are indicated for each principal component. Variation among species and regions in the first three PC axes is shown in panels **(B–D)**. Significant differences between species (adjusted $P < 0.05$) are indicated by different lowercase letters above the brackets for each species. Similarly, regions labeled with different lowercase letters were significantly different in *post hoc* tests.

TABLE 4 | Linear model results for measures of variation in the general characteristics of indigobird chatter calls.

Response Variable	Effect	η^2	*F*-value	*P*-value	*Q*-value
PC1 (+chatter length, +no. syllables)	Species	0.03	2.53	0.06	0.14
	Region	0.04	3.25	**0.04**	0.11
	Morphology PC1	<0.01	1.11	0.30	0.44
	Morphology PC2	<0.01	0.01	0.93	0.96
	Elevation	<0.01	0.002	0.96	0.96
PC2 (−center frequency, −max frequency)	Species	0.05	8.53	**<0.0001**	**0.0003**
	Region	0.03	13.39	**<0.0001**	**<0.0001**
	Morphology PC1	<0.01	0.61	0.44	0.64
	Morphology PC2	<0.01	0.35	0.56	0.76
	Elevation	0.02	2.48	0.12	0.23
PC3 (+pace)	Species	0.13	5.08	**0.003**	**0.008**
	Region	0.06	1.79	0.17	0.29
	Morphology PC1	<0.01	0.19	0.66	0.86
	Morphology PC2	<0.01	0.08	0.78	0.88
	Elevation	0.05	7.02	**0.009**	**0.03**

Traits that contributed most (i.e., loadings > 0.4 or < −0.4) to each PC axis are indicated in parentheses below the respective PC. Positive and negative loadings mean that larger PC values are associated with larger or smaller trait values, respectively. P- and Q-values < 0.05 are shown in bold. F-value numerator degrees of freedom for each test: species = 3, region = 2, morphology PC1 = 1, morphology PC2 = 1, elevation = 1. Denominator degrees of freedom equals 105 for each test.

vocalizations by young indigobirds shapes the vocal behavior of males as well as the mate and host preferences of females. In theory (Gavrilets, 2003), these features of indigobird social behavior are sufficient to account for reproductive isolation of indigobirds associated with different hosts, facilitating rapid, sympatric speciation when a new host is colonized (Sorenson et al., 2003). This reproductive isolation is imperfect (Payne and Sorenson, 2004; Balakrishnan et al., 2009), however, and indigobird species have diverged in other traits that are clearly under genetic control, including male plumage and soft part colors and mimicry of the intricate nestling mouth markings of their respective hosts (Payne, 2005; Jamie et al., 2020). These traits likely reinforce reproductive isolation. This study poses the question of whether indigobirds may have also diverged in measurable aspects of their vocal behavior (perhaps associated with divergence in female preferences, which would be difficult to test). While it was already known that indigobirds' chatter calls are broadly similar and that their complex repertoires of NM songs evolve through cultural evolution and are highly labile over space and time, previous work has not rigorously tested for possible differences in more general measures of indigobird vocalizations (e.g., average repertoire size, song length, frequency, and pace). While any observed differences between species in these traits could be due to either genetic divergence, cultural evolution, or developmental effects (e.g., different host nest environments), the lack of such differences would allow genetic divergence to be ruled out.

Male indigobirds have large repertoires of intricate NM songs, which they share and presumably learn from local conspecifics, whereas the NM song repertoires of different indigobird species inhabiting a local area are entirely non-overlapping. Our results confirm the complete turnover of within species repertoires across short distances, resulting in landscapes filled with relatively small dialect neighborhoods, but also show that there is considerable variation in this pattern among species and localities. For example, *V. funerea* and *V. purpurascens* both occurred at relatively high density in the Ruvuma region, where their respective host species appear to be more evenly dispersed across the landscape than in other regions (DaCosta, personal observation). This presumably leads to higher connectivity of these local populations and more interaction among competing males, as evidenced by conspecific males > 15 km apart still sharing at least half their songs. In contrast, *V. codringtoni* males establish territories adjacent to riparian thickets, which provide the preferred nesting habitat of their host (*Hypargos niveoguttatus*), and are unevenly distributed in the region. This results in lower connectivity among *V. codringtoni* call sites and smaller dialect neighborhoods; males > 7 km apart were associated with different river drainages and did not share songs. Similarly, in the Iringa and Morogoro regions, song sharing between conspecifics often dropped to zero with distances greater than ∼5 km. This may be due to greater patchiness of suitable habitat for host species in these regions, leading to lower connectivity between call sites of their respective indigobirds.

Despite some variation, NM song dialect neighborhoods of all indigobirds are quite small on a regional scale, such that cultural transmission is unlikely to account for any consistent differences in NM song characteristics between indigobird species. Nonetheless, our analyses revealed significant species effects for all of the general measures of NM song variation we considered (**Table 3** and **Figures 4**, **5**). The NM song repertoires of *V. purpurascens* were the smallest and least diverse, but comprised longer songs with more syllables. Conversely, *V. chalybeata* and *V. codringtoni* had larger, more diverse repertoires featuring shorter songs with fewer syllables. *Vidua funerea* was intermediate for NM song repertoire size and for principal components that included song length and frequency measures (**Figure 5**). The apparent negative correlation between repertoire size and song length suggests a tradeoff between these traits, perhaps because the volume of brain nuclei involved in song learning or production imposes constraints on the overall complexity of the full repertoire (Devoogd et al., 1993). Particular NM song types are associated with courtship (Payne and Payne, 1977; Payne, 1979), and sexual selection in combination with this constraint may contribute to differences among species. For example, if female *V. purpurascens* prefer longer songs, this could result in smaller repertoires for this species. Sampling region was also a significant factor explaining variation in PC1 (song length, number of syllables, center frequency, maximum frequency) and PC3 (pace), with birds from Morogoro delivering shorter, lower frequency songs at a faster pace (i.e., syllables/second) (**Figure 5**). Call sites in Morogoro were at lower elevations,

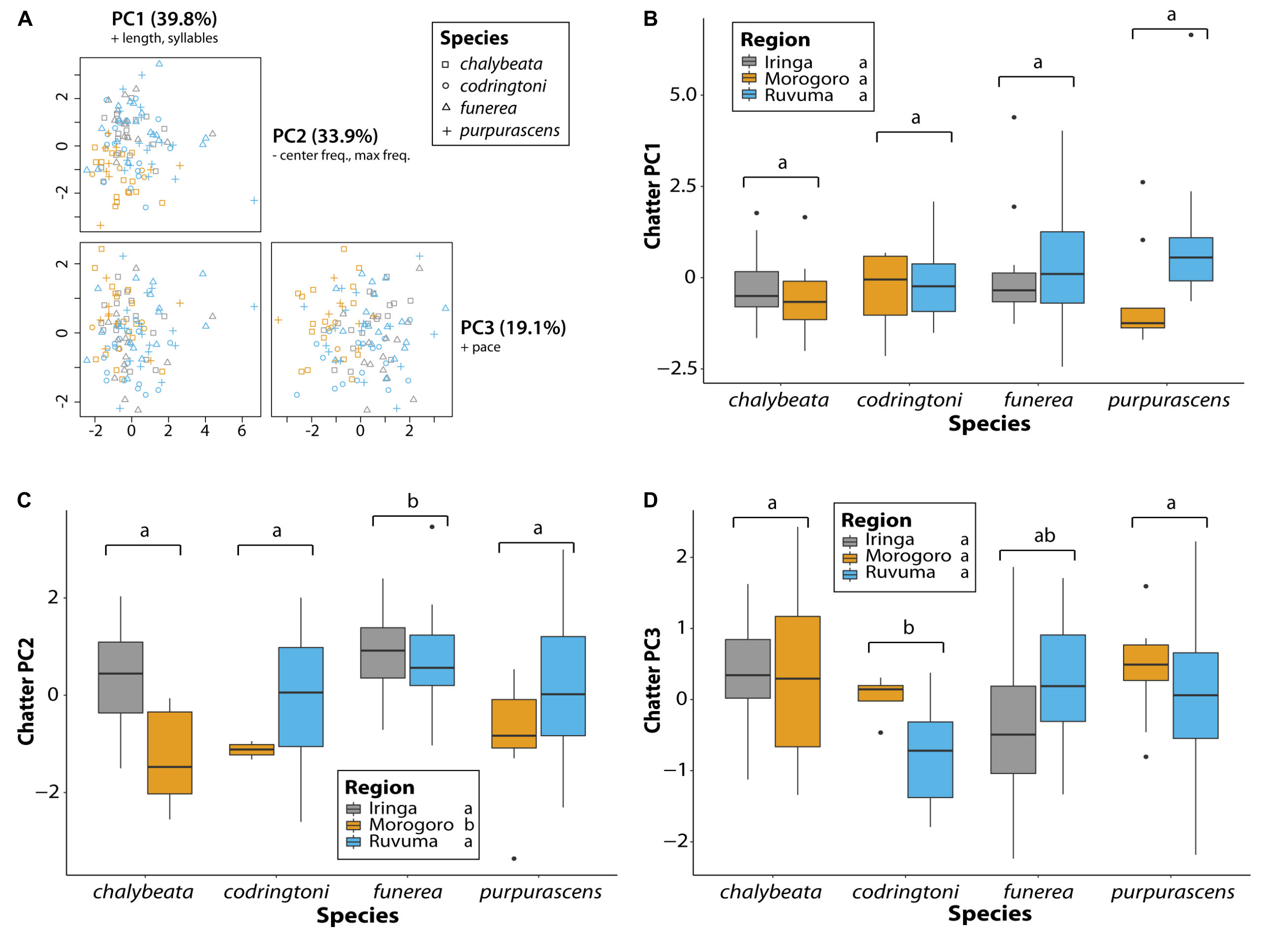

FIGURE 6 | Variation in chatter calls between indigobird species and sampled regions. Chatter length, number of syllables, pace, center frequency, and maximum frequency measurements were collapsed via a principal component analysis **(A)**. Variables with loadings > 0.4 (+) or < −0.4 (–) are indicated for each principal component. Variation among species and regions in the first three PC axes is shown in panels **(B–D)**. Significant differences between species (adjusted $P < 0.05$) are indicated by different lowercase letters above the brackets for each species. Similarly, regions labeled with different lowercase letters were significantly different in *post hoc* tests.

and indigobirds in this region were of smaller body size, but with relatively large bills and short tails (**Table 2** and **Figure 3**). These results support a role for local ecology in shaping morphometrics and in turn NM song characteristics, but for both of these principal components species identity explained a larger proportion of the variance. Moreover, species identity was the only significant factor in analyses of PC2 scores, as in analyses of repertoire size, and repertoire diversity. Thus, these results indicate consistent differences between indigobird species in NM song characteristics even though distant populations of the same species share no specific songs in common.

Despite the recent diversification of indigobird species, these differences might reflect divergent evolution of NM songs in much the same manner as indigobird species have diverged in other traits that likely play a role in mate choice and species recognition (i.e., plumage and soft parts colors). Alternatively, species differences in NM song attributes may reflect the phenotypic effects of being reared by different host species. In addition to possible effects on body size, learning, and mimicking the songs of a particular host species, a critical component of the social and breeding behavior of indigobirds (Payne, 1973, 1979; Payne et al., 2000), might influence the characteristics of the indigobird's NM songs. For example, males reared by hosts with small repertoires may have greater neural capacity available for acquiring and memorizing large repertoires of NM songs, and/or indigobirds associated with hosts that produce songs with particular characteristics (e.g., high frequency or rapid delivery of notes) might develop neural circuits that favor NM songs with similar attributes. Qualitative assessments provide some support for these possibilities. The red-billed firefinch (*Lagonosticta senegala*) has the smallest repertoire among the host species in Tanzania (Payne et al., 1992), and individual males of its indigobird parasite (*V. chalybeata*) had relatively large repertoires of NM songs (**Figure 4A**). Among the hosts of the indigobird species we sampled, the Peter's twinspot (*Hypargos niveoguttatus*) produces vocalizations with the highest frequencies, and the NM songs of their indigobird parasite (*V. codringtoni*) also tend to be higher in frequency as compared to other indigobirds

(**Figures 5B,C**). Jameson's firefinches (*Lagonosticta rhodopariea*) produce unusually rapid alarm calls with notes delivered at rates of 22+ per second (Payne, 1996). Males of the indigobird parasitizing this species (*V. purpurascens*) mimic these alarm calls precisely, and their NM songs have a fast pace in Morogoro but not in Ruvuma (**Figure 5D**). A rigorous test of whether learning and mimicking the vocalizations of particular hosts shapes indigobird NM songs in predictable ways should include quantitative analyses of both host and parasite song recordings from multiple localities, and sampling of all ten indigobird species and their respective hosts to increase the power of the analysis.

Generally, chatter calls were more similar across both species and sampled regions as compared to NM songs (**Table 4** and **Figure 6**). Species identity was a significant factor explaining variation in PC2 and PC3 scores, whereas sampling region was significant only for PC2. One notable species difference was a slower pace of chatter calls in *V. codringtoni* (**Figure 6D**), mirroring the slower pace of its NM songs (**Figure 5D**) and suggesting the possibility of a common underlying mechanism. Nonetheless, our findings are consistent with the conclusion of Payne (1973) that chatter calls are broadly similar across species, supporting the hypothesis that chatter may represent an innate signal or password (*sensu* Hauber et al., 2001) that allows young male indigobirds to recognize and associate with other indigobird males, and then, using host song mimicry to identify conspecifics, select appropriate tutors for acquiring their NM songs. While an alternative mechanism by which male indigobirds could acquire the appropriate local dialect is not obvious, we note that the above scenario has not been experimentally tested.

Our analyses revealed consistent differences among indigobird species in general features of their non-mimetic songs despite the fact that conspecifics, including those in relatively close geographic proximity (e.g., 5–20 km) and those in different regions, may have entirely different repertoires of specific songs (i.e., sequences of notes), and despite the influence of significant environmental effects likely mediated through effects on body size and relative bill size. Divergent selection on female preferences and/or male sound production might contribute to the observed differences, but we speculate that phenotypic plasticity and, more specifically, the effects of learning and mimicking the songs of different hosts likely explains most of the differences we observed. Notably, *V. funerea* and *V. purpurascens* are broadly syntopic at Ruvuma but are associated with different hosts and have NM song repertoires that differ significantly in the length and pace of songs (**Supplementary Figure 2**) as well as repertoire size and diversity (**Figure 4**). Notably, allopatric populations of *V. funerea* and *V. purpurascens* are similar to their respective conspecific Ruvuma populations in repertoire size and diversity. The two populations at Ruvuma, however, are morphologically and genetically indistinguishable (DaCosta, 2014), which is not typically the case for sympatric indigobird species. Thus, genetic divergence between these populations is unlikely as an explanation for the observed differences in song traits. Regardless of the relative contributions of genetic divergence and phenotypic effects, the species differences in song traits documented in this study may contribute to reproductive isolation among nascent and recently evolved indigobird species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Boston University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JD and MS conceived and designed the study, collected indigobird vocalization recordings, and wrote the manuscript. JD conducted data analyses. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.725979/full#supplementary-material

Supplementary Figure 1 | Repertoire of non-mimicry vocalizations from a representative *Vidua purpurascens* male from the Ruvuma region. **(A)** Sampling of 100 non-mimicry songs recovered a repertoire size of 14 songs. Numbers above songograms show the number of times each song appeared in the sample of 100. **(B)** Three representative chatters to display characteristics of the calls and variation in length.

Supplementary Figure 2 | Variation in measured characteristics of non-mimicry songs before variables were condensed with a principal component analysis.

Supplementary Figure 3 | Variation in measured characteristics of chatter calls before variables were condensed with a principal component analysis.

Supplementary File 1 | DaCostaSorenson_measurements.xlsx. Measurements of morphological, non-mimicry song, and chatter characteristics for each indigobird (n = 114) sampled in the study.

Supplementary File 2 | DaCostaSorenson_song_sharing.xlsx. Jaccard index calculations of non-mimicry song sharing among conspecific indigobirds, with a separate worksheet for each region.

Supplementary File 3 | DaCostaSorenson_PCA_results.xlsx. Results (rotations, importance of components, and eigenvalues) for PCA analyses of morphology, chatter, and NM songs.

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The Role of Intra-Clutch Variation of Magpie Clutches in Foreign Egg Rejection Depends on the Egg Trait Considered

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The existence of a coevolutionary process between avian brood parasites and their hosts predicts a lower intra-clutch variation in egg appearance of host eggs among rejecters as this would favor egg discrimination of parasite eggs by hosts once parasitic egg mimicry had evolved. So far empirical tests of this prediction have ignored the fact that different aspects of host egg phenotypes may differ in the relative role of environmental vs. genetic determination, and hence that the role of intra-clutch variation in egg rejection within a population cannot be invariant. Here, we estimated whether the intra-clutch variation in several aspects of host eggshell features is consistently associated to rejection of parasitic foreign eggs across years in a magpie host population parasitized by great spotted cuckoos. We innovatively estimated spottiness by means of the fractal dimension of eggs, which considers the homogeneity of spot pattern complexity in eggshells. Our results show that low intra-clutch variation in the blue-green coloration at the middle area of the eggs associated with a high chance of rejection, but only in one of the 3 years we conducted the study. In addition, females that rejected foreign eggs presented more homogenous spot patterns in their clutches as estimated by their fractal dimension than females that accepted experimental eggs, independently of the year of study. Finally, intra-clutch variation in egg volume of host eggs was not associated to rejection. Analyses at the individual level revealed that the relative role of genetic vs. environmental factors that determine egg phenotype would be feature-specific in magpies, females having a characteristic spottiness, but not color or volume, pattern. Our work stresses the importance of considering a holistic approach including several aspects of variation in host egg phenotype (size, color, and homogeneity of spot pattern), as some aspects might be more susceptible to selection through egg rejection than others, presumably because they are less influenced by variation in the environmental conditions. Moreover, our study highlights the importance of replication in studies on the adaptive value of host traits in egg rejection.

Keywords: brood parasitism, color, fractal dimensions, repeatability, egg phenotype and spottiness

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INTRODUCTION

Avian brood parasites impose dramatic costs on the reproductive success of their hosts, which has led to the evolution of counter-adaptations to circumvent them (Rothstein, 1990; Davies, 2000). One of the most widespread defenses against parasitism among hosts is the discrimination and rejection of parasite eggs from their nests. Egg discrimination reduces the harm caused by brood parasitism, and selects for egg mimicry in the parasite eggs to counter host defenses. Once brood parasites have evolved eggs that mimic those of their hosts, a further step in the arms race would be the evolution of a smaller degree of intra-clutch variation in the appearance of host eggs, as this could facilitate hosts to discriminate parasitic eggs (Victoria, 1972; Øien et al., 1995; Soler and Møller, 1996; Moskát et al., 2008). After more than two decades of empirical work examining the key prediction of this hypothesis (the intra-clutch variation hypothesis) in different host-brood parasite systems, the literature is equivocal regarding whether homogeneity in host egg appearance favors or not the discrimination of parasitic egg. Some studies have shown that females with lower intra-clutch variation were more likely to reject artificial foreign eggs as it would be expected (Stokke et al., 1999; Soler et al., 2000; Peer et al., 2010; Wang et al., 2016), but others found the opposite trend (Lotem et al., 1995; Avilés et al., 2004). Noteworthy, in some studies host intra-clutch variation was no associated with the probability of rejecting model foreign eggs (Karcza et al., 2003; Croston and Hauber, 2015). Discrepancy in the pattern also arises when the eggs used in experiments were conspecific or real cuckoo eggs (Procházka and Honza, 2003; Moskát and Lovászi, 2004; Stokke et al., 2004; Cherry et al., 2007; Landstrom et al., 2010; Polačiková et al., 2011; Abernathy and Peer, 2014).

Most of these studies have in common that they have been performed in one single population and study year providing a "snapshot" of the role of intra-clutch variation in rejection of foreign eggs. This approach implicitly ignores the fact that the different features of the avian egg phenotype, such as egg-shell color, degree and pattern of spottiness and egg volume, although partly genetically determined (Gosler et al., 2000; Liu and Cheng, 2010), also depend on climatic conditions, female condition or local availability of dietary components necessary for the synthesis of pigments included in the egg-shell (Moreno and Osorno, 2003; Avilés et al., 2007; Hubbard et al., 2010; Berkowic et al., 2015). The relative role of the genetic vs. the environmental component might hence be feature- or species-specific, which may have hampered our abilities to obtain reliable and reproducible patterns about the role of egg homogeneity for rejection. The effect of environmental conditions on egg phenotypes may even differ between brood parasites and their hosts rendering changes in mimicry that may potentially affect rejection (Avilés et al., 2007). Therefore, if, as it is common in terrestrial environments, conditions change from 1 year to another, the role of intra-clutch variation in egg rejection might differ between breeding seasons and for the different aspects of the egg's phenotype. So far, very few studies have addressed changes in eggshell color or spottiness in time (Geltsch et al., 2017) in the context of the coevolution of host and parasite eggs.

Illustrating this issue, two studies on the role of intra-clutch variation in egg rejection in magpie (*Pica pica*) hosts have given opposite results (Soler et al., 2000; Avilés et al., 2004). Although both studies used different methods to estimate intra-clutch variation (photography vs. spectrophotometry), neither was replicated raising the possibility that differences between studies were merely due to environmental effects. Replication is important to draw general patterns and reach well-founded conclusions, and although replicates are not common in behavioral ecology research (Kelly, 2006, 2019; Nakagawa and Parker, 2015); they have often led to different and inconsistent results (Kelly, 2006). Replication has allowed the identification of factors that may affect egg rejection in magpies, such as female age (Molina-Morales et al., 2014; Martínez et al., 2020), that were previously discarded (Soler et al., 2000). Here, aiming to qualify the importance of replication in the assessment of cuckoo-host evolutionary hypotheses, we have estimated homogeneity (that is, intra-clutch variation) in host egg phenotype and tested egg rejection in a magpie population parasitized by the great spotted cuckoo (*Clamator glandarius*) during 3 consecutive years. We estimated the homogeneity of egg volume based on egg measurements, homogeneity of eggshell coloration using spectrophotometry, and, innovatively, estimated homogeneity of spottiness using the fractal dimension of eggshells calculated from pictures of the clutches. The fractal dimension (FD hereafter) is a parameter that measures complex patterns in objects whose value is influenced by properties that include number, length, turgidity, and connectivity of elements within a given object (Mandelbrot, 1983). FD is scale invariant providing a single value that summarizes the way the pattern "behaves" across scales (Mandelbrot, 1983; Jovani et al., 2013), and it has been used to describe complex color patterns or designs in living organisms, such as the complex color patterns of sexual ornaments in birds (Pérez-Rodríguez et al., 2013; Cantarero et al., 2018), the porosity of avian eggs (Zhang and Wang, 2012) or the homogeneity of spottiness in bird eggs and the amount of protoporphyrin deposited on the eggshell (Gómez et al., 2019, 2021).

Our goal is twofold: we examine the association between intra-clutch variation and egg rejection using different aspects of egg phenotype and multiple years, taking a more holistic approach to the problem. In order to better understand previous inconsistent results and the between-year variability in our results, we also study the variability of egg features across years, and analyze the repeatability of these in a subset of females that bred more than 1 year in our population, as a basis to determine trait-specific sensitivity of eggs to environmental variation. We predict that high homogeneity in egg appearance (low intra-clutch variation) is associated with rejection of foreign eggs when the traits are less plastic and more female-specific.

METHODOLOGY

Study Area and System

The study was conducted in La Calahorra (37° 10′ N, 3° 03′ W, Hoya de Guadix, Granada, Southern Spain) during the years 2016–2018. It is a patchy area of about 12 km^2

where groves of almond trees (*Prunus dulcis*), in which magpies preferentially build their nests, are very common. Magpies are territorial, sedentary, and socially monogamous long-lived passerines (Birkhead, 1991). In our study area magpies lay one clutch during April-May, and are the main host of the great spotted cuckoo (*Clamator glandarius*).

Monitoring Magpie Individuals and Nests

We started to monitor the nests once the first pair starts nest building, usually at the beginning of March and continue until July when the breeding season ended. Once a nest is found we visit it once a week to detect egg laying. Nests were found by careful inspection of all trees in the area, GPS positioned and then visited at 5 days intervals. In order to determine whether nests were parasitized, during egg laying and hatching, they were visited every 2–3 days. We considered that nests were parasitized when at least one cuckoo egg was found in them. Magpies in our study area only reject about 5% of real cuckoo eggs (see Soler et al., 1995), and so the risk of not detecting parasitized nests (because magpies had rejected the cuckoo eggs quickly) is very low. The information recorded on each nesting attempt included laying date (that we expressed as the number of days from the first of April), number of cuckoo and magpie eggs, and number of cuckoo and magpie nestlings that fledged. Once the clutch was completed we registered several measures that have been used to calculate intra-clutch variation in egg appearance (see below). We monitored 13 nests in 2016, 53 nests in 2017 and 71 in 2018.

Egg Rejection Experiment

We tested magpie response to mimetic model eggs to classify females as acceptors or rejecters (e.g., Soler and Møller, 1990; Soler et al., 1999). Mimetic model eggs were made of plaster of Paris mixed with white glue and painted with acrylic paints. Model eggs were made to resemble the cuckoo eggs in appearance, size, and mass to the human eye, although they do not perfectly match the color of real great spotted cuckoo eggs when measured with a spectrophotometer (see Molina-Morales et al., 2014; **Figure 1**). We introduced one mimetic model egg during magpie egg laying and revisited the nest after 6–7 days. Previous work in our magpie population has shown that 75% of all rejection of artificial models occurs in the first 24 h after parasitism and that after 72 h all eggs have been rejected (Avilés et al., 2004). The response was coded as rejection if the model egg disappeared from the nest or acceptance if the model egg was incubated with the host's clutch. It could be argued that the day on which the model egg is introduced into the clutch could affect females' evaluation of intra-clutch variation if intra-clutch variation estimated on few host's eggs was not representative of intra-clutch variation estimated on the entire host clutch. To rule out this possibility, we analyzed the level of correlation between intra-clutch variation estimated on three randomly selected eggs and intra-clutch variation estimated from the whole clutch across a sample of 90 clutches. Since intra-clutch variation in the three parts of the eggs is correlated (**Supplementary Table 1**) we performed the analyses using the measurements taken the sharp pole. The level of correlation was superior to 0.63 for the three PCs suggesting that intra-clutch variation for some eggs is similar to the one calculated for the entire clutch (PC1 $r = 0.63$, $t_{90} = 7.70$, $P \leq 0.001$; PC2 $r = 0.69$, $t_{90} = 9.04$, $P \leq 0.001$; PC3 $r = 0.70$, $t_{90} = 9.19$, $P \leq 0.001$). Therefore, the fact that females may have partially or fully evaluated the clutch should not affect our conclusions.

FIGURE 1 | Magpie clutches parasitized by Great spotted cuckoo. The picture shows magpie eggs, real cuckoo egg, and experimental model eggs. **(A)** Eight magpie eggs, one great spotted cuckoo egg and mimetic model egg in the lower right corner. **(B)** Seven magpie eggs, one great spotted cuckoo egg near to mimetic model egg in the lower right corner.

Intra-Clutch Variation in Egg Appearance

We considered intra-clutch variation in three aspects of avian egg phenotype known to influence foreign egg rejection in different brood-parasite hosts systems, namely egg volume (Marchetti, 2000), spottiness (Moskát et al., 2008), that we innovatively quantify using the Fractal dimension and, egg coloration (Rothstein, 1982; Soler et al., 2000; Lyon, 2003).

- Intra-clutch variation in egg volume was estimated as the standard deviation of egg volume (calculated on length and width of each egg applying Hoyt, 1979 formula) in each clutch.

- Intra-clutch variation in FD of spottiness. Once a magpie clutch was completed it was photographed using a CANON 350D digital camera (Canon Inc., Tokyo, Japan). Images were stored in jpeg format and a metric reference (pixel to mm conversion factor) was automatically obtained by extracting a red patch of known size present in all the images. Settings were adjusted automatically depending on light conditions. We were not able to work in the real color domain (equivalent reflectance images) because neither raw images were available nor gray targets of known reflectance were present in the images in order to linearize them. Therefore, we opted for transforming the images into their CIE $L^*a^*b^*$ 1976 [ISO 11664-4:2008(E), 2007] versions and operated only over the lightness channel L^*. To process these images, we modified the spot detection algorithm included in SpotEgg (Gómez and Liñán-Cembrano, 2017) by defining a local threshold for every pixel in the lightness channel. For every pixel, we created an adaptive threshold

(Bradley and Roth, 2007) to determine whether the pixel belongs to the background or spot domains in which, instead of the most common 1/8th of the size of the image to determine the size of the windows that define the region where local thresholds are calculated, we considered 1/20th of the length of each egg, for a larger sensitivity. The obtained spot images were latter filtered in size using mathematical morphology operations to eliminate spots whose larger axis is smaller than 1/200 the length of the egg. Finally, the black and white spot images were passed to the Fractal Dimension (FD) calculation function in SpotEgg which employs the Minkowski–Bouligand definition (Schroeder, 1991) to determine FD, commonly known as the box-counting technique.

-Intra-clutch variation in egg coloration. At the same time we took photographs, we also measured egg coloration by spectrophotometry in the field. We obtained reflectance spectra in the 360–740-nm range from 888 eggs of 137 clutches, using a Konica Minolta CM250 spectrophotometer. Color was always measured five times; the first one in the sharp pole, three measures in the area in-between the two poles and another one in the blunt pole; each measure was circa 1 mm^2. We calculated the mean value from the three measures in the area in-between poles (middle area hereafter). Measurements were relative and referred to a standard white reference (WS-2) and to the dark. Reference white and dark calibrations were made before the beginning of the measurements of each clutch.

We have used Principal Component Analyses (PCA) for describing variation in eggshell color based on reflectance data (Cuthill et al., 1999; Cherry and Bennett, 2001). A PCA was performed on raw reflectance data (five measures for each magpie egg) to reduce the number of correlated variables (39 variables as reflectance values were taken at 10 nm intervals between 360 and 740-nm range) into a few orthogonal variables summarizing color variation (see for instance Cuthill et al., 1999; Avilés et al., 2006). PCA allowed us to distinguish between achromatic "brightness" variation represented by the first principal component (PC1) and chromatic variation represented by PC2 and PC3 (Endler and Thery, 1996). Together these three first components explained 99.82% of the total variance in spectra of magpie eggs. PC1 was flat and described achromatic variation explaining 94.9% of the overall variation. PC2 and PC3 were not spectrally flat and together they accounted for 85.1% of the chromatic variance (see **Figure 2**). PC2 had high and positive loadings at short wavelengths and high negative loadings at long wavelengths and could therefore classify the eggs sampled along a gradient of long ultraviolet-red reflectance. PC3, however, had high positive loadings approximately at the blue-green (475–550 nm) wavelength and thus could be described as a bluish-greenness gradient. To estimate the degree of intra-clutch variation in coloration, we calculated the standard deviation of brightness (PC1 scores), ultraviolet-red color (PC2 scores) and blue-green color (PC3 scores) based on average values per egg in each magpie clutch.

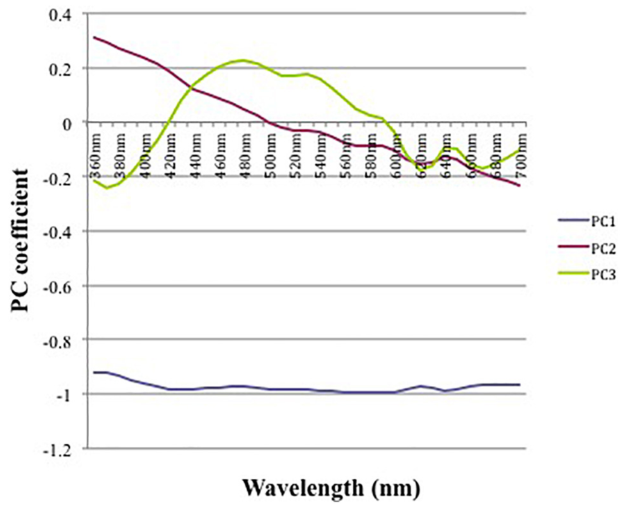

FIGURE 2 | Principal components in relation to wavelength, derived from reflectance spectra from magpie eggs in each clutch. PC1 describes achromatic variation explaining 94.9% of the overall variation. PC2 and PC3 accounted for 85.1% of the chromatic variance. PC2 indicates ultraviolet-red colors and PC3 blue-green colors.

Statistical Analyses

Aiming to qualify the environmental nature of egg homogeneity we fitted four Mancova models. First, we considered intra-clutch variation in volume and FD as dependent variables and included clutch size and laying date as predictors and year as a fixed factor. In a second Mancova model we considered intra-clutch variation in all color (PCs) as dependent variables, and the predictors in the first model. Finally, we repeated the same model structure but using the mean values of egg volume and FD in a third Mancova model, and the mean values of color, in a fourth Mancova model, respectively. Moreover, we analyze within-individual patterns in intra-clutch variation on a subset of 21 females that bred more than once in our population and calculated repeatability of intra-clutch variation for each trait using a variance components analysis. All together these analyses aimed to help us to qualify the potential for selection of different egg features by estimating to what extent the different features are environmentally vs. genetically determined.

We fitted binomial generalized linear models (GLM) using SAS (SAS Institute, Cary, NC, United States) with rejection as dependent variable to analyse the role of intra-clutch variation in the different features considered. We run separated models for egg volume, FD of spottiness and color as predictors because a preliminary model including variation in these features, study year and their interactions did not converge. All models included study year as a fixed term and the interaction between study year and intra-clutch variation in the corresponding trait. We also entered standardized laying date as covariate, to control for possible differences in female quality (Thorley and Lord, 2015). In this system, parasitized nests have smaller clutch sizes because cuckoo females break some host eggs while parasitizing (Soler et al., 1996; Molina-Morales et al., 2013). Indeed, in this study the clutch size of parasitized nest was significantly smaller than that of non-parasitized ones [$F_{(1,\ 135)} = 17.56$ $P = 0.00005$; Non-parasitized nests, mean = 6.4, SE = 0.11

n = 103; Parasitized nests mean = 5.38, SE = 0.26 n = 34). Besides be linked to parasitism, clutch size may *per se* affect intra-clutch variation (de Hierro and De Neve, 2010). Therefore, to avoid collinearity we opted to include clutch size, instead of parasitism, as covariate.

The model analyzing the influence of intra-clutch variation in the FD also included average spottiness as a covariate given that it is known that the FD depends on the degree of spottiness (Pérez-Rodríguez et al., 2013; Gómez et al., 2021). When dealing with color variation, we fitted separated models for the blunt pole, the sharp pole and the middle part to avoid collinearity issues, given that the degree of correlation between measures of intra-clutch variation in egg color at the three egg areas were highly positively correlated to each other (Polačiková et al., 2011; see **Supplementary Table 1**).

RESULTS

Overall, we assessed the response toward model eggs in 137 magpie breeding attempts in the 3 years of study. In this study, 30 out of 137 (21.89%) mimetic model eggs were rejected (2016: 23.07%; 2017: 18.8%; 2018: 23.9%).

Yearly and Individual Variation in Egg Traits

Although intra-clutch variation in egg volume and FD [$F_{(4, 248)}$ = 0.443, P = 0.777] and color features (PCs) [$F_{(18, 248)}$ = 0.704, P = 0.805] did not change between the years (**Supplementary Table 2**), the average phenotype of magpie eggs in the population changed (**Supplementary Table 3**). Specifically,

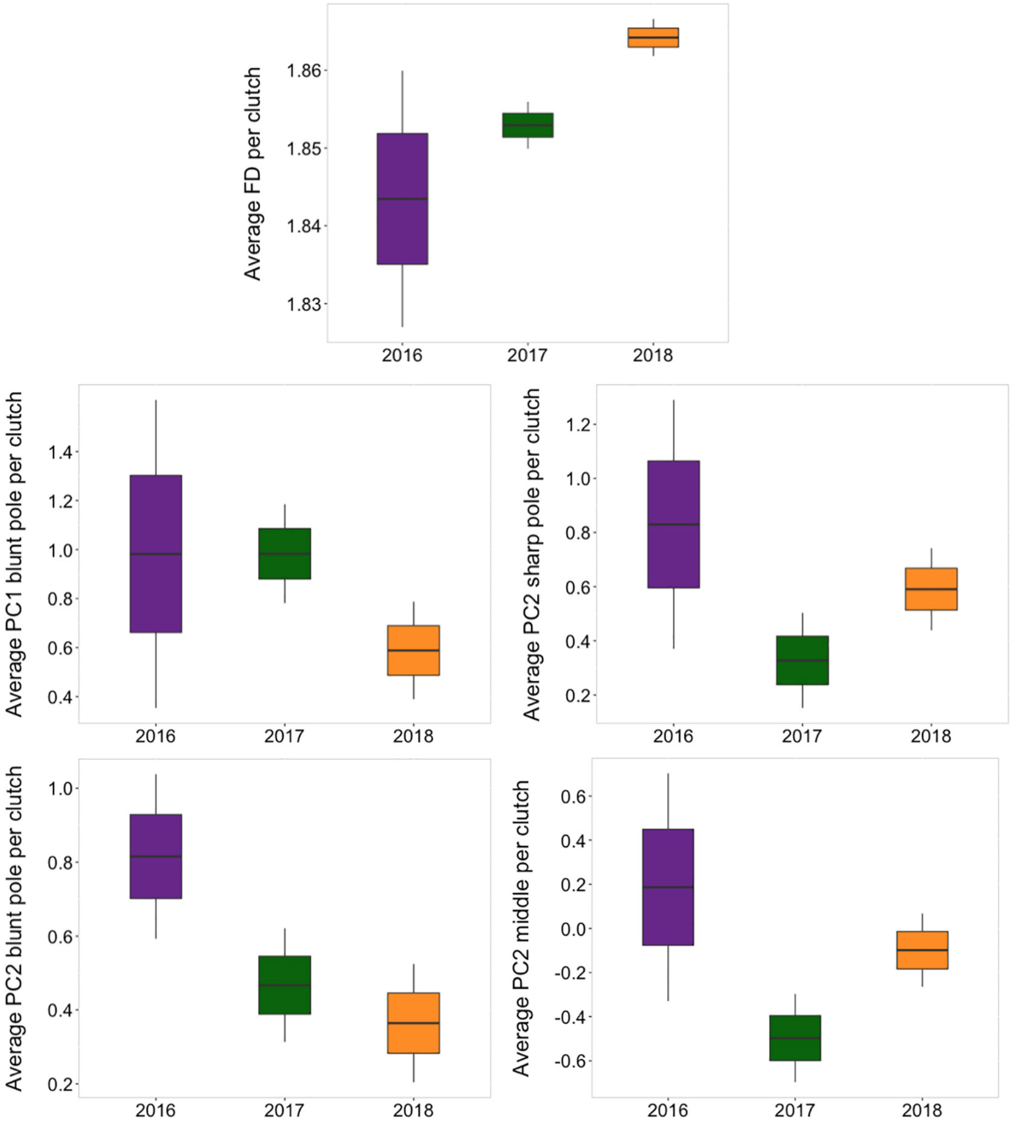

FIGURE 3 | Yearly variation in average values per clutch of eggshell features. Horizontal line represents the mean value, boxes standard error and line bars that indicate confidence intervals (95%).

we found that the average coloration of eggs (ultraviolet-red reflectance of the sharp pole and the middle area and brightness of the blunt pole) [$F_{(18,\ 248)} = 2.655$, $P < 0.001$], and the mean FD of spottiness of the eggshell, but not the mean volume, changed between years [$F_{(4,\ 248)} = 6.70$, $P < 0.001$] (**Supplementary Table 3** and **Figure 3**).

Analyses on the subset of females breeding more than once in the population revealed a low repeatability in the intra-clutch variation in egg volume and in the ultraviolet-red and blue-green colors of the eggshell ($r < 0.6$, $p > 0.05$). However, intra-clutch variation in brightness of the blunt pole was repeatable ($r = 0.49$, $F = 2.92$, df = 20, $p = 0.009$), as well as intra-clutch variation in FD ($r = 0.58$, $F = 3.84$, df = 20, $p = 0.0017$).

Intra-Clutch Variation in Egg Traits and Rejection in Different Years

Intra-clutch variation in the FD (**Table 1**) and in green-blue coloration in the middle area of the egg (**Table 2**) were associated with egg rejection of foreign eggs, and these patterns were in some cases affected by study year.

The probability of rejection was not associated with homogeneity in egg volume within the clutches (**Table 1**). However, the clutches of acceptors showed higher values of intra-clutch variation in the FD of their eggs than those of rejecters, irrespective of the year of study (**Table 1** and **Figure 4**). Regarding eggshell color variation, rejection was not related to variation in the sharp and blunt poles (**Table 2**). However, intra-clutch variation in blue-green color (i.e., PC3 score) in the middle area of the eggs differed between acceptor and rejecter magpies in interaction with the year (**Table 2** and **Figure 5**). Rejecters showed significantly lower intra-clutch variation in blue-green color than acceptors only in 2017 (**Figure 5**).

DISCUSSION

Our results show a clear, consistent relationship between intra-clutch variation in magpie egg phenotype and the probability of egg rejection by females. Moreover, we have demonstrated the importance of using different aspects of egg phenotype and of replicating the experiments over the years to better understand this association.

Victoria (1972) proposed that high homogeneity in the appearance of host eggs could be under strong selection in parasitized host populations because it would make easier the detection of noticeable features of parasite eggs by comparison of hosts own eggs with foreign ones (Øien et al., 1995; Soler and Møller, 1996). However, support for this hypothesis has been inconsistent, with studies showing opposite results (see section "Introduction"). Here we have analyzed whether intra-clutch variation in egg appearance changes and if this relates to host egg rejection in magpie hosts by considering variation in several traits and regions of host eggs. We have found that intra-clutch variation in egg appearance (volume, eggshell color, FD, see **Supplementary Material**) does not significantly differ between

TABLE 1 | Intra-clutch variation in egg appearance in relation to rejection of model eggs.

Fixed effects	Level	β (95%CI)	*F*	*df*	*P*
Model 1: Egg volume as predictor of rejection					
Intercept		−2.25 (−4.98 to 0.48)			
Clutch size		0.17 (−0.22 to 0.57)	0.78	129	0.37
Laying date		−0.18 (−0.75 to 0.39)	0.39	129	0.53
Year	2016	−1.47 (−4.45 to 1.50)	1.4	129	0.25
	2017	−1.42 (−3.27 to 0.44)			
	2018	0.00			
Intra-clutch variation volume		<0.001 (−0.0005 to 0.0006)	3.06	129	0.08
Intra-clutch variation volume*Year	2016	0.002 (−0.002 to 0.007)	1.57	129	0.21
	2017	0.002 (−0.0007 to 0.005)			
	2018	0.00			
Model 2: Fractal dimension as predictor of rejection					
Intercept		0.46 (−3.04 to 3.97)			
Clutch size		0.02 (−0.39 to 0.43)	0.01	121	0.91
Laying date		−0.18 (−0.86 to 0.50)	0.27	121	0.60
Year	2016	2.11 (−3.29 to 7.52)	1.01	121	0.37
	2017	−1.77 (−5.17 to 1.63)			
	2018	0.00			
Intra-clutch variation FD		**−168.84 (−358.18 to 20.51)**	**4.04**	**121**	**0.04**
Intra-clutch variation FD *Year	2016	−273.86 (−918.21 to 370.49)	0.57	121	0.56
	2017	81.80 (227.15 to 390.76)			
	2018	0.00			
Spottiness		**0.67 (0.09 to 1.25)**	**5.22**	**121**	**0.02**

Results of GLMs testing for differences in intra-clutch variation in egg volume, fractal dimension and spottiness in relation to rejection. Because color variables were correlated, we analyzed each area of the egg with an independent model. The covariates laying date and spottiness were standardized to improve interpretability. Estimates and interval confidence (95%) are given. Year reference level: 2018. Significant terms are highlighted in bold.

TABLE 2 | Intra-clutch variation in egg appearance in relation to rejection of model eggs.

Fixed effects	**Level**	**β (95%CI)**	***F***	***df***	***P***
Model 1: Egg color in the blunt pole as predictor of rejection					
Intercept		−2.16 (−5.35 to 1.03)			
Clutch size		0.15 (−0.27 to 0.57)	0.49	123	0.37
Laying date		−0.36 (0.97 to 0.25)	1.34	123	0.53
Year	2016	−122.82 (−2489.51 to 2214.16)	0.86	123	0.25
	2017	0.82 (−2.18 3.83)			
	2018	0			
Intra-clutch variation PC1		1.42 (−1.17 to 4.02)	0.01	123	0.92
Intra-clutch variation PC2		0.11 (−2.56 to 2.78)	0.01	123	0.91
Intra-clutch variation PC3		−1.96 (−5.49 to 1.57)	0.01	123	0.92
Intra-clutch variation PC1*Year	2016	−169.86 (−3256.47 to 2916.75)	0.16	123	0.85
	2017	1.18 (−2.96 to 5.32)			
	2018	0			
Intra-clutch variation PC2*Year	2016	256.39 (−4197.69 to 4710.46)	0.88	123	0.41
	2017	−3.82 (−2.96 to 5.32)			
	2018	0			
Intra-clutch variation PC3*Year	2016	108.1 (−2018.07 to 2234.28)	0.04	123	0.95
	2017	−0.94 (−7.51 to 5.64)			
	2018	0			
Model 2: Egg color in the middle area as predictor of rejection					
Intercept	level	−2.18 (−5.42 to 1.05)			
Clutch size		0.17 (−0.25 to 0.60)	0.67	123	0.42
Laying date		−0.20 (−0.81 to 0.41)	0.41	123	0.52
Year	2016	−3.33 (−13.93 to 7.27	2.39	123	0.09
	2017	4.65 (0.15 to 9.15)			
	2018	0			
Intra-clutch variation PC1		−0.59 (−3.64 to 2.47)	0.60	123	0.44
Intra-clutch variation PC2		0.77 (−1.64 to 3.20)	1.03	123	0.31
Intra-clutch variation PC3		−0.30 (−3.55 to 2.94)	0.65	123	0.42
Intra-clutch variation PC1*Year	2016	7.08 (−4.95 to 19.11)	0.73	123	0.48
	2017	−0.38 (−5.39 to 4.63)			
	2018	0			
Intra-clutch variation PC2*Year	2016	−9.03 (−21.27 to 3.20)	1.10	123	0.34
	2017	0.13 (−4.11 to 4.37)			
	2018	0			
Intra-clutch variation PC3*Year	**2016**	**5.97 (−7.88 to 19.82)**	**3.08**	**123**	**0.04**
	2017	−11.94 (−22.36 to −1.51)			
	2018	0			
Model 3: Egg color in the sharp pole as predictor of rejection					
Intercept	level	−1.56 (−4.86 to 1.74)			
Clutch size		0.2 (−0.22 to 0.63)	0.89	123	0.35
Laying date		−0.13 (−0.70 to 0.44)	0.21	123	0.65
Year	2016	1.58 (−9.73 to 12.90)	0.04	123	0.96
	2017	0.017 (−2.99 to 3.03)			
	2018	0.00			
Intra-clutch variation PC1		1.61 (−1.24 to 4.46)	1.44	123	0.23
Intra-clutch variation PC2		0.017 (−2.64 to 2.67)	2.47	123	0.12
Intra-clutch variation PC3		−4.09 (−8.19 to −0.003)	0.40	123	0.53
Intra-clutch variation PC1*Year	2016	1.56 (−7.92 to 11.06)	0.10	123	0.90
	2017	−0.45 (−4.46 to 3.55)			
	2018	0.00			
Intra-clutch variation PC2*Year	2016	−16.02 (−35.38 to 3.33)	1.43	123	0.24
	2017	0.46 (−3.35 to 4.28)			
	2018	0.00			
Intra-clutch variation PC3*Year	2016	8.62 (−4.77 to 22.01)	0.95	123	0.39
	2017	−0.87 (−7.46 to 5.72)			
	2018	0.00			

Results of GLMs testing for differences in intra-clutch variation in egg color (PC1 achromatic, PC2 ultraviolet-red, PC3 blue-green) in relation to rejection. Because color variables were correlated, we analyzed each area of the egg with an independent model. The covariates laying date and spottiness were standardized to improve interpretability. Estimates and interval confidence (95%) are given. Year reference level: 2018. Significant terms are highlighted in bold.

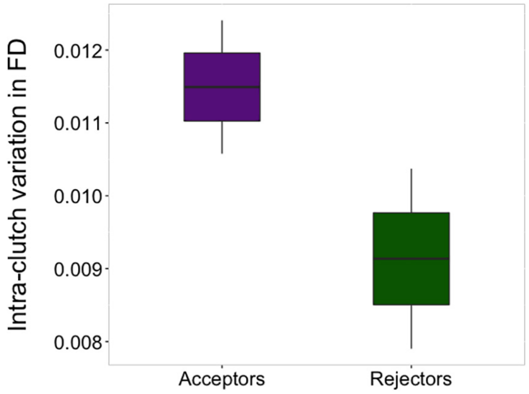

FIGURE 4 | Differences in intra-clutch variation in the fractal dimension between acceptor and rejecter magpies. Horizontal line represents the mean value, boxes standard error and line bars that indicate confidence intervals (95%).

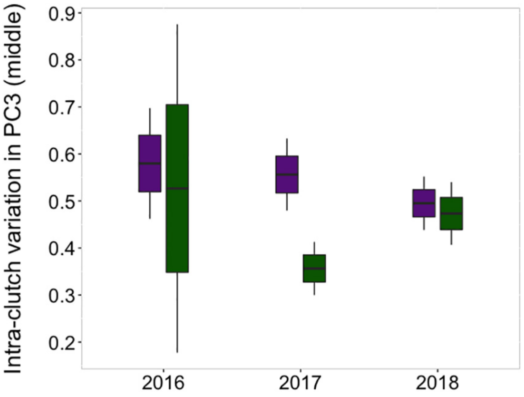

FIGURE 5 | Differences in intra-clutch variation in blue-green egg coloration in the middle area of the eggs between acceptor and rejecter magpies in three different years. Green boxes correspond to rejecter individuals and purple ones correspond to acceptors.

years but that the average values of some components of color and spottiness pattern varied, suggesting that variability of these eggshell features would have a strong environmental component. In addition, we also found that within-individual consistency differ for the different egg features, with brightness and patterns of spottiness being the features most typical of individuals. By using different host egg traits (size, color and Fractal Dimension) sampled across several years we have revealed that not all these traits show a consistent relationship with egg rejection, and that some of them have a higher potential to be subjected to selection pressures arising from rejection. In fact, it is intra-clutch variation in one of the more female-specific traits (patterns of spottiness as estimated by FD), which consistently affects egg rejection.

Our results confirm a key prediction of the co-evolutionary arms race hypothesis, namely that a low intra-clutch variation favors discrimination and rejection of foreign eggs in magpie for those traits that are more female-specific. Besides, these results are in accordance with a recent and innovative work using machine learning algorithms (as a proxy of a bird observer), which showed that birds may use primarily the most repeatable eggshell features (those with low intra-clutch variation) to recognize their own eggs and reject the foreign ones (Gómez et al., 2021). These findings stresses that the role of intra-clutch variation in rejection is contingent on the particular features of the egg phenotype considered, as some might show a higher degree of environmental determination whereas other are more female-specific.

Intra-clutch variation in blue-green color of the middle area of the eggs has an effect in the rejection behavior only one of the years, when rejecter females laid eggs more homogeneous in the blue-green coloring of their middle part. The importance of intra-clutch variation in blue-green coloration in the rejection of model eggs in magpies has been previously described (Avilés et al., 2004), although in that study higher intra-clutch variation in host egg appearance associated with rejection was found. The difference between the two studies may be due to several factors. First, it may be a consequence of methodological discrepancies, since we took measurements of the egg in three different areas, whereas the previous study considered the egg as a whole. Considering specific areas of the eggshell surface has been shown to be important in some studies; for example, both within clutch variation in the blunt pole (Spottiswoode and Stevens, 2010), or both egg poles (Polačiková et al., 2011). However, this possibility seems unlikely because in no case we found a negative association between homogeneity in blue-green color and rejection for the considered areas of the egg in this study. Secondly, Avilés et al. (2004) sampled the population only in one breeding season, whereas this study comprises three different breeding seasons. This is particularly important when host traits and behaviors may express plastically as it is known to happen for some host traits (Avilés et al., 2007; Hubbard et al., 2010; Molina-Morales et al., 2014; Berkowic et al., 2015; Martínez et al., 2020), and in particular for blue-green color of the eggshell (Soler et al., 2008). Alternatively, the inconsistency could be related with a different age structure in the population in different years. If intra-clutch variation covaries with female age, as it has been proposed for other species (Lotem et al., 1995; Siefferman et al., 2006), different age-structures in the population may render different rejection probabilities. Indeed, it has been shown that probability of rejection increases with age in magpies (Molina-Morales et al., 2014; Martínez et al., 2020). Although we do not know whether intra-clutch variation in blue-green color changes with age in magpies we have found that this feature was not repeatable. Another explanation is that differences in environmental conditions may favor or disfavor the ability to find food to obtain the pigmentation needed to color the eggs (Moreno and Osorno, 2003; Moreno et al., 2006; Morales et al., 2011). The intra-clutch variation in brightness is repeatable showing that this particular trait does not depend on environmental conditions.

In this work we have introduced a novel feature (in the context of foreign egg rejection experimental work) that we hypothesized can influence egg rejection in magpies, the fractal dimension of eggshell spottiness. The previous study that model host recognition used machine learning algorithms and characterized the patterning of spottiness using the FD of spottiness (Gómez et al., 2021), found that, among the several variables related with spottiness, the FD was the most important for recognition and almost the most repeatable within the clutch. Here, we have shown that this feature influences rejection in the same way in all the study years. Individuals that rejected the foreign eggs were those whose intra-clutch variation in fractal dimension was lower, which means that all the eggs within the clutch were similarly homogeneous. Fractal dimension characterizes the complexity of a spatial pattern in a given object or trait, and increases with surface complexity (Gómez et al., 2019). Fractal dimension of spottiness is indicative of the distribution of spots across the eggshell and is positive related with the amount of protoporphyrin (Gómez et al., 2019), the pigment that constitutes them. Previous studies have demonstrated that fractal dimension of coloration of the bib in partridges was related to body condition and immune responsiveness (Pérez-Rodríguez et al., 2013). Because intra-clutch variation in avian eggshell pigmentation has also been related to female quality (De Coster et al., 2013; Minias et al., 2020), it could be argued that rejecter females are individuals in a better condition or higher quality. In any case, our results agree with the expectation that the value of intra-clutch variation in determining egg discrimination should be larger for those traits, which are less environmentally driven.

CONCLUSION

Summing up, our work stresses the importance of considering a holistic approach including several aspects of egg phenotype and replication in studies on the adaptive value of host traits in egg rejection. Host may counteract parasite egg mimicry evolving very homogeneous eggs in such a way that intra-clutch variation in egg appearance is minimal.

Our results may suggest that this evolutionary process will not affect all egg traits with the same strength. Features such as color, or others, which may relate in their expression to the age and/or physical condition of the female at a given time, would be less likely used as cue for parasite egg recognition. However, the homogeneity of eggshell patterns (here quantified through FD) could have been favored by natural selection as a signature that facilitates the identification of own eggs. We must also acknowledge that the degree of difference between foreign and host eggs could affect the likelihood of rejection of foreign eggs. Nonetheless our study was based on rejection of mimetic models harboring little variation among them. Future experimental studies should ideally use real cuckoo eggs or models with variable levels of mimicry with host eggs to ascertain whether the role of intra-clutch variation in rejection is mediated by the degree of matching between parasite and host eggs. Plastic expression of the different aspects of the egg phenotype might, however, be species-specific or population-specific, and would need to be evaluated as a key premise in future tests of the intra-clutch variation hypothesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Also, a dataset is available at https://doi.org/10.6084/m9.figshare.16811494.v1.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Etica de la Universidad de Granada.

AUTHOR CONTRIBUTIONS

MM-M and JA conceived the idea and analyzed the data. MM-M led the writing of the manuscript. MP and JGM performed the experiment in the field work. JG and GL-C carried out the images processing and performed the analyzes related to fractal dimension. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.702637/full#supplementary-material

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The Perceptual and Cognitive Processes That Govern Egg Rejection in Hosts of Avian Brood Parasites

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Hosts of avian brood parasites are under intense selective pressure to prevent or reduce the cost of parasitism. Many have evolved refined egg discrimination abilities, which can select for eggshell mimicry in their parasite. A classic assumption underlying these coevolutionary dynamics is that host egg recognition depends on the perceivable difference between their own eggs and those of their parasite. Over the past two decades, the receptor noise-limited (RNL) model has contributed to our understanding of these coevolutionary interactions by providing researchers a method to predict a host's ability to discriminate a parasite's egg from its own. Recent research has shown that some hosts are more likely to reject brown eggs than blue eggs, regardless of the perceived differences to their own. Such responses suggest that host egg recognition may be due to perceptual or cognitive processes not currently predictable by the RNL model. In this perspective, we discuss the potential value of using the RNL model as a null model to explore alternative perceptual processes and higher-order cognitive processes that could explain *how* and *why* some hosts make seemingly counter-intuitive decisions. Further, we outline experiments that should be fruitful for determining the perceptual and cognitive processing used by hosts for egg recognition tasks.

Keywords: brood parasitism, color categorization, egg rejection, opponent channels, receptor-noise limited model

INTRODUCTION

Avian brood parasitism is an alternative reproductive strategy where one female lays her eggs in another bird's nest, imposing the costs of rearing her young on a set of foster parents (Stevens, 2013). In response, hosts often evolve egg recognition abilities as a major line of defense against parasitism, which, in turn, can select for improved eggshell mimicry in their parasite and instigate a coevolutionary arms race (Dawkins and Krebs, 1979; Davies and Brooke, 1989; Stoddard and Stevens, 2011). Such interactions have provided researchers with a tractable system to examine parasitic relationships and coevolution (Dawkins and Krebs, 1979; Davies and Brooke, 1989; Hauber et al., 2015b).

The perceptual and cognitive processes governing host egg recognition are central to understanding host decision-making and coevolutionary arms races (Dawkins and Krebs, 1979;

Davies and Brooke, 1989; Hauber et al., 2015b). Classic theory assumes that hosts can select for eggshell mimicry in the parasite by rejecting parasite eggs they perceive as dissimilar to their own (Dawkins and Krebs, 1979). Pioneering work by Stoddard and Stevens (2011) applied an avian receptor noise limited (RNL) model (Vorobyev and Osorio, 1998) to the common cuckoo, *Cuculus canorus,* and their respective hosts. They found that hosts with strong recognition abilities were parasitized by cuckoos that laid eggs with refined eggshell mimicry (as predicted by the RNL model). However, while useful for predicting discriminability between stimuli (Olsson et al., 2018; Price et al., 2019), the RNL model's original intent was to make predictions with which to compare behavioral responses and then infer color processing mechanisms (Osorio and Vorobyev, 2018). Contrary to traditional expectations (Brooke and Davies, 1988; Reeve, 1989), Hanley et al. (2017) illustrated that some hosts reject brown model eggs more than blue model eggs, even when those eggs were equally dissimilar to the hosts' own. In this experiment, model eggs were painted colors that specifically aligned with perceived variation in natural eggshell color (from blue-green to brown; Hanley et al., 2015). This finding has been meta-replicated in five hosts from three continents, each of which face distinct forms of parasitism (Abolins-Abols et al., 2019; Hanley et al., 2019b; Manna et al., 2020); however, not all hosts will accept any egg models painted "blue" (Langmore et al., 2005; Begum et al., 2012; Hanley et al., 2019a; Abernathy et al., 2021). These counter-intuitive and, at times disparate, findings raise the question, "why have these hosts deviated from our null expectations"?

Here, we explore alternative visual-cognitive processes that may explain such deviations from the expected RNL model. Specifically, we describe color vision in birds by explaining how color is both received and perceived. We then outline the discrimination challenge facing hosts and describe a higher-level cognitive process (categorization) that hosts may use to process this color information during decision-making (Harnad, 1987; Shepard, 1987; Goldstone and Hendrickson, 2009). Our goal is to contextualize previous findings of strikingly similar color-biased rejection behaviors by describing egg recognition tasks from color reception through perception (**Figure 1**). Finally, we provide potential experimental frameworks to aid future investigations into the perceptual and cognitive processes used by hosts for egg discrimination tasks.

Color Reception

Birds are thought to have excellent color vision (Jacobs, 1981; Goldsmith, 1990; Hart, 2001; Ödeen and Håstad, 2003). They possess four types of single-cone photoreceptors, sensitive to ultraviolet (or violet), short, medium, or long wavelength light (Hart, 2001). Avian color reception is thought to be based on the stimulation of these single cones (Hart, 2001; Ödeen and Håstad, 2003), where estimates of quantum catches (i.e., the number of photons being absorbed by the cone from the stimulus) approximate the stimulation of each photoreceptor:

$$Q_i = \int_{300}^{700} R_i(\lambda)\, S(\lambda) I(\lambda) O(\lambda) d\lambda$$

Where Q_i represents the quantum catch for receptor i, R is the sensitivity of photoreceptor i after accounting for oil droplet transmittance, S is the reflectance of a stimulus, I is the irradiance in photon flux, and O is the ocular media that narrows the sensitivity of photoreceptors in birds (Bowmaker et al., 1997; Vorobyev, 2003). By convention, we refer to the quantum catches of the ultraviolet/violet-, short-, medium-, and long-wavelength sensitive photoreceptors as Q_u, Q_s, Q_m, and Q_l, respectively.

Color Perception

Before a color can give rise to a perceptual experience in an organism, the color that is received by the eye must be transformed and transmitted to the brain (Osorio et al., 1999; Kelber, 2016; Price et al., 2019). This is done by forming opponent channels that contrast received quantum catches against each other (**Figure 1**), a process that underlies color discrimination in a range of organisms (Osorio et al., 1999; Ventura et al., 2001; Rocha et al., 2008; Kelber, 2016; Price et al., 2019). In theory, related opponent channels [e.g., $(Q_u+Q_l) - (Q_s+Q_m)$ and $(Q_s+Q_m) - (Q_u+Q_l)$] can produce signals that vary in sign (positive or negative) and therefore may result in two different responses. Unfortunately, the actual neural mechanisms of each channel—and, indeed, the total number of channels used—are unknown in birds (Kelber, 2016; Price and Fialko, 2018; Vasas et al., 2018; Price et al., 2019). This makes it challenging to model the actual signals reaching the avian brain.

To circumvent this difficulty, Vorobyev and Osorio (1998) developed the RNL model that predicts discriminability between stimuli. In this model, the actual opponent channels are unimportant and the sign of the signal makes no difference on model output (Vorobyev and Osorio, 1998). This model assumes that visual signals and discrimination thresholds are determined by photoreceptor noise (Vorobyev and Osorio, 1998), that there are one fewer opponent channels than photoreceptor types, and that altering light levels do not change perceived differences between stimuli (Kelber, 2016). The RNL model predicts differences between stimuli in just noticeable differences (JNDs), classifying stimuli as either discriminable (>1 JND) or not discriminable (<1 JND). Although in practice, it is generally recognized that the theoretical threshold of 1 JND is likely overly conservative in most natural contexts, and more realistic thresholds may be set ∼2 JNDs (Spottiswoode and Stevens, 2010, 2012; Stevens et al., 2013). This model is appealing because it accurately estimates visible contrast, at least in some contexts (e.g., brightly lit conditions), provided reflectance spectra for two stimuli (e.g., those in a detection or discrimination task), spectral sensitivity estimates of each of the organism's photoreceptor types (Price et al., 2019), and the irradiance of the environment (Endler, 1993) are available.

However, there are certain conditions that may impact the RNL model's ability to accurately predict discriminability. For example, this model does not currently account for how visual contrast against the background impacts the thresholds beyond which two stimuli can be discriminated (i.e., for discrimination tasks). When viewing colorful stimuli, most animals will compensate for differences in light conditions through a process known as chromatic adaptation (Lind, 2016; Price et al., 2019).

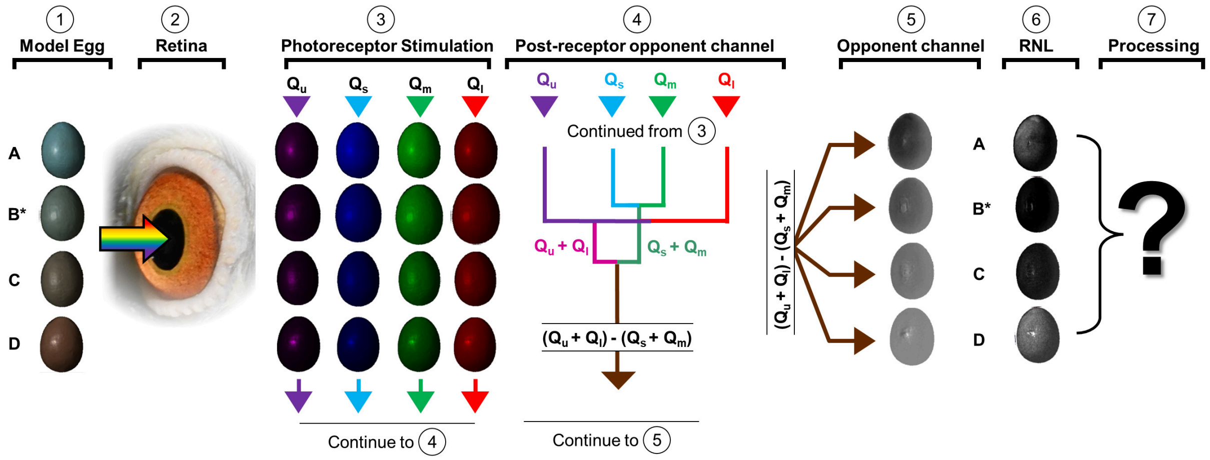

FIGURE 1 | The pathway to perceived color variation involves the capturing of light reflected by stimuli such as model eggs (column 1) by the retina (column 2), the resultant photoreceptor stimulation (column 3), and the formation and transmission of an opponent signal to the brain (column 4). We can make predictions about perceived egg color that likely result from these opponent channels (column 5), possibly differing from the predictions of the receptor noise-limited (RNL) model (column 6). Ultimately, we have much to learn about the cognitive processes that hosts use to act on color information (column 7). Here we show model eggs (labeled A-D in column 1), where egg B is considered an "own" egg template (to which others will be compared, indicated with asterisk). The inset eggs under "photoreceptor stimulation" represent the quantum catches (see section "Color Reception" for details) under standard daylight conditions, following previously published methods (Stevens et al., 2007). The post-receptor processes (columns 4–7) are less well studied in birds, and birds may rely on specific opponent channels that weigh the contribution of specific photoreceptors in distinct ways. We show one such opponent channel (column 5) that is produced based upon the quantum catches of each egg model type (each set of arrows describes the process), when the sign of the signal (positive or negative) and may result in distinct responses to stimuli that differ from our null expectation under the RNL model (column 6, black = 0 JND and white ≈5 JND) where each egg is compared against egg B. That perceived color information may then be used *via* unknown (question mark) higher order processes. Inserted photograph of the avian eye (in column 2) was modified (cropped, flipped, rotated, and vignetted) from "Eyes and eyelids" by PigeonsAreAwesome under the CC BY-SA 4.0 license.

In this process, when the background predominantly reflects long-wavelength light (typical in birds' nests), the viewer will upregulate short-wavelength receptor signals (e.g., blue) and downregulate long-wavelength receptor signals (e.g., brown; Price et al., 2019). Consequently the predicted *detection* of blue-green eggs in brown nests is, rightly, improved (higher JND) by accounting for the nest background (Price et al., 2019). However, animals tend to perform better on *discrimination* tasks (e.g., comparing a parasite's egg and a host's egg) when stimuli are more similar to the background (e.g., two brown eggs on a brown nest) and poorer when stimuli are more different from the background (e.g., two blue eggs on a brown nest); thus, chromatic adaptation can effectively alter the threshold necessary to differentiate stimuli (Krauskopf and Gegenfurtner, 1992; Lind, 2016; Price et al., 2019). Currently, the RNL model does not account for these effects for discrimination tasks.

Higher-Order Processing

Most studies on color discrimination assume that animals use low-level cognitive mechanisms simply based on discrimination thresholds (Kelber and Osorio, 2010); however, previous research has provided evidence of decision-rules based on higher-level cognitive mechanisms in invertebrates, fish, mammals, and even in birds (Tapper and Halpern, 1968; Sandell et al., 1979; Nelson and Marler, 1989; Poralla and Neumeyer, 2006; Ham and Osorio, 2007; Benard and Giurfa, 2008; Avarguès-Weber et al., 2011; Lachlan and Nowicki, 2015; Renoult et al., 2015; Caves et al., 2018). One such higher-level cognitive process is categorization, which occurs when responses to stimuli vary less within categories than between categories (Repp, 1984; Harnad, 1987; Treisman et al., 1995). This is known as a boundary effect, which is a hallmark test of categorical perception and can only be assessed measuring responses across a phenotypic range that spans both sides of a decision boundary (Harnad, 1987; ten Cate and Rowe, 2007; Kelber and Osorio, 2010; Hauber et al., 2015b). Categorization is thought to increase the speed, accuracy, and certainty of choices, while reducing the requirements for neural processing (Nelson and Marler, 1989; Kepecs et al., 2008), particularly useful for performing unfamiliar tasks or when information is uncertain (Dukas and Waser, 1994; Benard et al., 2006; Kepecs et al., 2008). Although the potential for hosts to use color categorization in egg recognition has been discussed previously (Spottiswoode and Stevens, 2010; Hanley et al., 2017), and tests have illustrated a clear decision boundary (see figures S3 and S4 from, Hanley et al., 2017), no study has yet confirmed that hosts use categorization for egg recognition (Green et al., 2020).

DISCUSSION

By providing reasonable predictions about perceivable differences between host and parasite eggs, the RNL model has proven a crucial method for testing certain hypotheses surrounding co-evolutionary arms races between host perception and parasite egg phenotypes (Spottiswoode and Stevens, 2010; Stoddard and Stevens, 2011). Comparisons between the predictions of the RNL model and actual host responses can demonstrate higher-order processes, such as categorization (Nelson and Marler, 1989; Caves et al., 2018), or help determine the discrimination thresholds necessary for specific egg recognition tasks (Lind, 2016; Olsson et al., 2020). Such experiments would refine our understanding of the perceptual and cognitive processes that underly egg recognition decisions (e.g., opponent channels, chromatic adaptation, and categorization) and advance our understanding of host-parasite coevolutionary dynamics. Here we outline tests necessary to explore hitherto untested perceptual and cognitive processes, which may explain why some hosts' behaviors appear to deviate from the expectations of the RNL model (e.g., rejecting brown but accepting equally dissimilar blue eggs).

Host Selection for Proposed Tests

The experiments we outline below are ideally suited for hosts traditionally considered intermediate rejecters (Rothstein, 1975; Davies and Brooke, 1989; Peer and Sealy, 2004), rather than hosts that unilaterally accept or reject eggs. Additionally, these tests are suited for hosts with intermediate, rather than extreme, eggshell colors so that experimental egg model stimuli can be realistically bluer or browner than the host's. In such an experiment, a researcher would insert a single egg model, either experimental or control, into each host's nest and record their response (reject/accept) within a predefined period (Canniff et al., 2018). We advocate that researchers estimate avian-perceived coloration of each host egg (rather than average host color as in Hanley et al., 2017), experimental egg, and nest, in addition to measuring the irradiance at each nest, as these data are vital for interpreting host behavioral responses.

Perceptual Mechanisms

Egg rejection studies using egg models varying in coloration along a continuous range would be particularly useful for determining whether a host's response is governed by a particular opponent channel (Hanley et al., 2017, 2019b; Abolins-Abols et al., 2019; Manna et al., 2020). Such experiments (**Figure 2A**) would allow a researcher to determine whether host response is better predicted by opponent channel(s) or discriminable differences predicted under the RNL model and also whether responses vary sharply anywhere along that color range (decision boundary, see below). This could be a fruitful line of research, since past studies have found that individual photoreceptor quantum catches provided better predictions of host response than discriminable differences predicted under the RNL model (Cassey et al., 2008; Hauber et al., 2020). If hosts used a common opponent channel, that may explain why disproportionate rejections of brown eggs have been found in a range of hosts (Stokke et al., 2007; Cassey et al., 2008; Soler et al., 2012; Hanley et al., 2017, 2019b; Abolins-Abols et al., 2019; Manna et al., 2020). If such a common channel exists, and is used for egg recognition, it most likely broadly distinguishes short- from long-wavelength dominated colors (e.g., blue and brown, respectively). Still, while uncovering a specific shared opponent channel used by diverse hosts would be exciting, other aspects of visual perception might also explain host behaviors that deviate from our null expectations.

Chromatic adaptation to the (typically) brown nest background provides one such intriguing possibility. Lind (2016) demonstrated that the thresholds necessary for color discrimination can shift depending on the contrast between the color of the stimuli and the nest background. Thus, hosts with blue-green eggs would face a comparatively more challenging task when discriminating a blue egg (i.e., higher discrimination threshold) than discriminating a brown egg (i.e., lower discrimination threshold). Consistent with this observation, hosts found to disproportionately reject brown egg models laid (at least moderately) blue-green eggs (Honza et al., 2007; Cassey et al., 2008; Soler et al., 2012; Hanley et al., 2017, 2019b; Abolins-Abols et al., 2019; Manna et al., 2020). Although past experiments found that nest contrast did not influence host egg recognition (Aidala et al., 2015; Hauber et al., 2015a), a blue nest lining did increase performance on an egg discrimination task by 25% in the blue-green egg laying American robin *Turdus migratorius* (Aidala et al., 2015). We feel this is still a promising line of research, particularly if future studies employ an experimental manipulation of nest background (Aidala et al., 2015) alongside sufficient variation in the color of egg stimuli to determine threshold values (sensu Lind, 2016). We suggest measuring the discrimination thresholds by recording behavioral responses to sets of blue-green and brown egg stimuli sufficiently diverse to surpass threshold levels (e.g., 66.7% rejection rates; see **Figure 2B**) on high and low contrast nest backgrounds.

Cognitive Mechanisms

If host responses show evidence of a sharp decision boundary (**Figure 2A**), subsequent experiments should test for color categorization. We suggest deploying discrete sets of egg models of carefully designed colors (Nelson and Marler, 1989; Cheke et al., 2006; Ham and Osorio, 2007; Caves et al., 2018), rather than eggs with continuously varying colors (sensu Hanley et al., 2017). For example, egg sets could be bluer (set 1) and browner (set 2) than the hosts' own (**Figure 2C**), but of identical brightness. Within each set, one stimulus (stimulus 1) should be relatively similar (e.g., 3 JND) to the host's own egg (the control stimulus) and differ from the next stimulus (stimulus 2) in the set by an identical degree (e.g., 3 JND). Importantly, sets of stimuli should span the previously detected decision boundary, and there must be comparable perceivable differences within and between categories. While our focus is on colors ranging from blue-green to brown, other color ranges can be explored and more colors can be added as necessary. Color categorization would be detected if differences in host responses are significantly greater across the category boundary (see above) than within either category. Although this is a necessary follow-up test to confirm color categorization in hosts (Green et al., 2020), it may not reveal the basis for such categorical behavior. Typically, categorical behavioral responses in an experiment such as the one we propose (**Figure 2**) would imply hosts assign categorical labels to distinguishable egg stimuli (e.g., egg sets); however, identical responses may result from other processes (e.g., chromatic adaptation altering discrimination thresholds for certain discriminations tasks;

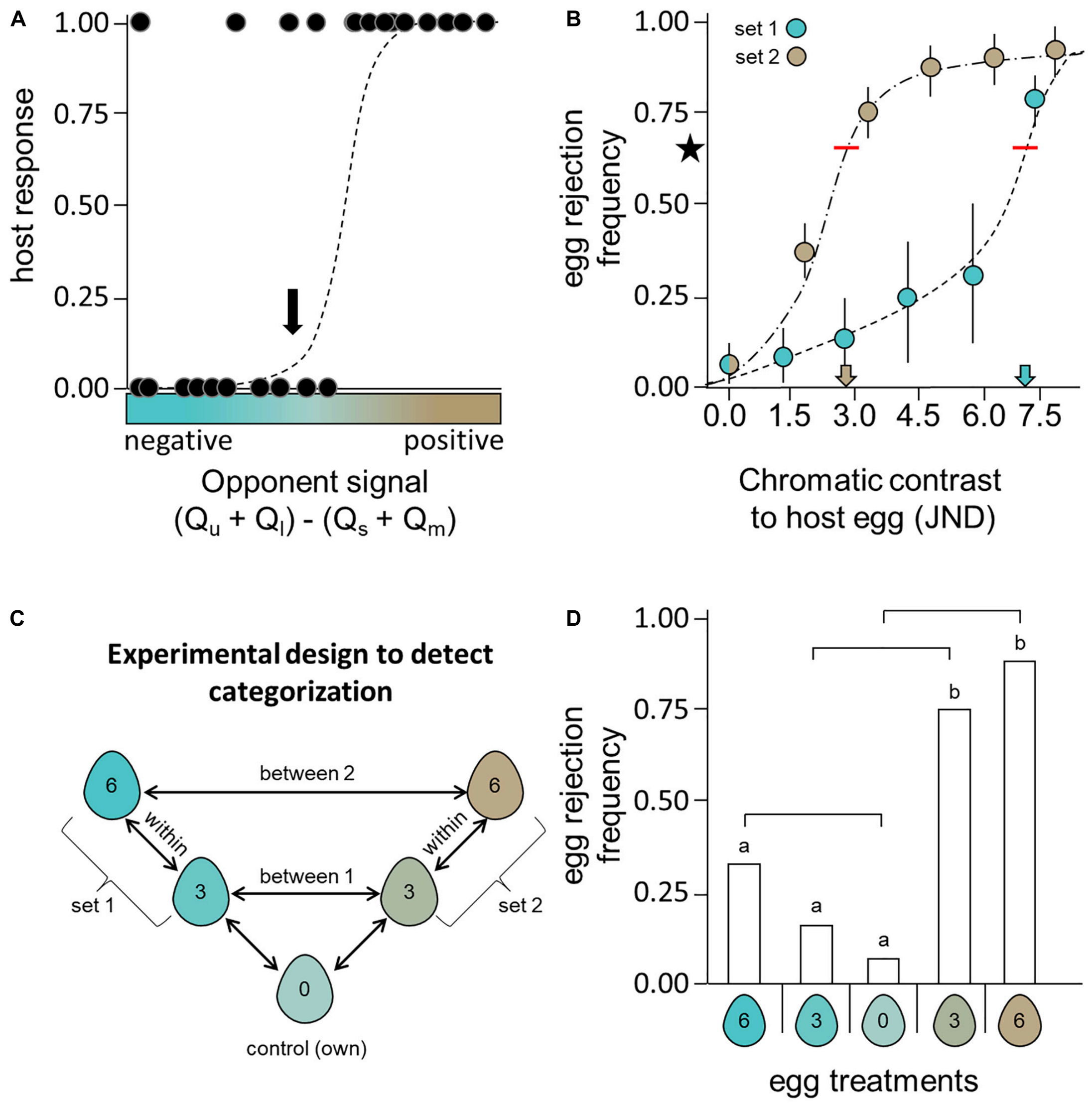

FIGURE 2 | Here we illustrate expected results from an experiment **(A)** investigating host response to eggs (acceptance = 0, rejection = 1) painted colors ranging from bluer (negative) to browner (positive) than the hosts' own eggs (solid line on x axis). Egg stimuli are plotted by their opponent channel signal, $(Q_u + Q_l) - (Q_s + Q_m)$, such that negative and positive values represent blue and brown egg colors, respectively. In this example, hosts reject eggs bluer than their own even less frequently than eggs with better color matches (as seen in *Turdus* thrushes, Hanley et al., 2017). We fit a hypothetical logistic regression (dashed line) to these data. These predictions would be compared against the predictions of an RNL model. For reference, an arrow indicates the color that most closely matches the host's own egg color. Such egg models **(B)** that vary from the host's own egg color (chromatic contrasts in JND) can be deployed in nests to measure the discrimination thresholds for eggs bluer (set 1) or browner (set 2) than the host's own. For a host with a moderately blue-green egg, we expect that chromatic adaptation to a natural brown nest background could shift the discrimination threshold (blue and brown arrows on x-axis), such that threshold necessary to detect 66.7% of egg models (red lines) vary for each set of stimuli. Here error bars represent variation across multiple experiments (e.g., spanning years or populations). Finally, we illustrate an **(C)** experimental schematic and set of **(D)** expected results for tests of color categorization. Eggs from each egg set, would vary by consistent intervals. The numbers in egg icons represent the chromatic contrast to the host's own egg, with the control model identical in color to the host's own. To detect categorization, the **(D)** differences between categories (egg sets) would have to be larger than differences within. We illustrate hypothetical results [identical to panel **(B)**] and the *post hoc* significance of these comparisons (above bars) that would document categorical behavior, emphasizing particularly informative comparisons using lines above the bars. These illustrate hypothetical results based on past results from hosts that lay blue-green eggs (e.g., *Turdus* thrushes: Hanley et al., 2017); however, these designs would be particularly informative on a host population that has a distinct egg polymorphism (relatively blue or relatively brown) as in the Daurian Redstart *Phoenicurus auroreus* (Yang et al., 2016; Zhang et al., 2021a,b).

Figures 2B,C). Regardless of the underlying mechanism of categorical behaviors (e.g., Caves et al., 2020), such rejection behavior would select for eggshell phenotypes in novel and unappreciated ways.

Expected Evolutionary Outcomes

Importantly, these perceptual and cognitive processes (i.e., chromatic adaptation, categorization) may all impact host egg recognition, and may not be mutually exclusive. Each can be tested by comparing host behavior to the predictions of the RNL model, and would impact host-parasite dynamics in interesting ways. For example, we would expect that heavily parasitized host populations that lay blue-green eggs would evolve even bluer eggs than unparasitized populations, under either categorization or chromatic adaptation, due to their preferential rejection of brown eggs (Soler et al., 2012; Hanley et al., 2013). In these cases, host discrimination might not only select for parasite eggshell mimicry, but also for shifts of the discrimination threshold itself (**Figure 2B**). We argue that these aspects of perception and cognition are as vital to coevolutionary arms races as the egg traits that are selected. Furthermore, we expect that the cognitive mechanisms underlying host egg recognition will be more plastic (Buchanan et al., 2008) than their egg and visual morphologies, which are, respectively, constrained and conserved traits (Ödeen and Håstad, 2003; Hanley et al., 2015). Focusing research attention on these more labile cognitive processes will likely be fruitful for determining the role hosts play in shaping coevolutionary dynamics.

Conclusion

In this perspective, we propose that hosts' discontinuous responses to continuous variation in eggshell color could be explained by more explicit perceptual (opponent channels, chromatic adaptation) and cognitive (categorization) models. By outlining fruitful directions for future research, we encourage researchers to use the RNL model as a null model with which to compare other higher-level processes. Avian brood parasitism provides an ideal system to not only learn about parasitism and coevolution (Soler and Soler, 2000; Stoddard and Hauber, 2017), but also the hitherto unidentified perceptual and cognitive mechanisms (Stoddard and Hauber, 2017) used by wild birds. By developing experiments explicitly designed to discern the underpinnings of avian vision, we can learn more about how birds interpret the world around them, an understanding that is vital to grasping their past, present, and future evolutionary trajectories (Endler and Mielke, 2005; Endler et al., 2005; Stevens, 2011), as well as to informing current conservation efforts (Blackwell et al., 2012; Dominoni et al., 2020). It is our hope that this manuscript will catalyze future investigations into the cognitive processes that underly perception and decision-making in broad taxonomic groups and contexts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the perspective contain no data, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DH provided the concept. DH, SR, and JV proposed experimental designs. All authors worked to develop and revise the writing.

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The Evolution of Predator Resemblance in Avian Brood Parasites

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Predators have profound effects on prey behavior and some adult brood parasites use predator resemblance to exploit the antipredator defenses of their hosts. Clarifying host perception of such stimuli is important for understanding the adaptive significance of adult brood parasite characteristics, and the mechanisms by which they misdirect hosts. Here I review the literature to explore the adaptive basis of predator resemblance in avian brood parasites, and natural variation in host responses to these stimuli. I also provide a framework for the information ecology of predator resemblance, which is based on the principles of signal detection theory and draws from empirical evidence from the common cuckoo, *Cuculus canorus*, as the most widely studied system. In this species, visual and acoustic hawk-like stimuli are effective in manipulating host defenses. Overall, contrasts across host responses suggest that different modalities of information can have independent effects on hosts, and that predator resemblance takes advantage of multiple sensory and cognitive processes. Host perception of these stimuli and the degree to which they are processed in an integrated manner, and the physiological processes underlying regulation of the responses, present new avenues for brood parasitism research.

Keywords: adaptive resemblance, imperfect mimicry, eavesdropping, perception, predator-prey, mimicry, communication, cuckoo

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INTRODUCTION

Predation is a powerful force of natural selection. As a consequence, systems for detecting, recognizing and responding to predators are widespread (Lima and Dill, 1990; Ruxton et al., 2018; Leavell and Bernal, 2019). In the Kalahari Desert, you might witness a fork-tailed drongo aggressively dive-bombing a tawny eagle (*Dicrurus adsimilis,* and *Aquila rapax,* respectively). At roughly sixty times lighter, why does the drongo risk harassing this gigantic predator? Typically, approaching large predators can be explained by rewards shared across the prey community *via* mobbing defenses (Shields, 1984; Caro, 2005). By contrast, rodents that actively approach predatory cat cues derive no benefit for themselves or conspecifics. This fatal attraction occurs under *Toxoplasma gondii* infection, which alters predator aversion in intermediate rat hosts. The manipulative endoparasite benefits from increased transmission to the stomach of a cat (the definitive host; Berdoy et al., 2000). Indeed, cheats regularly benefit from stimulus ambiguity in the world of predator-versus-prey, harnessing mimicry and misdirection to mislead (Ruxton et al., 2018; Leavell and Bernal, 2019). Particularly infamous amongst cheats are the brood parasites, and here, I review the role of predator resemblance in facilitating brood parasitism.

Hosts of brood parasites suffer reduced reproductive success *via* two main routes: the premature death of their young, and by misdirected parental effort (Rothstein, 1990; Kilner, 2005; Kilner and Langmore, 2011; Mark and Rubenstein, 2013). To avoid these costs, hosts have evolved adaptations in defense against brood parasitism. In response, counteradaptations (to avoid host detection and enhance parasitism success) are the corresponding adaptations in antagonistic coevolution between host and parasite, that explain the evolution of, for example, extreme egg mimicry (Spottiswoode and Stevens, 2010). Indeed, egg mimicry has long provided textbook examples of host-parasite coevolution (Rothstein, 1990; Davies, 2000; Feeney et al., 2014). By contrast, characteristics at the adult stage can be shaped by ecology beyond the host's nest. In some cases, adult characteristics appear to capitalize on a fate worse than brood parasitism for the host parent: that of falling prey to a predatory hawk (Lima and Dill, 1990; Davies, 2000).

Is Hawk-Like Resemblance Adaptive for Cuckoos?

Some adult brood parasitic cuckoos (Cuculinae) share characteristics with raptors; an observation that has long enchanted natural historians (Wallace, 1889; Chance, 1940). Phenotypic overlap could have arisen through convergent evolution, or *via* antagonistic coevolution with hosts (Payne, 1967; Craib, 1994). Comparative analyses across cuckoos suggest brood parasitic species are more likely to resemble predatory birds than species exhibiting parental care (Payne, 1967; Krüger et al., 2007). Moreover, predator-like characteristics appear to have evolved among cuckoos after brood parasitism arose (Krüger et al., 2007), suggesting that predator resemblance is adaptive. Importantly, predator resemblance could influence host behavior in three ways. First, by provoking escape responses, which extends opportunity to access the nest for egg-laying (Welbergen and Davies, 2008, 2011; York and Davies, 2017). Second, by deterring hosts from mobbing, which could provide greater access to nests, or avoid the costs of being mobbed (Welbergen and Davies, 2008, 2011; York and Davies, 2017). Indeed, host mobbing defenses are important since highly vocal nest-defending reed warblers (*Acrocephalus scirpaceus*) are parasitized least (Campobello and Sealy, 2018), mobbing can result in cuckoo injury or death (Molnár, 1944; Wyllie, 1981; Davies and Brooke, 1988; Šulc et al., 2020), and mobbing can alert neighbors, thereby increasing local nest-guarding and egg-rejection defenses. That said, mobbing can also be costly for hosts, and can increase parasitism and depredation (Smith et al., 1984; Krama and Krams, 2005; Davies and Welbergen, 2009; Campobello and Sealy, 2018). Third, predator resemblance could also influence hosts by misdirecting their defenses from clutch- to self-protection, thus increasing antipredator vigilance while reducing investment in egg rejection defenses (York and Davies, 2017).

Elegant experiments by Davies and Welbergen indicated that visual sparrowhawk, *Accipiter nisus*, resemblance is adaptive in host interactions with common cuckoos (*Cuculus canorus;* hereafter "cuckoo"; Duckworth, 1991; Davies and Welbergen, 2008). Barred chest plumage deters non-host parids from approaching, and reduces mobbing defenses in reed warbler hosts—as expected if they fear and avoid hawks (Welbergen and Davies, 2008, 2011). Indeed, warblers were more aggressive in mobbing cuckoos that appeared less hawk-like (where chest-barring was concealed; Welbergen and Davies, 2011), thereby confirming that hawk resemblance is perceived and effectively enhances brood parasitism success. However, not all individuals are fooled by the imperfect visual resemblance. Hosts mobbed cuckoos three times more intensely than hawks, demonstrating a capacity for discrimination, which is important because it provides the necessary basis for antagonistic coevolution (Duckworth, 1991; Welbergen and Davies, 2011; Yu et al., 2017). Some regularly and infrequently parasitized species will aggressively mob cuckoos despite their hawk-like appearance (Trnka and Prokop, 2012; Lyon and Gilbert, 2013; Ma et al., 2018). These apparently aggressive species may have overcome cuckoo hawk resemblance by being highly discriminating, driven by intense social learning under high parasitism prevalence. Alternatively, aggressive mobbing of a hawk could represent a cost of visual hawk-like characters (Lyon and Gilbert, 2013). As illustrated by the tawny eagle and drongo, prey will occasionally mob even very large predators vigorously, but this is not the case across all prey species and is rare among reed warblers (Welbergen and Davies, 2009). Indeed, baseline responses to predators are an important consideration in cuckoo-host dynamics and in particular, with regards to local variation in predator presence.

Female cuckoo calls share characteristics with those of Accipiter hawks (Newman, 2013; Liang, 2017; York and Davies, 2017). Similarities between female cuckoo and sparrowhawk calls provoke antipredator behavior to a similar extent both among hosts and non-hosts (York and Davies, 2017). Furthermore, in a number of cuckoo host species, female cuckoo and sparrowhawk calls similarly supress host defenses against brood parasitism, supporting the view that perceiving a female cuckoo call as that of a hawk manipulates a trade-off between behaviors that promote self-protection versus those that support parental investment (York and Davies, 2017; Roncalli et al., 2019; Marton et al., 2021; Shen et al., 2021). In the case of reed warblers, rejection defenses were suppressed, by contrast, great reed warbler, *A. arundinaceus*, mobbing responses were dampened following exposure to female cuckoo calls, which suggests the effects of these calls can supress both major lines of defense against brood parasitism. Whether these calls also increase opportunities for brood parasitism by increasing nest access is unknown (York and Davies, 2017; Marton et al., 2021). Given that diverse hawk-like stimuli are salient and provoke varied responses among hosts, we turn our attention to their evolution in the context of wider information ecology.

How Does Hawk Resemblance Evolve in Cuckoos?

Communication involves a signal that is emitted by a sender to influence a receiver, and in turn, the response of the receiver determines signal efficacy, ultimately providing a net fitness payoff to both parties (Bradbury and Vehrencamp, 1998; Maynard-Smith and Harper, 2003). Information is also available in the form of cues; mere byproducts of an organism existing in the environment (e.g., body shape, locomotory sounds), that are not emitted to influence a receiver and, importantly, cannot evolve independently from the characteristic about which they provide information (Maynard-Smith and Harper, 2003; Stevens, 2013). Crypsis scrambles information that could be extracted from cues, thereby concealing the organism from the detection systems of a natural enemy or victim, and is not widely considered to be a form of communication (Stevens, 2013; but see: Ruxton et al., 2018). By contrast, mimicry biases the characteristics of a mimic to correspond with information that is emitted by a model. In wrongly identifying the mimic as the model, the target receiver (the dupe) releases a benefit for the mimic while paying some form of cost for being misled (**Figure 1**; Dalziell and Welbergen, 2016; Font, 2019). Mimetic traits persist where there is a net fitness benefit for receivers, since correct detection of a true signal from the model is nevertheless advantageous (Stevens, 2013; Font, 2019). While examples of both signal and cue mimicry have been identified, the basis for their origin and maintenance are frequently debated and revised (Stevens, 2013; Dalziell and Welbergen, 2016; Jamie, 2017; Ruxton et al., 2018; de Jager and Anderson, 2019; Font, 2019). As such, the compelling diversity of adaptive resemblance continues to provide fascinating conceptual advances.

In cuckoos, visual hawk-like resemblance includes a number of features: overall shape and size, flight pattern, yellow legs and eye rings, and conspicuous barred chest plumage (Davies, 2000; Davies and Welbergen, 2008; Welbergen and Davies, 2011). Other predators that exhibit disruptive patterns (e.g., leopard spots or tiger stripes) are conspicuous in some contexts, but under dappled lighting or high speed motion, such patterns make it challenging to detect the boundaries of the predator's form (Stevens, 2013). The barred chest plumage of hawks and cuckoos is salient for prey, and the extent of chest barring modifies prey responses to hawks (Davies and Welbergen, 2008; Welbergen and Davies, 2011). The chest barring of the cuckoo could be considered a deceptive signal (because there is a fitness benefit in resembling the hawk) and is generally classed as Batesian mimicry (Welbergen and Davies, 2011). However, the resemblance is not based on a signal because the chest barring is cryptic plumage, therefore the conventional basis of mimicry is absent (**Figure 1A**). Additionally, a key criteria of Batesian mimicry is the assumption that the mimic imposes costs for the model (Stevens, 2013) and such a mechanism to enforce costs on hawks is difficult to envisage, and in fact, any habituation to hawk-like characteristics due to cuckoo exposure would be beneficial to hawks. Perhaps the least troublesome approach is to place hawk-like barring cues within the broader umbrella of adaptive resemblance (**Figure 1B**; Starrett, 1993).

Female brood parasitic cuckoos also exhibit plumage polymorphisms. While it appears that the existence of polymorphisms in female cuckoos is not aligned with mimicking an alternative raptor model (Trnka et al., 2015), cuckoos with hawk-like features are more likely to be polymorphic (Thorogood and Davies, 2013a). Plumage polymorphism is effective because hosts use social learning to identify the gray cuckoo morph as a fake, which incurs a frequency-dependent fitness benefit for the rarer rufous morph (Thorogood and Davies, 2012). It is also possible that rufous females benefit from reduced costs of being mobbed, due to any costs associated with hawk-like appearance (Lyon and Gilbert, 2013). This additional benefit for the hepatic morph would therefore be frequency-dependent in relation to host baseline aggression toward hawks.

The ecological basis for the resemblance between female cuckoo and hawk calls differs from that underlying visual resemblance. Adult hawks produce calls in the context of communication between the pair, accompanying provisioning visits during courtship and chick rearing (Newton, 1986). Consequently, these calls are conspicuous signals and prey can benefit from interceptive eavesdropping, since hawk calls are indicative of ongoing local predation risk (Peake, 2005; Ridley et al., 2014; Billings et al., 2015). Given that small passerines are the main target of sparrowhawk hunts, female cuckoo hawk-like calls exploit an interceptive eavesdropper with dishonest information; which can be considered a distinct role in communication ecology (**Figure 1C**). The benefits of hawk-like female cuckoo calls may originate from signals used in intraspecific communication (York and Davies, 2017; York, 2018). Cuckoos of both sexes are responsive to playbacks of adult cuckoo calls (Moskát and Hauber, 2019). The existence of multi-function signals is widespread in animal communication, since once a beneficial signal is produced, additional benefits reinforce its advantage (Bradbury and Vehrencamp, 1998). Although adult Cuculinae vocalizations are not fully described, those with sex differences in adult calls are brood parasitic (Payne, 2005; Kim et al., 2017).

The flexible and ephemeral nature of behavioral signaling means that female cuckoo calls can be produced with the most beneficial timing to influence relevant audiences (Chance, 1940; Wyllie, 1981; York and Davies, 2017). Their brevity may explain limitations for hosts to develop counter-responses through learning (York and Davies, 2017). Opportunities for individual and social learning of calls by hosts could be more scarce than for visual characteristics, since associative learning requires a mobbing or active demonstrator to be effective (Campobello and Sealy, 2011a; and references therein). If learning does occur, temporal variation in parasitism intensity may account for between-year variation in host defenses (Campobello and Sealy, 2011a; Thorogood and Davies, 2013b). Populations with higher frequencies of parasitism where cuckoos are more abundant (e.g., Moskát et al., 2008; Campobello and Sealy, 2011b) could provide greater opportunities for habituation or learning processes.

How Do Hosts Respond to the Hawk-Like Stimuli of Cuckoos?

Processing Hawk-Like Stimuli

Signal detection theory has long provided a basis for understanding animal communication and sensory ecology (Duncan and Sheppard, 1965; Wiley, 2006). How well prey can separate a hawk from background environmental noise (detection) is analogous to how well a host can discern between

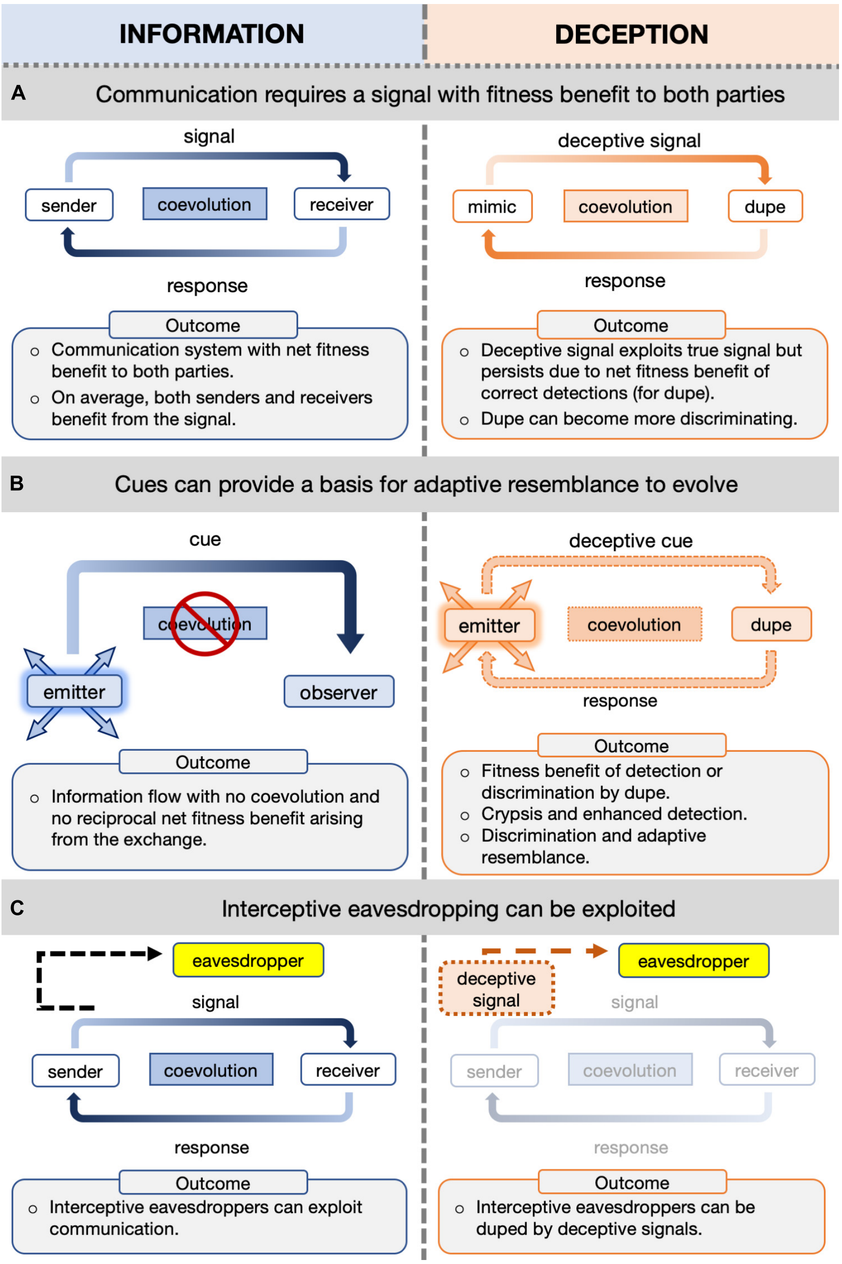

FIGURE 1 | Schematics to illustrate potential roles and relationships in adaptive resemblance. Mutually beneficial or neutral information (left column) and deceptive information (right column) for: **(A)** communication (L) versus deceptive mimicry that exploits an existing relationship (R)—on average it benefits the signaller to signal and the recipient to respond due to net fitness benefits of correct detections; **(B)** information from cues can provide the basis for other forms of adaptive resemblance to evolve, without relying on an established signaller-receiver relationship; **(C)** interceptive eavesdroppers can exploit an existing communication relationship, and in turn are susceptible to deceptive signals with overlapping characteristics.

cuckoo and hawk (discrimination), since both processes are influenced by sensory limits and performance (Stoddard and Stevens, 2011). We currently have a limited understanding of the perceptual processes underlying host responses to adult cuckoos.

Both the hawk-like barred chest plumage and conspicuous yellow eyes of cuckoos provoke responses from hosts (Davies and Welbergen, 2008; Trnka et al., 2012). How host responses relate to whether the characteristic of the predator model shared by the cuckoo is either contextually cryptic (barred plumage), or conspicuous (yellow skin) deserves further consideration. It is possible that these two types of information differ in perceptual processing. Detection of barring might be influenced by perceptual filtering, and its effect could be distance dependent, resulting in camouflage at distance and conspicuousness at close range, as occurs in some species (Stevens, 2013; Ruxton et al., 2018). Another interesting possibility is that the effect of barring could be conspicuous when static but cryptic in motion, since the same stimulus can vary in effect depending on observer context (Caro et al., 2013). The extent of this effect could depend on the exact plumage barring and degree of similarity to the model hawk, indeed, cuckoos do tend to be more similar to sympatric hawk species (Gluckman and Mundy, 2013). Future experimental work would fruitfully examine the integration of information to determine host perceptual processing speed and response thresholds to variation in these cryptic and conspicuous stimuli.

For hosts that appear less susceptible to the repellent effects of hawk-like appearance, vocal resemblance might provide cuckoos with another important mechanism for accessing host nests and avoiding the costs of being mobbed (Marton et al., 2021). The call may frequently follow the otherwise secretive and rapid behavioral sequence of a female gliding down to lay in the nest (Chance, 1940). Hosts observing this event may therefore be exposed to several hawk-like stimuli (body form and flight, plumage and pattern similarities, and calls) in sequence. Sequential exposure could either additively reinforce, or compensate for deficiencies in the others, depending on host discriminatory rules, iterative sampling rate, and multidimensional integration of predator stimuli (Leavell and Bernal, 2019). The effects estimated from experimental studies on singular hawk-like stimuli might therefore represent an underestimation of effects generated by an animated and multimodal live bird. Alternatively, cuckoo hosts might rely largely on discrimination rules based on single traits in isolation due to effects such as overshadowing (Kazemi et al., 2014).

Responding to Hawk-Like Stimuli

Host responses to hawk-like stimuli likely depend on species-specific baseline thresholds for predator detection and behavioral responses on detecting a predator. Prey can initiate several response types on detecting a predatory threat. Heightened vigilance combined with freezing can avoid localization by the predator, or fleeing the location can occur in the absence of, or in immediate response to, attempted attack (Ruxton et al., 2018). Alternatively, prey can aggressively mob predators, whereby easily localizable individuals approach and make violent contact with the predator (Shields, 1984; Caro, 2005). Cuckoo hosts use this range of behavioral defenses toward cuckoos, therefore it is important to examine whether host responses reflect discrimination, or costs of hawk-like resemblance (Davies and Welbergen, 2008; Lyon and Gilbert, 2013).

Detection thresholds can be modulated by individual factors (personality, state, age) and extrinsic environmental variation (Ruxton et al., 2018; Römer and Holderied, 2020). Similarly, discrimination thresholds are modulated with local parasitism or predation risk, which determines the trade-off between the costs of false alarms and correct detections for hosts in a given population (Welbergen and Davies, 2008, 2009; Davies, 2011). Behavioral responses to stimuli are regulated by neuroendocrine and endocrine mechanisms, and predator stimuli can provoke acute stress responses with sustained effects (Clinchy et al., 2013). Brood parasitism can influence stress physiology (Mark and Rubenstein, 2013), and hawk-like stimuli could contribute to modulation of this pathway. By doing so, brood parasites indirectly affect host risk-assessment physiology, which is analogous to the endoparasites that influence physiology underlying the risk-taking decisions of their host, and thereby promote parasite transmission (Poulin, 2010). Future studies could fruitfully examine the physiological mechanisms underlying responses to hawk-like stimuli, and the consequences for host life-history trade-offs. One important consideration is how such mechanisms interact with defense against other threats (e.g., egg predators; Campobello and Sealy, 2010, 2011a; Lawson et al., 2021).

DISCUSSION

The intriguing absence of predator resemblance among other avian brood parasites remains unexplained. Parasitic cowbirds (Icteridae) exhibit a number of general adaptations to mitigate host defenses, but lack resemblance of predators that prey on adult hosts (Lawson et al., 2021). This may be a consequence of phylogenetic or body size constraints. Hawk resemblance is also relatively rare even among cuckoos (of 141 species, 17% “hawk-like,” 28% with barred plumage; Thorogood and Davies, 2013a). Another possibility for adult brood parasites is aggressive mimicry, whereby the brood parasite resembles an innocuous model (Feeney et al., 2015). This form of resemblance could be more common than is widely appreciated, and deserves further attention.

Overall, predator resemblance allows cuckoos to exploit hosts and to enhance brood parasitism by taking advantage of multiple sensory and cognitive processes. The hawk-like stimuli of brood parasitic cuckoos appear to defy satisfactory labeling using established frameworks for mimicry. I hope that considering their placement in the context of communication ecology as adaptive resemblance, as described here, will prove useful.

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Decoupled Acoustic and Visual Components in the Multimodal Signals of the Common Cuckoo (*Cuculus canorus*)

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Because of its parasitic habits, reproduction costs of the common cuckoo (*Cuculus canorus*) are mostly spent in pre-laying activities. Female costs are limited to searching host nests and laying eggs, whereas, males spend time in performing intense vocal displays, possibly with territorial purpose. This last aspect, together with a sexual plumage dimorphism, points to both intra- and inter-sexual selections operating within this species. One element triggering sexual selection is a differential fitness accrued by different phenotypes. Before analyzing possible sexual selection mechanisms operating in cuckoos, it is therefore necessary to verify whether there is a variability among male secondary characters by describing and quantifying them. Here we aimed to quantitatively characterize the main two potential candidates of sexual selection traits, i.e., calls and displays, shown by males at perches. During the 2019 breeding season, in a site within the Po Plain, we both audio and video recorded cuckoo males at five different perches. We analyzed acoustic variables as well as display sequences searching for potential correlations. We found a significant variation among calls that could be clustered into four vocal types. We also found that no visual displays were associated with vocal displays; cuckoo males were either vocal and motionless or soundless and active. We discuss our results under the perspective of the potential value of sexual selection in brood parasites and its role in its parasitic habit.

Keywords: cuckoo, sexual selection, courtship rituals, bioacoustics, multimodal signals

INTRODUCTION

The common cuckoo (*Cuculus canorus*) is an obligatory brood parasite, as it lays its eggs in nests of other species who raise its young until fledging at the expenses of their own offspring (Chance, 1940; Wyllie, 1981). While this reproductive strategy results in saving an important amount of energy devoted to parental care, both female and male cuckoos are greatly engaged in pre-laying reproductive activities (Nakamura and Miyazawa, 1997; Davies, 2015). Since the beginning of the reproductive season, females constantly search for the "right" nest to parasitize

(Reboreda et al., 2017), whereas, males are often observed on obvious perches while performing long and repetitive displays.

In the context of sexual selection, acoustic signals as much as courtship rituals, can provide information regarding male quality over which potential receivers, i.e., females and males, may assess mating opportunities and confrontation prospects (Searcy and Andersson, 1986). Over evolutionary time, signals have been selected to convey honest communication serving a dual function, mate attraction and territory defense (Searcy and Andersson, 1986; Benedetti et al., 2018).

Little attention, however, has been given to the potential multimodal nature of these signals in the common cuckoo. Multimodal signals are defined as signals whose components are received by different sensory systems. They are widely diffused in the animal world and have been mainly investigated in the context of sexual selection, such as during courtship displays (Mitoyen et al., 2019). The exact function and selective role of multimodal signals is, however, not agreed upon, with several non-exclusive explanations proposed. Multimodal signals for example might have evolved to convey redundant information, with multiple components having the same meaning and retaining it also if emitted separately (Hebets and Papaj, 2005; Mitoyen et al., 2019). This is known as the back-up signal hypothesis and it is linked to environmental variability: the more channels are used in communications, the least probable it is that the signal might be missed (Johnstone et al., 1996).

On the other hand, the presence of multiple components might allow the sender to emit information about completely different topics. If that was the case, multimodal signals might evolve whenever there are different targets for the signals – for example, during simultaneous inter and intrasexual competition – or when the message itself is comprised of information about distinct aspects of the sender individual (e.g., both its position and its quality). In this case coordination between components is crucial: simultaneity or sequentiality between components might convey messages different than the sum of their parts (emergent properties, Rowe and Guilford, 1996). Part of the reason for the lack of consensus on the function of multimodal signals is that trait-based experiments tend to separate them in their single components (Groot et al., 2021). This can lead to biased or partial conclusions about the responses to these signals. Research involving common cuckoos has greatly focused on their calls, some of which have been found to be signals involved in both intra (Moskát et al., 2017, 2018) and inter-sexual selection (Moskát and Hauber, 2021). However, to our knowledge no research has been conducted on the potential multimodal nature of cuckoo signaling, i.e., if there are other components accompanying the acoustic display.

Behavioral displays have been examined in other brood parasite species, specifically in bronzed cowbirds (*Molothrus aeneus*) (Friedmann, 1929) and pin-tailed whydah (*Vidua macroura*) (Shaw, 1984), and described as a courtship ritual soliciting females at a close range. In both species, it has been described as pre-copulatory displays composed of airborne and terrestrial elements (Clotfelter, 1995). Cuckoo males perform their vocal displays from perches shared with females, who gain convenient point of view to find nests to parasite. Cuckoo males become territorial with its vocal properties (Moskát et al., 2017), but whether visual displays also serve this function has not yet been tested nor is there any evidence that they can attract a mate or transmit social information. The only clue comes from an experiment aimed to verify male cuckoo ability to discriminate different female morphs. Cuckoos attempted to mate with a decoy without any preliminary attempt to perform any courtship, indicating that male displays may not be used to attract females (Lee et al., 2019).

Unlike vocalizations, visual displays are, thus, completely unexplored in cuckoos, as there is no ethogram or evidence showing inter-individual variability or advanced hypotheses on their functions. Hypothetically, given that male cuckoo acoustic displays elicit a response from both males and females, a visual display might appear unneeded, or present only as a redundancy. However, given the extended amount of time this species devotes to the acoustic displays, it might be possible that a visual display might be a less generic, more targeted message for a specific set of other individuals – for example, a female approaching or a challenging male –. This might point to a sequentiality in the two displays, with the visual component following the acoustic one.

We studied both acoustic and visual displays in the common cuckoo, aiming: (i) to tentatively reveal how many individuals resided in the area by identifying vocal types (VTs, i.e., male individuals differing by their vocal features) and to determine VTs spatial preferences among their perches; (ii) to describe a cuckoo male ethogram and quantify consistent behavioral sequences; (iii) to verify the presence of multimodal visual/acoustic signals in male cuckoo displays and to characterize their relationship, and (iv) to verify whether there are VT-specific behavioral rituals.

MATERIALS AND METHODS

Study Area

We studied cuckoo rituals from May to August 2019 within the Mirandola Plain, a 500 hectares area recently naturalized in a marsh area with large reedbed, regularly monitored by volunteers of the SOM (Modena Ornithological Station). Today the whole area is a Special Protection Area hosting a very high biodiversity (Lui and Giannella, 2003), including dense populations of reed and great reed warbler (*Acrocephalus scirpaceus* and *A. arundinaceus*, respectively), two of the most parasitized species in Italy (Campobello and Sealy, 2009) with abilities to social learn antiparasitic defenses (Campobello and Sealy, 2011). At the beginning of the season, we identified five perches, for brevity Vantage Points, VPs (VP1, VP9, VP16, VD, and T; **Supplementary Figure 1**), where cuckoos performed vocal and visual displays most often. Distances between perches averaged 364 m (range 160–1,189 m).

Acoustic and Video Recording

We recorded cuckoo calls and displays with three video cameras (Sony DCR-DVD650, Canon LEGRIA HF R86, Nikon D330). We recorded cuckoo calls also with a ZOOM H4 digital recorder (ZOOM Corporation; parameters: 44. 1 kc/s sampling rate, 16bit depth) connected to a directional microphone (Audio Technica AT815b). We modified a tripod so that it could hold simultaneously the microphone and one of the video cameras. Other two tripods held one video camera each. With favorable weather conditions, thus with no precipitations or strong wind, each day, we placed each of three tripods in front of a randomly chosen VP and left recording for approximately 3 h.

Call Description

Among all cuckoo calls only the most common one, the *cu-coo* call, has been shown to possess individual specific properties. This advertising call consists of two notes and is employed by cuckoos to recognize familiar individuals, such as close neighbors (Moskát et al., 2017). Both notes of *cu-coo* call are individual-specific (Moskát et al., 2018), in particular with the call frequency, duration and, especially, the maximum frequency of the first syllable being the most characterizing variables (Zsebõk et al., 2017). It is therefore possible to discriminate individuals starting from the analysis of their vocalizations by using acoustic variables as discriminatory elements (**Table 1**; Lei et al., 2005; Li et al., 2017; Zsebõk et al., 2017). In addition to the *cu-coo* call, males possess a richer vocal repertoire than the single multi-purpose call uttered by females, the *bubbling* call. Other male calls include a slight different variant of the advertising call, the *cu-cu-coo* call, and two calls used more less frequently, the *gowk* and *gou* calls (Lei et al., 2005; Moskát and Hauber, 2019). Contrarily to the *cu-coo* call, there is no evidence that all the other calls are characterized by an inter-individual variability.

Acoustic and Video Analysis

Acoustic Analysis

We analyzed a total of 27 h 03' of tracks recorded by both microphone and video cameras. Recordings from video cameras were extracted with the Video Pad-Video Editor program. After a preliminary visual screening of collected recordings, we identified several *cu-coo* and *cu-cu-coo* call sequences (**Supplementary Figure 2**). The other calls of the cuckoo male repertoire were rarely used, making their analysis difficult if not impossible. We detected, however, a call resembling both the *gowk* (Moskát and Hauber, 2019) and *gou* (Lei et al., 2005) calls but, given the scant sample size and the lack of reference on its vocal characterization (**Table 1**), we preferred to assign it a new name (*bark* call) and provide characterization details to assist future comparisons (**Supplementary Figure 3**).

We used the terms *call* and *syllable* only when analyzing the sequences including *cu-coo* and *cu-cu-coo* calls. Specifically, the syllable was one single element, the calls the set of several syllables (i.e., the *cu-coo* together form one call, with *cu* the first syllable - S1 and *coo* the second– S2), whereas the whole sequence was composed of all call repetitions divided by a pause of less than 2 s as shown in **Supplementary Figure 2**. To find cuckoo sequences, we visually inspected spectrograms using Raven Pro 1.5.0 (Yang and Center for Conservation Bioacoustics, 2014; Cornell Lab of Ornithology, Ithaca, NY, United States) with the following settings: brightness 48, contrast 69, spectrogram window size at 3,268 points.

To analyze time-and-frequency parameters, we selected only the *cu-coo* and *cu-cu-coo* sequences characterized by high intensity and absence of overlap with other signals. We manually selected a maximum of three calls per sequence selecting those with a better quality on the base of the spectrogram inspection (brightness 48, contrast 85, FFT spectrogram window size at 1,329). Then we analyzed acoustic variables that were previously used in other studies (Lei et al., 2005; Wei et al., 2014; Li et al., 2017; Zsebõk et al., 2017): for each syllable (i) syllable length (ΔT, i.e., ending– starting times), (ii) minimum frequency (F_{min}), (iii) maximum frequency (F_{max}), (iv) bandwidth (ΔF, i.e., maximum – minimum frequencies), (v) peak frequency (F_{peak}, i.e., frequency with the maximum energy); for the intra-call syllables: (viii) pause between two adjacent syllables (T_{pause}), and (ix) difference between maximum frequencies of two adjacent syllables (Δ F_{max}).

Behavioral Analysis

Out of a total of 9 h 19' of video recordings, cuckoos were present in only 2 h 50' distributed in 42 video clips. We identified 16 behaviors within three behavioral categories and behaviors within each category were mutually exclusive (see section "Statistical Analysis" and section "Behavioral Analysis," **Table 2**). We coded all behaviors using Boris v.7.9.22 software (Friard and Gamba, 2016), while slowing down the speed of video-recording by 50%. All video clips showed one focal individual, a cuckoo male, at the time. To investigate whether the single behavioral events – i.e., any instance of focal individual activity or its absence – were replicated in consistent behavioral sequences, all behavioral data were further summarized in three categories: "Posture," "Movement," and "Vocalization." Behavioral events within the same category (states) were considered mutually exclusive; all events within each category of each behavioral sequence belonged to one of the following states.

Posture category was defined as the general positions that individuals assumed when perched, with two states: either (i) *Lax* individual keeps their wings lower than the tail, the wingtips pointing down and the rump up; or (ii) *Non-Lax*, individual either moves or perches with their wings above the tail. Movements were divided in three states: (i) *Still*, i.e., not performing any movement, (ii) *Tail swing* individual moves its tail (either *tail swing left to right* or *tail swing up*): we pooled *tail swing left to right* together with *tail swing up* as the latter behavior happened almost always simultaneously with the former (92.5% of the time, see **Table 2**); (iii) *Active*, which includes several other types of movements (i.e., *head movement*, *autogrooming*, *moving alongside the branch*, *rotating the body*; see **Table 2**). Vocalizations were summarized in two states: (i) *calling* and (ii) *silent* (since *bark call* represented only the 0.05% of the total vocalizations, for the purpose of this analysis, we decided to pool all calls together). For further rationale behind these categorizations, see section "Results" and section "Behavioral analysis" and **Table 2**.

Statistical Analysis

Acoustic Analysis

To test whether we could discriminate different vocal types among all selected sequences, we averaged the syllable acoustic variables per song, then we quantified the intra-song coefficient of variation (CV) as $CV = 100*[1+1/(4*n)]*SD/mean$, where

TABLE 1 | Summary of bioacoustics studies on cuckoos listed by their calls.

Reference	Call	Site	Study period	Marked individuals? Y/N	Spectrogram? Y/N	Inter-individual vocal differences?
Xia et al., 2019	*Cu-coo*	China	2017–2018	Y	Y	–
Moskát et al., 2018	*Cu-coo*	Hungary	2017	Y	Y	Y
Li et al., 2017	*Cu-coo*	China	2016	Y	Y	Y
Moskát et al., 2017	*Cu-coo*	Hungary	2016	Y	Y	–
York and Davies, 2017	*Cu-coo*	United Kingdom	2016	N	Y	–
Zsebõk et al., 2017	*Cu-coo*	Hungary	2013	N	Y	Y
Jung et al., 2014	*Cu-coo*	South Korea	2012	N	Y	Y
Wei et al., 2014	*Cu-coo*	Asia and Europe	2012–2013	N	Y	–
Fuisz and de Kort, 2007	*Cu-coo*	Hungary	1998–2003	N	Y	–
Lei et al., 2005	*Cu-coo*	Asia and Europe	1997–1998	N	Y	Y
Xia et al., 2019	*Cu-cu-coo*	China	2017–2018	Y	Y	–
Lei et al., 2005	*Cu-cu-coo*	Asia and Europe	1997–1998	N	Y	–
Moskát et al., 2021	*Cu-kee*	Hungary	2020	N	Y	–
Moskát and Hauber, 2019	*Gowk*	Hungary	2018	N	N	–
Lei et al., 2005	*Gowk*	Asia and Europe	1997/1998	N	Y	–
Lei et al., 2005	*Gou*	Asia and Europe	1997/1998	N	Y	–
Moskát and Hauber, 2019	*Bubbling*	Hungary	2018	N	Y	–
Xia et al., 2019	*Bubbling*	China	2017–2018	Y	Y	–
York and Davies, 2017	*Bubbling*	United Kingdom	2016	N	Y	–
Lei et al., 2005	*Bubbling*	Asia and Europe	1997–1998	N	Y	–

TABLE 2 | Summary of behavioral events recorded during the video analysis.

Category	State	Behavioral event	% of events in category	Description
Position	Lax	Lax	94.29	Keeping wings lower than the tail, the wingtips pointing down, and rump up
	Non-lax	Non-lax	6.61	Either moving or perching with wings above the tail
Movement	Tail swing	Tail swing up	4.49	Moving tail up and down
		Tail swing left-to-right	16.04	Moving tail left to right
	Active	Move from branch	0.09	Moving from one perch to another
		Turn around	0.96	Fast spinning around on the same spot
		Rotating body	0.57	Slow spinning around on the same spot
		Auto grooming	3.05	Preening own feathers
		Fly away	1.13	Away from the video frame and out of sight
		Head movement	32.43	Moving head in whatever direction
		Arriving	0.26	Arriving within the video frame and perching
	Still	Still	48.82	Completely motionless
Vocalization	Calling	*Cu-coo* call	49.30	Two-syllable call uttered by males
		Bark call	0.26	Guttural call uttered by males
		Duet	0.57	Two cuckoo males uttering calls one after the other. in an alternate mode
	Silent	Silent	49.87	Completely soundless

Events were divided in three categories (position, movement, and vocalization). Each categories included a sequence of self-excluding states. States, in turn, were built by pooling together behavioral events that were similar (e.g., tail swing up and tail swing left-to-right) or extremely rare.

n is the sample size (following Zsebõk et al., 2017). We then selected the acoustics variables characterized by a low level of CV (less of 3%) and rescaling them to prevent biases due to data overdispersion (Zuur et al., 2007). By using SPSS software (SPSS Institute Inc., Chicago, IL, United States), we conducted a hierarchical cluster analysis (Yim and Ramdeen, 2015) with the squared Euclidean distance on the rescaled variables. All acoustics variables previously selected were tested with Kruskal-Wallis rank sum test and Dunn's test to verify whether they differ among the vocal types.

Spatial Preferences

To test whether the vocal types were found significantly different in specific VPs, we conducted χ^2 tests on contingency tables built with both the number and the percentages of each vocal type found in each VP. These analyses were run with Statistica 10 (StatSoft Inc, 2001).

Behavioral Analysis

Analyses of this section were performed by using R version 4.0.3 (R Foundation for Statistical Computing; R Core Team, 2014). Each video was decoded as a three-categories behavioral sequence, i.e., a sequence of events (42 sequences, 54.57 ± 99.58 [mean ± SD] events). Each event was characterized by three states, one for each category: for example, event 1 of sequence 1 could be lax/active/silent, followed by event 2 of sequence 1 lax/active/calling, followed by event 3 of sequence 1 lax/still/calling, and so on. From these three-categories behavioral sequences we created a contingency table with co-occurrences of Posture, Movement and Vocalization, and we tested the significance of these co-occurrence with Fisher exact test, adjusted with False Discovery Rate [fdr, packages Multcompview (Graves et al., 2015) and GmAMisc (Alberti, 2020)].

To further analyze associations between behavioral displays and vocalizations, behavioral sequences were also modeled with a hidden Markov chain model (package seq HMM, Helske and Helske, 2017). This analysis allows to search for Hidden Markov States (HMSs), i.e., partial sequences and co-occurrences of states that might be repeated within a sequence. In the models, we used Movement and Vocalization categories, because the Posture category was represented by the only state (*Lax*) in the vast majority of behavioral events. We created the model with the function "build_hmm" and fitted it with function "fit_model" (Helske and Helske, 2017). The model estimated several parameters through Maximum Likelihood: initial probabilities (probability to be in a specific HMS at the beginning of the sequence), transition probabilities (the probability to pass from one HMS to the others) and emission probabilities (the probability of a state in each category to be associated with an HMS). This analysis appeared particularly suited to our dataset as it can estimate more than one category (or channel) at the time, i.e., it could detect pattern of association and transition even between states not belonging to the same category, thus forming multi-category HMSs. We selected the number of HMSs via BIC-selection and fitted the model with best BIC estimates (Helske and Helske, 2017). No covariates were included.

Finally, to detect possible differences in the behavioral displays of the vocal types, we performed a finer scale Symmetry test of the two contingency tables (Alberti, 2020) considering the four most common behavioral states of those sequences: *cu-coo call*, *tail swing left to right*, *tail swing up*, and *bark call*.

RESULTS

Vocal Types and Spatial Preferences

We counted 800 call sequences, including 368 recorded in the morning and 432 in the afternoon. For characterization, we manually selected 550 *cu-coo* calls, for a total of 1,100 syllables and then we characterized the syllables (S1 and S2) by their acoustic variables in terms of mean ± SD. The acoustic variables with low CVs values and therefore used to identify the potential different vocal types were the following: F_{min}, F_{max}, F_{pmax} for both the first (S1), and second syllable (S2). Entering these six variables into a cluster analysis and by using a cut-off limit of 4, we identified four groups and one isolated call which was excluded for the following analyses (**Figure 1**). The cluster dendrogram shows four different groups, thus four vocal types (VTs), that we labeled Red, Blue, Green and Yellow. The Kruskal-Wallis test showed significant differences between the four clusters for each variable (all tests, χ^2 = 46.4–60.8 [min-max], $P < 0.001$, $N = 79$, df = 3). In more detail, the Duncan tests showed, first, that both syllables mirrored exactly the differences and similarities resulted in each of the variables (**Supplementary Table 1**), and, second, that although all four groups were significantly different for most of the variables, the only two vocal types that differed significantly for all of them were the Blue and the Red (**Figure 2**). Out of the four vocal types, only the Red one was ubiquitous, thus not showing specific VP preferences (χ^2 = 7.38, $P = 0.117$, $N = 29$). The Blue was found mostly on VP9 (χ^2 = 33.72, $P < 0.001$, $N = 36$), whereas the Green (χ^2 = 20.36, $P < 0.001$, $N = 11$) and Yellow (χ^2 = 16.00, $P = 0.003$, $N = 4$) preferred the VP1. These significant values, however, disappeared when we analyzed the percentages of the number of times vocal types spent in each VP, indicating they had no spatial preferences (all tests, χ^2 = 0.25—4.00, $P > 0.05$, **Figure 3**).

Behavioral Analysis

We determined 2,292 behavioral events distributed in 42 sequences, each one characterized by three states according to the three categories: Posture, Movements, or Vocalizations. Within the Posture category, cuckoo males spent most of their time perching in *lax* posture (94.3% of all the events in the combined behavioral sequences). During our recordings, they often emitted a *cu-coo* call (49.3% of the events), very rarely a *bark* call (0.2% of all events), and otherwise they stayed silent (50.5% of the events). As for Movement, individuals either perched *still* (48.8% of the events), performed *tail swing* (16.2% of the events) or other movements (*active*, 34.9% of the events, see **Table 2** for further details).

We found a positive association between *lax* posture and *calling*, with the *non-lax* posture being associated with *silent* (Fisher's exact test adjusted with fdr, $P < 0.001$, **Figure 4A**). *Active* was associated with *non-lax* posture while both *still* and, more weakly, *tail swing* were associate with *lax* (Fisher's exact test adjusted with fdr, $P < 0.001$, **Figure 4B**). We also found a positive association between perching *still* and *calling* and between staying *silent* and *active* (both Fisher's exact test adjusted with fdr, $P < 0.001$, **Figure 4C**). The opposite associations – *calling* and being *active*; perching *still* and staying *silent* – were accordingly negative (both Fisher's exact test adjusted with fdr, $P < 0.001$). *Tail swing* showed weaker associations, positive with staying *silent* and negative with *calling* (both Fisher's exact test adjusted with fdr, $p < 0.001$, **Figure 4C**). All these results together indicate that cuckoo males adopted either a still lax posture while uttering *cu-coo* calls or an active perched position while being silent. Swinging tail was the only movement cuckoo males weakly associated with either a lax posture or being silent.

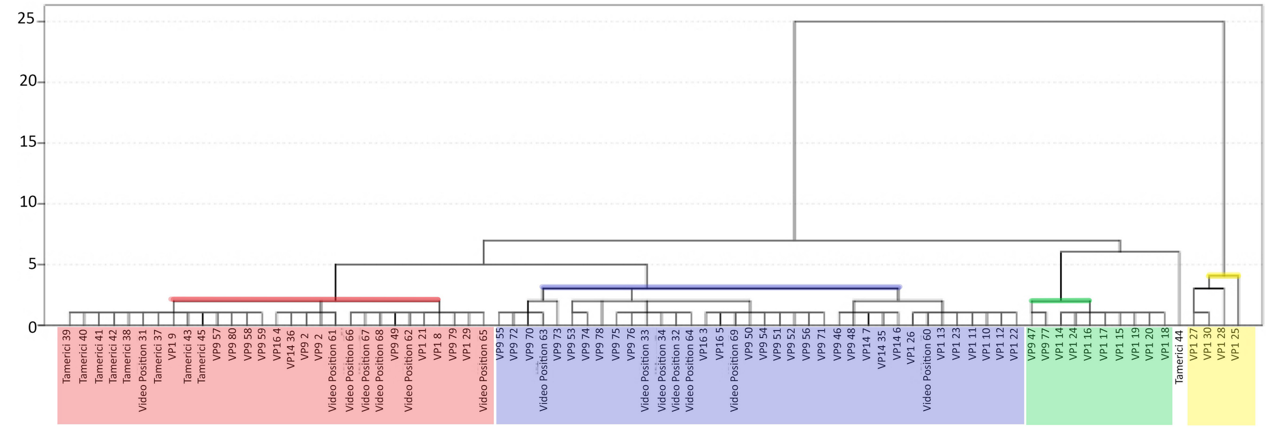

FIGURE 1 | Dendrogram of the hierarchic cluster analysis based on mean values of each acoustic variable (see text) quantified in each song. Labels at the bottom show the vantage point where the audio recording took place and the song identification number. The y-scale represents the rescaled distance for the four vocal types, Red, Blue, Green and Yellow, here identified by using a distance cut-off of 4 (Yim and Ramdeen, 2015).

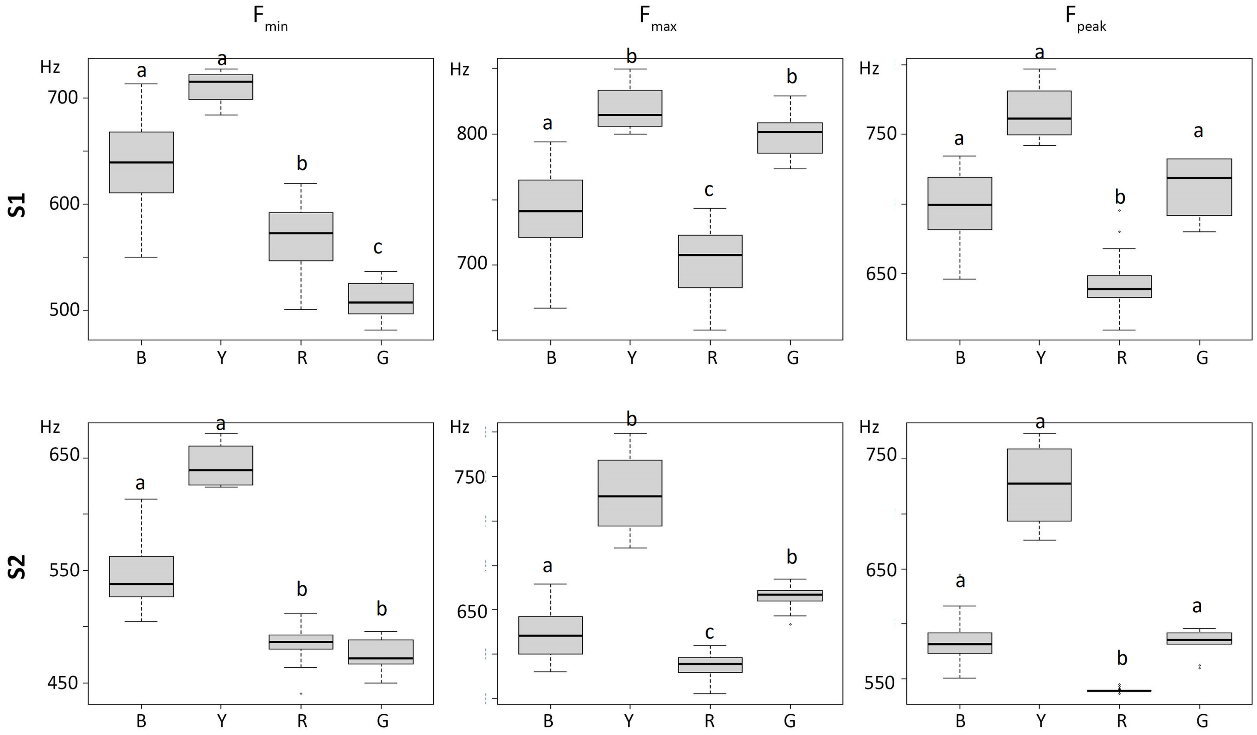

FIGURE 2 | Boxplot of F_{min}, F_{max}, F_{pmax} variables for both the first (S1), and second syllable (S2) for the four vocal types identified by the cluster analysis (R, red; B, blue; G, green; Y, yellow). Different letters indicate significant differences between groups revealed by Dunn's test ($P < 0.05$, for detailed statistics see **Supplementary Table 1**).

The BIC of the Markov chain model was lowest with four hidden states. After fitting the model, hidden Markov state 2 (HMS2) had the lowest initial probabilities (<0.001; State 3 had highest initial probability (0.692) while HMS1 and HMS4 had initial probabilities of, respectively, 0.163 and 0.145 (**Table 3** and **Figure 5**). In general, as expected from the results of the contingency association tables, in the model HMS3 had high emission probabilities for *silent* and *active* (respectively 1.000 and 0.870, **Figure 5**, Green) while HMS4 had high emission probabilities for *calling* (0.998) and *still* (0.990) (**Figure 5**, Blue). HMS1 described individual staying *silent* (0.988) and performing *tail swing* (0.869) (**Figure 5**, Light Gray), while HMS2 –the least probable – represented individuals performing a *tail swing* (1.000) and also *calling* (0.793) or stayed *silent* (0.207) (**Table 3**

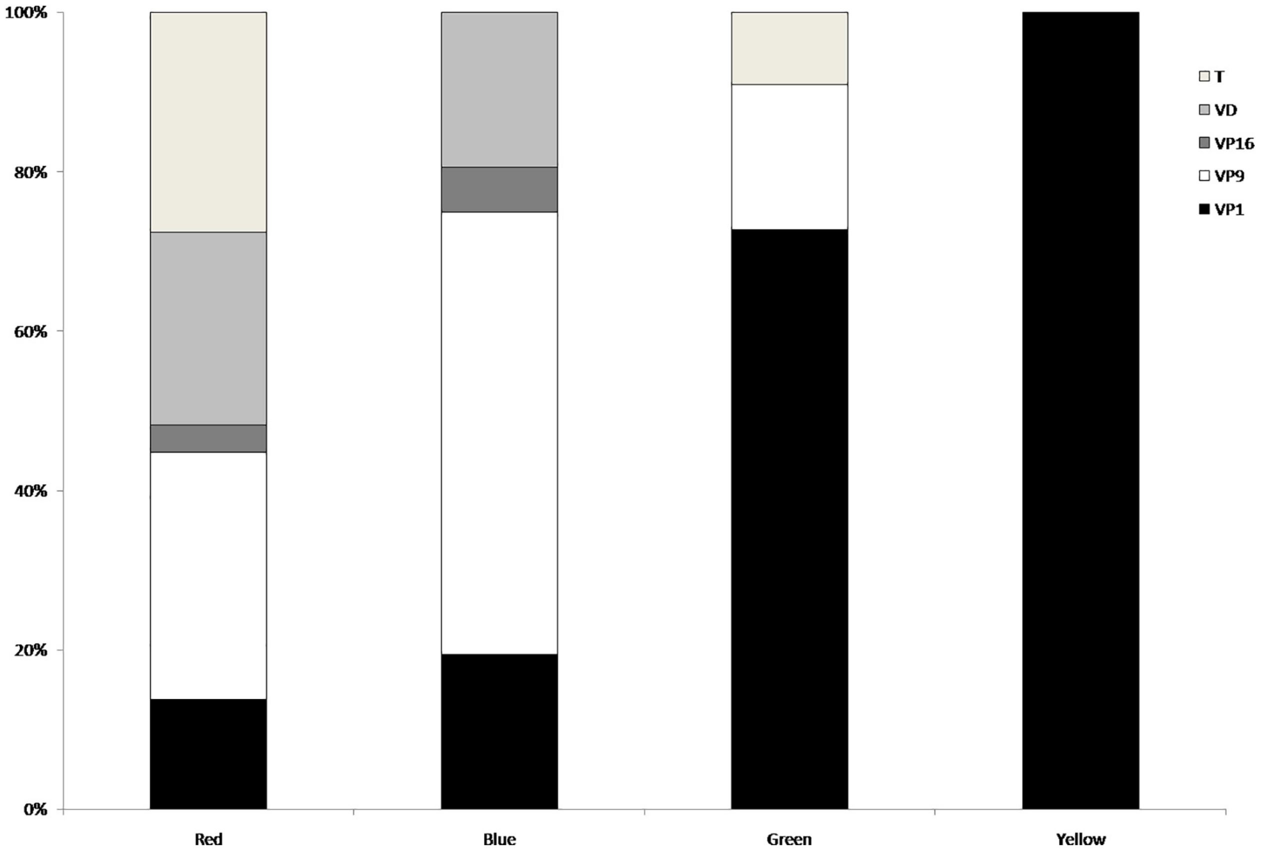

FIGURE 3 | Percentages of the number of times each of the four vocal types (red, *N* = 29; blue, *N* = 36; green, *N* = 11; and yellow, *N* = 4) was found on each of the five Vantage Points (T, VD, VP16, VP9, and VP1).

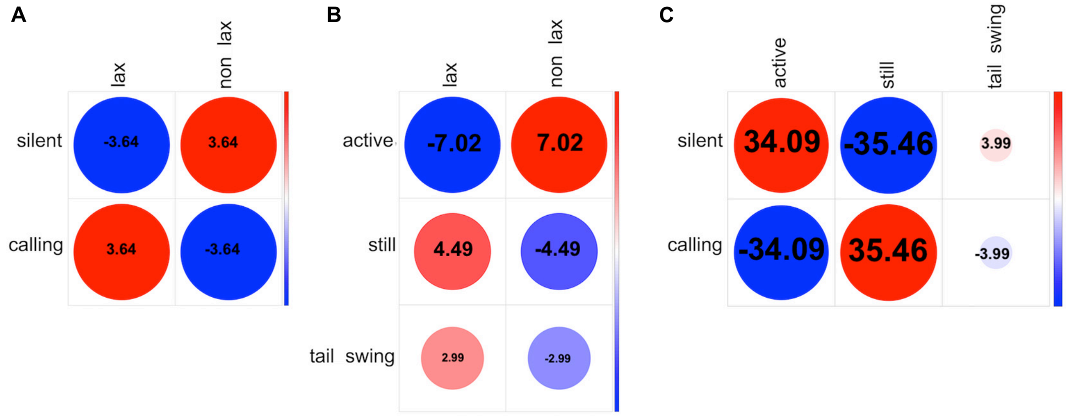

FIGURE 4 | Strength of association between states (behavioral events) of different categories. The charts show Pearson's Standardized Residuals (numbers within the circles), obtained through χ^2 test of independence on permuted dataset. Greater positive deviation from independence (positive association) is indicated with darker hues of RED; greater negative deviation from independence (negative association) is indicated with darker hues of BLUE. Only residuals higher than threshold of 1.96 are shown. **(A)** Association between Vocalization and Posture categories. **(B)** Association between Movement and Posture categories. **(C)** Association between vocalization and posture categories.

and **Figure 5**, Red). In general, the hidden Markov models provided further confirmation that individuals either perched silent and active or were still and calling. These last results also showed that transition probabilities underscored a generally very high (0.960 < x > 0.7992, **Table 3**) chance that each hidden state repeated itself with a low probability to switch to another one.

TABLE 3 | Hidden Markov model parameters, obtained through maximum likelihood.

		Initial probabilities			
		HMS1	**HMS2**	**HMS3**	**HMS4**
		0.163	0.000	0.692	0.145
		Transition probabilities			
	From				
	To	**HMS1**	**HMS2**	**HMS3**	**HMS4**
	HMS1	0.960	0.010	0.030	0.000
	HMS2	0.007	0.934	0.059	0.000
	HMS3	0.004	0.003	0.799	0.194
	HMS4	0.000	0.008	0.162	0.830
		Emission probabilities			
Vocalizations		**Categories**			
	State	**Silent**	**Cu-coo**		
	HMS1	0.988	0.012		
	HMS2	0.207	0.793		
	HMS3	1.000	0.000		
	HMS4	0.002	0.998		
Movements		**Categories**			
	State	**Still**	**Tail swing**	**Active**	
	HMS1	0.089	0.869	0.042	
	HMS2	0.000	1.000	0.000	
	HMS3	0.130	0.001	0.870	
	HMS4	0.990	0.000	0.010	

Initial state probabilities, transition probabilities, and emission probabilities for each hidden Markov state are reported.

Thus, we did not find any evidence of an association between behavioral displays and calling, nor of other specific patterns within the sequences of behavioral events.

The relatively low number of videos associated with Red and Blue vocal types did not allow an in-depth analysis, and care should be used when interpreting even significant results. With this being said, we found that the Blue VT performed more *tail swing up* than Red VT with respect to total events (Nominal Symmetry Test adjusted with fdr through 100000 Montecarlo simulations, $P = 0.011$). There was also a slight (non-significant) difference in their emission of *bark* call with Red VT performing some, while Blue VT never did (Nominal Symmetry Test adjusted with fdr through 100000 Montecarlo simulations, $P = 0.25$).

DISCUSSION

Despite their complexity, cuckoo vocal features allowed us to discriminate different vocal types among the cuckoo males during their breeding period. While literature widely suggests that vocal types in "cu-coo" calls might be linked to inter-individual differences in male cuckoos (Lei et al., 2005; Li et al., 2017; Moskát et al., 2017, 2018; Zsebõk et al., 2017) we could not verify this, as we did not individually mark the multiple males frequenting the area. Although all but one vocal types seemed inclined to prefer specific perches, this apparent spatial preference disappeared as their sample size increased. This result suggests that no territorial behavior takes place among competing males, or at least, that vantage points are not territories to be defended. Perched on bare branches of trees beside or within reedbeds, cuckoo males showed two main behavioral states where vocal and motor activities were decoupled, as if they were almost mutually exclusive. Thus, vocal cuckoos were motionless whereas active cuckoos were soundless. Each behavioral state also tended to repeat itself with a low probability to switch into the other. Lastly, our results were not conclusive on the possible differential behavioral patterns adopted by each vocal type.

Cuckoo territoriality is suggested occurring in both sexes. In Eastern European populations, males utter *cu-coo* calls as a signal of territorial defense and aggression (Moskát et al., 2017, 2018; Tryjanowski et al., 2018) during the breeding season and when intrasexual aggression rate is high (Nakamura and Miyazawa, 1997). In a Japanese population, males are reported to engage in both aggressive interactions with other males and sexual contacts with females in singing areas, thus specific perches where dominant males actively sing and defend these areas from other males (Nakamura and Miyazawa, 1990, 1997). The size of singing areas of different males decreases as the density of males increases. Singing areas they might overlap with each other especially when they are within territories frequently visited by females because of a high presence of host nests. There are two causes, not necessarily mutually exclusive, explaining this territory overlapping. First, the presence of sub-dominant males sneaking some singing activity while dominant males are absent (Nakamura and Miyazawa, 1997) and, second, the polygamous nature of cuckoo males who can travel long distances to increase their mating opportunities with different females (Marchetti et al., 1998). Our results, apparently suggesting the lack of male territoriality, could instead be the outcome of either contexts driving toward singing area overlapping, thus subdominant males roaming across the entire study site and/or males searching different females to mate.

If male calls are mostly related to territoriality, our hypothesis aimed to verify a simultaneous visual component might be associated to them, indicating the presence of a multimodal visual and acoustic display. Our results did not show this being the case, as cuckoo males either call or move while were on their perches, indicating that male cuckoos do not possess a multimodal display. In fact, we argue that our results show the absence of, first, a display with simultaneous visual and acoustic components and, second, a display with a fixed and repeated alternation of the two.

There could be several explanations for a decoupling between calls and behavioral displays. First, the voice of the cuckoos is sufficiently powerful that, depending on environmental conditions, can be heard at several kilometers of distance (Meshcheryagina and Opaev, 2021). While a male sings from an obvious perch, both potential mates and competing males can hear his call from afar, well before coming into contact with the vantage point. This implies that displaying while calling could

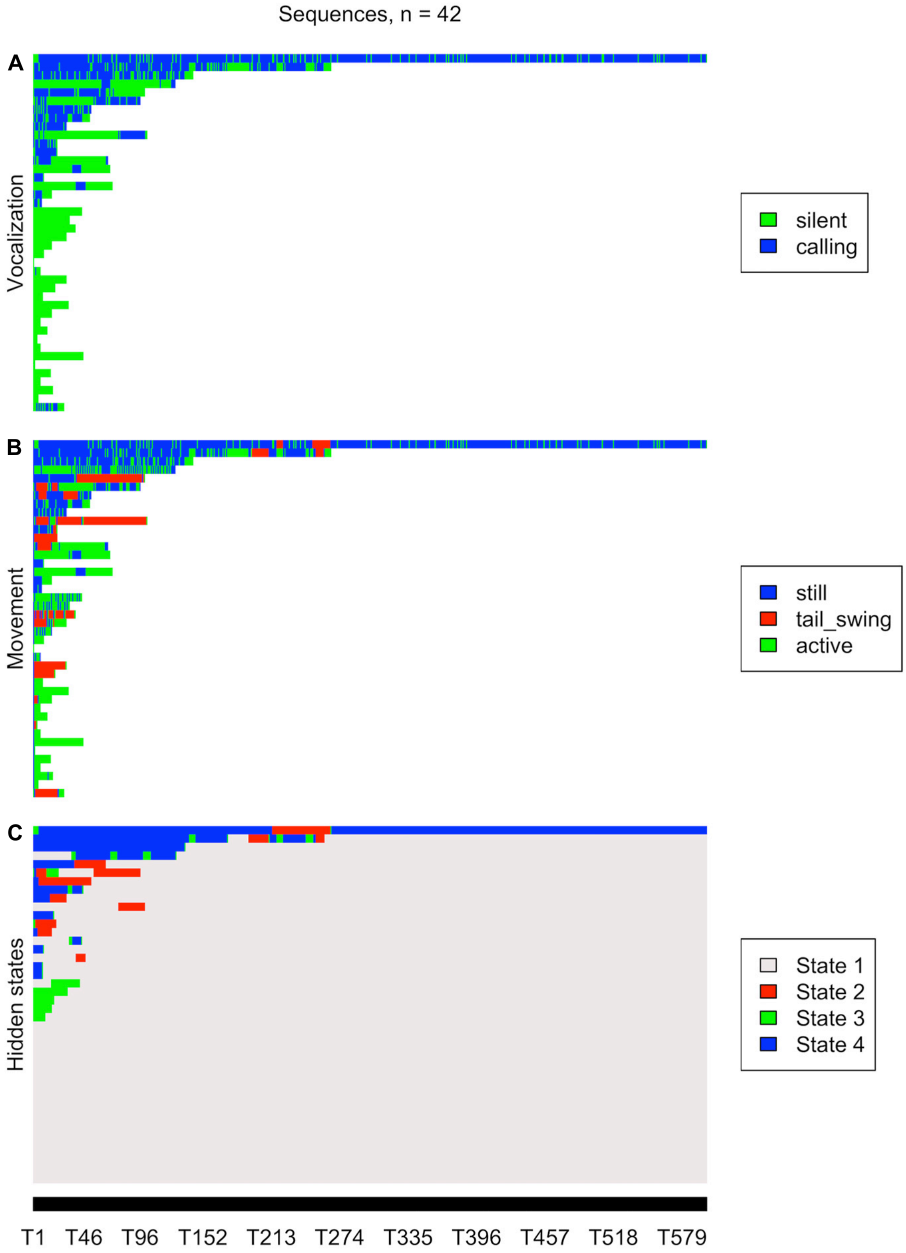

FIGURE 5 | Graphical representation of behavioral sequences of events within different categories (i.e., vocalization and movement). Each horizontal bar represents one behavioral sequence (*n* = 42, length 1–601 events). We attributed a color to each state and its behavioral events. Behavioral sequences of **(A)** vocalization events. **(B)** Movement events. **(C)** Hidden Markov states (HMS) showing that cuckoo males either tend to perch motionless while repetitively calling (HMS4) or are active while being silent (HMS3).

be broadly useless, a dangerous waste of energy (Cooper and Goller, 2004), and that the *cu-coo* call could be seen as a long-distance signal, meant to attract or repel conspecifics. Thus, there would not be the need of a visual display, neither as backup, nor to provide different information – the acoustic display of the common cuckoo is informative for both male and female conspecifics –.

Regardless from their association with calls, behavioral displays in general may play their function only if they are shown in close proximity of another individual (Bradbury and Vehrencamp, 1998). In our study, in the behavioral state in which cuckoo males moved silently, the movements involved cannot be described as potential displays for mate attraction or territoriality as they were generic movements (e.g., grooming). The only possible candidate to play a role as part of a ritual was the tail swings (Andersson and Iwasa, 1996). These resulted more evenly distributed, without a strong association with the *cu-coo* calls.

In social Cuculids, only anecdotic evidence is available for precopulatory displays (McNair, 1991; Merrett, 2014), whereas other displays have been investigated when used in communal chores (Strong et al., 2018) or toward their hosts (Davies, 2011). We cannot exclude that, since all of our video recordings showed only one male at the time, visual display might still have a role in the common cuckoo when directly confronted with a conspecific or as a response to heterospecifics. In other species acoustic and visual signals are assessed sequentially, depending on the range of the sensory system. For example, male sage grouses (*Centrocercus urophasianus*) attract females with their calls to their display site (Gibson and Bradbury, 1985); island flycatchers (Monarchidae) assess rival conspecifics first acoustically and then visually (Uy and Safran, 2013). Our results could be consistent with a visual component being employed and assessed only if the vocal component has managed to attract conspecific close enough, which, during our experiments, never happened at the VPs. An ideal test of tail swing function should aim to increase the sampling effort so to acquire recordings with the focal individual in close proximity to other conspecifics, both males and females.

CONCLUSION

Calls and visual displays are perfect candidates of traits on which sexual selection might operate as they may serve to compete for a territory (i.e., intra-sexual selection) or attract a mate (inter-sexual selection, Andersson and Iwasa, 1996; Bradbury and Vehrencamp, 1998). To our knowledge, our study is the first attempt in cuckoos to determine whether both behavioral traits show an inter-individual variability within population, the first necessary step to successively determine differential fitness of male ritual phenotypes. While we determined differential vocal types, our data did not allow to detect whether each one adopted differential rituals. In future studies, efforts should be directed to collect an adequate number of audio and video recordings of interactions between different individuals. These observations would allow the analysis of both intra- and intersex interactions, which may serve to better explain the apparent lack of associations between calls and visual displays we found in cuckoo males. This is a quite unusual condition in birds whose courtship rituals are often found expressed boldly together (Cooper and Goller, 2004). While the most frequent call, the *cu-coo* call, has been suggested to have a territorial function (Moskát et al., 2017), we found an apparent lack of territoriality of each vocal type that, on the contrary, did not appear to prefer specific perches for their vocal displays. Investigating the function of the cuckoo calls should not conducted independently from the forces selecting for call composition. The structure of the cuckoo song has been shown to depend on the probability to be mobbed by other species (Benedetti et al., 2018), suggesting that interspecific communication takes place selecting not only for individual traits but also for their extended phenotypic version (Campobello et al., 2015). Thus, potential selective factors, such as host density and host species availability, should be taken into account to examine the whole multimodal signals in cuckoos.

All the above-mentioned future directions involve activities that require a considerable field effort. The effort, however, would be proportionate to the value of the knowledge we could acquire about the mechanisms operating on the sexual selection of this species, that besides being part of one of the best coevolutionary models (Davies and Brooke, 1988; Campobello and Sealy, 2018) is also a declining bioindicator species (Tryjanowski and Morelli, 2015).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data can be found here: https://doi.org/10.6084/m9.figshare.16884925.

ETHICS STATEMENT

The animal study was reviewed and approved by ISPRA, via Vitaliano Brancati, 00144 Roma.

AUTHOR CONTRIBUTIONS

DC conceived and designed the study. ME collected the data. MC and GB analyzed the acoustic recordings. BT analyzed the video recordings. ME, MC, BT, and DC wrote the manuscript. SM, LS, and MD contributed to the manuscript and approved the submitted version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.725858/full#supplementary-material

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Tricking Parents: A Review of Mechanisms and Signals of Host Manipulation by Brood-Parasitic Young

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Obligate avian brood parasites depend entirely on heterospecific hosts for rearing their offspring. From hatching until independence, the young parasites must deal with the challenge of obtaining sufficient parental care from foster parents that are attuned to provisioning their own offspring. Parent-offspring communication is mediated by complex begging displays in which nestlings and fledglings exhibit visual (e.g., gaping and postures) and vocal (e.g., begging calls) traits that serve as signals to parents to adjust and allocate parental effort. Parasites can manipulate host parental behavior by exploiting these stable parent-offspring communication systems in their favor. During the past 30 years, the study of host exploitation by parasitic chicks has yielded important insights into the function and evolution of manipulative signals in brood parasites. However, despite these major advances, there are still important gaps in our knowledge about how parasitic nestling and fledglings tune into the host's communication channels and the adaptive value of the visual and acoustic signals they exhibit. Here we review the literature pertaining to host manipulation by parasitic young, focusing on four non-mutually exclusive mechanisms (i.e., host chick mimicry, begging exaggeration, host-attuned begging calls, and sensory exploitation) and the function and evolution of the signals involved, with the aim to summarize and discuss putative adaptations for stimulating parental feeding and escaping host discrimination. Finally, we bring some concluding remarks and suggest directions for future research on the ways in which brood parasites adapt to the communication systems of other birds to exploit the necessary parental care.

Keywords: brood parasitism, parent-offspring communication, begging, mimicry, host manipulation, sensory exploitation

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INTRODUCTION

To reproduce successfully, females of heterospecific brood parasites must locate nests of suitable host species and return to them at the appropriate time to lay their eggs. Once the eggs hatch, the parasitic offspring face the challenge of obtaining adequate levels of parental care from foster parents that are attuned to provision their own progeny. In parasite species that evict or kill all host eggs and nestlings soon after hatching ("nest mate evictors"), the chicks must deal with the problem of stimulating sufficient provisioning in the absence of any host nest mates. In species that do not eliminate their nest mates ("non-evictors"), chicks have the dual problem of eliciting parental feedings and competing for food within mixed broods. Given these selective pressures, it is not surprising that parasitic young have evolved behavioral and morphological traits that effectively serve to manipulate host parental behaviors in their favor (Davies, 2000; Soler, 2017). The well-known image of a tiny adult reed warbler (*Acrocephalus scirpaceus*) diligently feeding an enormous and completely alien common cuckoo (*Cuculus canorus*) chick depicts perfectly the kind of manipulative abilities that have intrigued naturalists since ancient times (Davies, 2000).

Host manipulation by parasitic chicks involves the exploitation of stable communication systems that play a role in solving parent-offspring conflict (Godfray, 1995). In birds, dependent young communicate their needs by means of complex begging displays that combine visual (e.g., gaping and stretching) and acoustic (i.e., begging calls) signals. In conjunction, the multiple components of begging displays convey honest information about offspring attributes, such as their need and condition, that care-giving adults can use to adjust their provisioning effort and to allocate food within the brood (Burford et al., 1998; Kilner et al., 1999; Leonard and Horn, 2001; Moreno-Rueda et al., 2009). Young of obligate brood parasites can "tune" their begging signals into these communication channels to secure the necessary parental care. Host exploitation can be achieved by means of two main kinds of adaptations, according to Davies (2011). Trickery adaptations are those that have coevolved with the host's counter-defenses against brood parasitism and allow parasitic chicks to be accepted by hosts as if they were their own (Davies, 2011). Tuning adaptations refer to those that help ensure the success of parasitic offspring once they have been accepted by hosts (Davies, 2011). This distinction may become diffuse if mistuning in the parasites ultimately results in discrimination by hosts (Davies, 2011), yet it provides a useful conceptual framework to examine the tactics whereby parasite young deceive their hosts and tap into their provisioning rules.

Several non-mutually exclusive mechanisms have been suggested in evictor and non-evictor parasites to exploit host parental behavior to their own benefit (Soler, 2017). Here, we focus on those that serve parasites to evade host discrimination (trickery), or to attune their begging signals to the communication systems or sensory biases of their hosts (tuning). Within this framework, trickery involves host chick mimicry, either as a counter-defense against active host rejection of non-mimetic young, or as an adaptation to avoid discrimination from hosts that deliver food preferentially in response to conspecific signals (Grim, 2005; Wang et al., 2020). In turn, we consider begging exaggeration, host-attuned begging calls and sensory exploitation as the main tuning mechanisms. The distinction between them is not clear-cut, but we use this categorization because it reflects three different, though non-mutually exclusive tactics deployed by parasites to manipulate host behavior through their begging displays. Begging exaggeration is widespread among parasite species and likely serve to exploit host biases for non-specific visual and acoustic features that signal offspring need. For example, parasitic chicks can beg more rapidly or intensively than host young to gain more resources from their foster parents (Redondo, 1993; Kilner et al., 1999; Dearborn and Lichtenstein, 2002). Host-attuned begging calls imply the ability of parasite chicks to learn to modify certain acoustic features in a host-specific manner to make their begging signals more profitable in any given host environment (Madden and Davies, 2006; Langmore et al., 2008). Finally, in sensory exploitation parasitic chicks exhibit morphological traits that are not actually used in host parent-offspring communication, but effectively stimulate the sensory system of the host (Tanaka and Ueda, 2005b). Begging exaggeration and sensory exploitation tactics are similar in that both rely on exploiting host's preexisting cognitive biases and can serve young parasites to compensate for deficient stimulation relative to a host's own brood. However, following the suggestion of Tanaka and Ueda (2005b), we consider them as distinct mechanisms based on whether parasitic chicks exhibit and amplify communication signals already present in host chicks (begging exaggeration) or they display traits that are absent in host young to provide additional begging stimuli (sensory exploitation). The mechanisms outlined above are not mutually exclusive since, for example, chicks of host-generalist parasites can beg exaggeratedly overall while varying their call structure or call rate according to the particular host environment (Madden and Davies, 2006; Tuero et al., 2016). Likewise, trickery and tuning adaptations for host manipulation may occur within a single parasitic species (e.g., Jamie et al., 2020).

Our aim here was to provide an updated review of how parasitic young exploit the honest parent-offspring communication systems of their hosts, either to escape host discrimination or to tap into host provisioning decisions in response to begging signals. To achieve this, we searched the primary literature (journal articles and book chapters found in Scopus and Google Scholar databases) for information on behavioral and morphological traits that could play a role as manipulative signals in species currently recognized as obligate brood parasites, considering the visual and acoustic sensory modalities. Of the 101 parasite species (Feeney et al., 2014; Gill et al., 2017), we found data on 13, including observational and experimental studies on begging behavior, quantitative assessments of visual and acoustic mimicry between parasites and hosts, and comparative studies (**Table 1**). We first summarize and assess the available information about mechanisms and signals for host manipulation found in parasitic nestlings and fledglings, according to our proposed categorization within the trickery-tuning framework. The species for which more than one mechanism was reported were included in each of the corresponding sections. Finally, we bring some concluding remarks and suggest directions for future research.

TRICKERY ADAPTATIONS IN PARASITIC YOUNG: HOST CHICK MIMICRY

Mimicry of host young has been suggested in several parasite species, but most available reports were based on anecdotal observations or subjective assessments of similarity by researchers (Grim, 2006; Jamie and Kilner, 2017). This is

TABLE 1 | Overview of relevant studies on mechanisms and signals for host manipulation by young of evictor and non-evictor brood parasites.

Mechanism of host manipulation	Sensory modality	Trickery/tuning adaptation	Manipulative signal	Parasite species	Host species	Type of study/Main methods	Effect on host behavior	References
1. Trickery mechanisms								
Host chick mimicry	Acoustic	Vocal mimicry	Call structure	Shining bronze cuckoo (*Chrysococcyx lucidus*)	Gray warbler (*Gerygone igata*)	Phylogenetic comparison	Not tested	Anderson et al., 2009
				Screaming cowbird (*Molothrus rufoaxillaris*)	Grayish baywing (Agelaioides badius)	Experimental/Playback supplementation	Increase provisioning relative to non-mimetic calls	Ursino et al., 2018
				Vidua finches (3 species)	Grassfinches (Family Estrildidae) – 3 hosts and 17 non-hosts	Descriptive/Bioacoustic analysis	Not tested	Jamie et al., 2020
	Visual	Visual mimicry	White down-feathers	Little bronze cuckoo (*Chrysococcyx minutillus*)	Large-billed gerygone (*Gerygone magnirostris*)	Experimental/Phenotype manipulation	Reduce chick rejection	Noh et al., 2018
			Gape pattern	Pin-tailed whydah (*Vidua macroura*)	Common waxbill (*Estrilda astrild*)	Experimental/Phenotype manipulation of host chicks	Induce higher provisioning	Schuetz, 2005
			Skin color, rictal flanges	Horsfield's bronze cuckoo (*Chrysococcyx basalis*), Shining bronze cuckoo (*Chalcites lucidus*), Little bronze-cuckoo (*Chrysococcyx minutillus*)	Fairy-wrens (*Malurus* sp., 2 species), yellow-rumped thornbill (*Acanthiza chrysorroa*), large-billed gerygone	Descriptive/Visual modeling	Not tested	Langmore et al., 2011
	Visual/acoustic	Multimodal mimicry	Skin color and begging calls	Horsfield's bronze cuckoo (*Chrysococcyx basalis*)	Superb fairy-wrens (*Malurus cyaneus*)	Experimental/Brood manipulation	Reduce rejection of cuckoo chicks	Langmore et al., 2003
			Host-like juvenile plumage and begging calls	Screaming cowbird (*Molothrus rufoaxillaris*)	Grayish baywing Agelaioides badius	Experimental/Bioacoustic analysis and brood manipulation	Avoid rejection of parasitic juveniles	De Mársico et al., 2012
			Host-like coloration, gape pattern and begging calls	Village indigobird (*Vidua chalybeata*)	Red-billed firefinch (*Lagonosticta senegala*) Goldbreast (*Amandava subflava*) Blue-capped cordon-bleu (*Uraegintus cyanocephalus*)	Experimental (in aviary)/cross-fostering	Increased survival	Payne et al., 2001
				Pin-tailed whydah (*Vidua macroura*)	Common waxbill (*Estrilda astrild*) Blue waxbill (*Uraeginthus angolensis*)	Experimental/cross-fostering	Improved survival of parasitic chick	Jamie et al., 2021
2. Tuning mechanisms								
Begging exaggeration	Visual/acoustic	Intense begging display	Long begging bouts	Great-spotted cuckoo (*Clamator glandarius*)	Magpie (*Pica pica*)	Experimental/Brood manipulation	No effect on nest provisioning; preferential allocation to cuckoo chick	Soler et al., 1995
			Begging intensity	Great-spotted cuckoo (*Clamator glandarius*)	Carrion crow (*Corvus corone corone*)	Descriptive/Video recording	Increase nest provisioning, but not food acquisition	Bolopo et al., 2015
				Screaming cowbird (*Molothrus rufoaxillaris*)	Grayish baywing (*Agelaioides badius*)	Experimental/Short-term need manipulation	Not tested	Lichtenstein, 2001a
				Brown-headed cowbird (*Molothrus ater*)	Yellow warblers (*Dendroica petechia*)	Experimental/Brood and short-term need manipulations	Increase nest provisioning	Lichtenstein and Dearborn, 2004

(Continued)

TABLE 1 | (Continued)

Mechanism of host manipulation	Sensory modality	Trickery/tuning adaptation	Manipulative signal	Parasite species	Host species	Type of study/Main methods	Effect on host behavior	References
					Field sparrow (*Spizella pusilla*) Red-winged blackbird (*Agelaius phoeniceus*) Brown-thrasher (*Toxostoma rufum*)	Experimental/Brood and short-term need manipulations	No effect	Rivers, 2007; Rivers et al., 2010
				Shiny cowbird (*Molothrus bonariensis*)	Rufous-bellied thrushes (*Turdus rufiventris*)	Experimental/Brood and short-term need manipulations	Increase nest provisioning	Lichtenstein, 2001b
					Southern house wren (*Troglodytes aedon*)	Experimental/Brood manipulations	No effect on food allocation	Bortolato et al., 2019
	Acoustic	Call exaggeration	Rapid call rates	Common cuckoo (*Cuculus canorus*)	Reed warbler (*Acrocephalus scirpaceus*)	Experimental/Playback supplementation	Stimulated provisioning	Davies et al., 1998; Kilner et al., 1999
			Tremulous begging calls	Shiny cowbird (*Molothrus bonariensis*)	Southern house wren (*Troglodytes aedon*)	Experimental/Playback supplementation	Stimulated provisioning	Gloag and Kacelnik, 2013
Host-attuned begging calls	Acoustic	Host-specific call features	Call rate	Common cuckoo (*Cuculus canorus*)	Reed warbler (*Acrocephalus scirpaceus*) Great reed warbler (*Acrocephalus arundinaceus*), Dunnock (*Prunella modularis*) Meadow pipit (*Anthus pratensis*)	Descriptive/Bioacoustic analysis	Not tested	Butchart et al., 2003
			Call rate and structure	Common cuckoo (*Cuculus canorus*)	Reed warbler (*Acrocephalus scirpaceus*) Dunnock (*Prunella modularis*) Robin (*Erithacus rubecula*)	Experimental/Cross-fostering, playback	Increased provisioning	Madden and Davies, 2006
					Great reed warbler (*Acrocephalus arundinaceus*) *Robin (Erithacus rubecula)* Rufous-tailed scrub robin (*Cercotrichas galactotes*)	Descriptive/Bioacoustic analysis	Not tested	Soler, 2017
					Great reed warbler (*Acrocephalus arundinaceus*) Reed warbler (*Acrocephalus scirpaceus*)	Experimental/Cross-fostering, bioacoustic analysis	Not tested	Samaš et al., 2020
				Great-spotted cuckoo (*Clamator glandarius*)	Magpie (*Pica pica*), Carrion crow (*Corvus corone*)	Experimental/Cross-fostering, bioacoustic analysis	Not tested	Roldán et al., 2013

(Continued)

TABLE 1 | (Continued)

Mechanism of host manipulation	Sensory modality	Trickery/tuning adaptation	Manipulative signal	Parasite species	Host species	Type of study/Main methods	Effect on host behavior	References
		Begging call plasticity	Call structure	Horsfield's bronze cuckoo (*Chrysococcyx basalis*)	Buff-rumped thornbills (*Acanthiza reguloides*)	Experimental/Cross-fostering, bioacoustic analysis	Not tested	Langmore et al., 2008
Sensory exploitation	Visual	Attractive traits	White gape papillae	Great -spotted cuckoo (*Clamator glandarius*)	Magpie (*Pica pica*)	Experimental/Signal manipulation	Stimulate nest provisioning and food allocation to cuckoo	Soler et al., 1995
			Bright red gape	Common cuckoo (*Cuculus canorus*)	Robin (*Erithacus rubecula*) Reed warbler (*Acrocephalus scirpaceus*) Dunnock (*Prunella modularis*)	Experimental/Signal manipulation in host chicks	No effect	Noble et al., 1999
			Yellow-wing patches	Horsfield's hawk-cuckoo (*Hierococcyx hyperythrus*)	Red-flanked bush robin (*Tarsiger cyanurus*)	Experimental/Signal manipulation	Stimulate provisioning	Tanaka and Ueda, 2005a
				Hodgson's hawk-cuckoo (*Hierococcyx nisicolor*)	Rufous-bellied niltava (*Niltava sundara*)	Descriptive/Video recording	Not tested	Luo et al., 2019

Mechanisms of host manipulation were grouped into two broad categories: "trickery mechanisms" refers to coevolved host chick mimicry in parasites in response to host discrimination; "tuning mechanisms" refers to the tactics through which parasitic young can attune their begging signals to the communication systems or sensory biases of their hosts. Tuning mechanisms are divided into begging exaggeration, host-attuned begging calls and sensory exploitation (see text for details). Sensory modality refers to whether the signal under study was visual, acoustic or both (i.e., the stimulus was the whole parasitic chick). Manipulative signal refers to the putative manipulative trait under study.

problematic in determining parasite trickery because humans and birds differ in their perceptual systems, especially regarding the visual modality (Hart et al., 2000a,b). In the past decades, it has been increasingly common to apply avian visual modeling techniques to quantify colors in birds (Vorobyev and Osorio, 1998). These techniques have been used to objectively assess the similarity between host and parasitic chicks as seen through a bird's eye, thus providing important insights into the function and evolution of visual trickery adaptations driven by host discrimination (Langmore et al., 2008, 2011; Anderson et al., 2009; Tanaka et al., 2011; De Mársico et al., 2012; Attisano et al., 2018; Jamie et al., 2020). In addition to objective measurements of similarity, experimental tests of the function of host resemblance in parasites, for example using controlled cross-fostering or playback trials, are critical to properly assess the existence of mimicry. In this section we focus on the relatively few well-documented examples of visual and vocal mimicry in parasites that can be regarded as coevolved adaptations to evade host discrimination against young unlike their own (Grim, 2006), including results from our own studies on fledgling mimicry in the host-specialist screaming cowbird (*Molothrus rufoaxillaris*). We summarize and discuss examples of trickery on a species-by-species basis, instead of focusing on traits, to bring a more integrative perspective about how host behavior has driven host chick mimicry in each case and drive the attention to the fact that mimicry can occur in multiple sensory modalities within any single species.

Bronze Cuckoos

Active host rejection of parasitic chicks occurs in hosts of Australasian bronze cuckoos (*Chrysococcyx* spp.). Superb fairy-wrens are primary hosts of the Horsfield's bronze cuckoo and known to reject cuckoo chicks by deserting them (Langmore et al., 2003). The main cue triggering this behavior is the presence of a single chick in the brood (Langmore et al., 2003), but fairy-wrens are less likely to abandon nests with Horsfield's bronze cuckoo chicks than with shining bronze cuckoo (*C. lucidus*) chicks (Langmore et al., 2003). This suggests that Horsfield's bronze cuckoos have evolved counter adaptations to evade host rejection. Consistent with this, chicks of this parasite species closely resemble the fairy-wren chicks in skin and rictal flange colors (Langmore et al., 2011), and they innately develop begging calls that match the acoustic structure of those of fairy-wren chicks as well (Langmore et al., 2008). Although it seems clear that host chick mimicry is adaptive for Horsfield's bronze cuckoos, more experiments are needed to further determine the role played by visual and vocal signals of parasitic chicks in deceiving super fairy-wrens. Evidence from another study indicated that fairy-wren hosts would be able to discriminate between their own and alien cuckoo chicks based on parent-specific call signatures that are transmitted to its offspring during the embryonic stage (Colombelli-Négrel et al., 2012).

The little bronze cuckoo (*C. minutillus*) is another species faced with host defenses against alien chicks. Two primary hosts in Australia, the large-billed gerygone (*Gerygone magnirostris*) and the mangrove gerygone (*G. laevigaster*), are able to reject cuckoo chicks by dragging them out of the nest, sometimes within

FIGURE 1 | Three examples of host chick mimicry in parasitic birds. **(A,B)** Little bronze cuckoo (*Chalcites minutillus*) chicks (*top*) are visual mimics of large-billed gerygones (*Gerygone magnirostris*) chicks (*bottom*). **(C)** Parasitic pin-tailed whydah (*Vidua macroura*) chicks (*left*) bear a close resemblance in mouth ornamentation to its common waxbill (*Estrilda astrild*) host. **(D)** Screaming cowbird (*Molothrus rufoaxillaris*) juveniles (*right*) mimic the plumage coloration of its primary host, the grayish baywing (*Agelaioides badius*). Photo credits: **(A,B)** Naomi Langmore, **(C)** Justin Schuetz, **(D)** Alec Earnshaw.

a few hours after hatching (Sato et al., 2010; Tokue and Ueda, 2010). Little bronze cuckoo chicks are striking visual mimics of gerygone chicks, closely matching their dark skin, multi-barbed white down-feathers and rictal flange color (Langmore et al., 2011; **Figures 1A,B**). Noh et al. (2018) showed that the number of down-feathers is a key trait used by gerygone hosts to discriminate between their own and alien chicks. Experimental trimming of down-feathers in cuckoo and gerygone chicks increased the likelihood of rejection relative to untrimmed chicks (Noh et al., 2018). This strongly suggests that host chick mimicry in little bronze cuckoos has evolved as a reciprocal adaptation against host recognition (Noh et al., 2018). However, the study also revealed that parasitic chicks do not fully match the recognition signals used by gerygones, since trimmed cuckoos were rejected at higher rates than trimmed host chicks (Noh et al., 2018). A recent study suggests that gerygone hosts could use the duration of the begging calls as a cue to spot and reject parasitic chicks and that cuckoo chicks more closely match the begging calls of host chicks at the age at which rejection typically occurs (Noh et al., 2021). More experiments that test if hosts cue also on acoustic or olfactory signals for making rejection decisions, and further examination of the similarity between little bronze cuckoos and host chicks in multiple sensory modalities, would provide new insights on this issue.

The shining bronze-cuckoo and its primary host in New Caledonia, the fan-tailed gerygone (*Gerygone flavolateralis*), provide an interesting example of parasite chicks that have seemingly evolved visual mimicry driven by an ongoing co-evolutionary arms race with their hosts (Sato et al., 2015). Fan-tailed gerygones have chicks of two distinct morphs, namely bright and dark, which can occur in monomorphic or polymorphic broods (Sato et al., 2015; Attisano et al., 2018). Shining bronze cuckoo chicks from New Caledonia are, at present, of a single bright morph (Sato et al., 2015; Attisano et al., 2018), though distinct yellow and dark morphs are known to occur in the Australian subspecies (Langmore et al., 2011). The cuckoo bright morph match closely the bright gerygone morph from an avian perspective, but it is also more similar to the dark host morph than the bright and dark host morphs are to each other (Sato et al., 2015; Attisano et al., 2018). Despite this similarity, from a sample of 15 parasitized gerygone nests in which the cuckoo egg hatched, hosts always rejected the cuckoo chick, usually within 24 h after hatching (Attisano et al., 2018). These observations suggest a true-recognition mechanism based on multiple sensorial cues underlying chick rejection behavior in gerygones, although the precise recognition signals have not been identified (Attisano et al., 2018). On the other hand, there are a few observations of shining bronze cuckoo fledglings being fed by fan-tailed gerygones at other sites in New Caledonia, suggesting that parasitic chicks can sometimes evade the refined discrimination abilities of its primary host (Attisano et al., 2018). Clearly, more research is needed to understand how gerygones can spot the cuckoo chick in their nests so precisely, and how cuckoo chicks occasionally manage to surpass this host defense. Shining bronze cuckoos from New Zealand were reported to bear begging call similarity with their gray warbler (*G. igata*) host (McLean and Waas, 1987), and a posterior comparative study involving gray warblers and 17 other native forest species of New Zealand further supports a close matching of host begging calls in shining bronze cuckoos (Anderson et al., 2009). Nevertheless, the adaptive value of this call similarity has not been determined, nor is it known whether a similar begging call matching occurs in other shining bronze cuckoo populations.

Brood-Parasitic Finches

The *Vidua* whydahs and indigobirds comprise 19 non-evictor species specialized in parasitizing grassfinches (family Estrildidae; Davies, 2000). Young estrildid finches are unique in that they have species-specific mouth ornamentations that are exhibited in begging displays (Payne, 2005). Most *Vidua* species lay eggs in nests of a single host and parasitic chicks often match the mouth markings and begging calls of their respective hosts (Nicolai, 1974; Payne and Payne, 2002; Payne, 2005; **Figure 1C**). However, it was not until recently that the similarity in begging signals between *Vidua* chicks and their hosts was assessed with quantitative and objective methods. Jamie et al. (2020) tested whether parasitic chicks of three *Vidua* species matched more closely the mouth patterning, gape color, begging calls, and postural displays of their respective hosts than they do those of other co-occurring grassfinch species. The results showed a closer phenotypic similarity between parasitic chicks and their hosts, supporting the idea that *Vidua* finches have evolved host-specific mimicry (Jamie et al., 2020). Interestingly, the study also revealed that host resemblance was not 100% accurate, since some *Vidua* chicks presented exaggerated traits relative to their hosts, such as enlarged palatal spots, longer begging calls and increased wing-waving behavior (Jamie et al., 2020). The idea that this "imperfect mimicry" could be adaptive by, for example, providing a supernormal stimulus that enhances parental provisioning warrants further investigation (Jamie et al., 2020).

Regarding the adaptive value of chick mimicry in *Vidua* finches, there is some evidence from cross-fostering experiments that lacking the species-specific signals can result in reduced survival of alien chicks in estrildid nests (Payne et al., 2001; Jamie et al., 2021). Recently, a field experiment demonstrated that reduced survival of cross-fostered pin-tailed whydahs (*V. macroura*) was the result of foster parents delivering less food to non-mimetic parasitic chicks compared to their own (Jamie et al., 2021). To date, there is no evidence that estrildid hosts actively reject chicks unlike their own; indeed, non-mimetic *Vidua* chicks do sometimes fledge successfully from nests of grassfinches other than their host, which helps to explain the occasional colonization of new host species in this parasite lineage (Sorenson et al., 2003, 2004). The study by Jamie et al. (2021) also showed that parasitic chicks did not modify the acoustic structure of their begging calls when transferred to nests of a non-host species. Innate call mimicry is expected in specialist brood-parasites if failure in exhibiting the appropriate begging signals results in fitness costs for parasitic chicks (Jamie and Kilner, 2017). Nonetheless, more experimental work is needed to disentangle how estrildid hosts integrate visual and vocal cues in chick discrimination. In a field experiment, Schuetz (2005) manipulated the gape flanges of common waxbill (*Estrilda astrild*) chicks (i.e., the natural host of pin-tailed whydahs), to test host response toward chicks with dissimilar gape morphology. Host chicks that had their flanges painted black suffered only a slight reduction in mass and skeletal growth compared to unmanipulated or sham-painted chicks (Schuetz, 2005). Altogether, these findings suggest that host manipulation by *Vidua* chicks involve multiple sensory modalities and, possibly, some signal exaggeration in addition to host-specific mimicry (Jamie et al., 2020). Such remarkable fine-tuning with respect to host begging signals has more likely been driven by a preexisting parental feeding preference in estrildid hosts for chicks bearing the elaborate traits specific to each species, rather than by the existence of active host defenses against brood parasitism (Hauber and Kilner, 2007; Jamie et al., 2021).

Screaming Cowbird

In theory, the co-evolutionary arms race between brood parasites and their hosts can encompass all stages of the nesting cycle (Soler, 2017). However, co-evolved adaptations during the fledgling stage are much less known (De Mársico et al., 2017). In this regard, the studies on host-parasite interactions between the screaming cowbird and its primary host provide the most compelling evidence to date for the evolution of host fledging mimicry in parasitic juveniles. Screaming cowbirds are host-specialists that mainly parasitize grayish baywings (*Agelaioides badius*) in southern South America. The parasitic young bear a striking resemblance to baywing offspring that cannot be attributed to common ancestry (Lanyon, 1992) and lasts until the former attain nutritional independence (Hudson, 1874; Fraga, 1998; Ursino et al., 2012; **Figure 1D**). Quantitative analyses have indicated that screaming cowbird fledglings would be indistinguishable from host fledglings from an avian perspective, and that they also closely match baywing begging calls (De Mársico et al., 2012). The function of this close similarity was tested by cross-fostering non-mimetic shiny cowbird (*Molothrus bonariensis*) chicks to baywing nests and comparing their fate to that of host and screaming cowbird young (Fraga, 1998; De Mársico et al., 2012). Baywings accepted any chick in their nests but stopped providing parental care to shiny cowbirds as

FIGURE 2 | (A) Shiny cowbird (*Molothrus bonariensis*) chicks (*center*) exhibit exaggerated begging displays in nests of a common host, the chalk-browed mockingbird (*Mimus saturninus*), where they compete strongly with host nest mates for parental feedings. **(B)** Whistling hawk-cuckoo (*Hierococcyx nisicolor*) chicks display a yellow wing-patch during begging that would serve to simulate an extra gape in host nests. **(C)** White palatal papillae of great-spotted cuckoo (*Clamator glandarius*) chicks (*center*) play a role in stimulating parental feedings from its magpie (*Pica pica*) host. Photo credits: **(A)** Vanina Fiorini, **(B)** Keita Tanaka, **(C)** Manuel Soler.

soon as they fledged, while they continued caring for screaming cowbird and their own fledglings for several weeks (Fraga, 1998; De Mársico et al., 2012). These results support the idea that the baywing-like appearance of screaming cowbird fledglings is a reciprocal adaptation in response to host rejection behavior (Fraga, 1998; De Mársico et al., 2012). A more recent study suggests that host discrimination against non-mimetic fledglings is context-dependent rather than based on an internal template of their own offspring's appearance, since baywings accept shiny cowbird fledglings when they were reared in the absence of host nest mates (Rojas Ripari et al., 2019a).

Disentangling the role of visual and acoustic signals for fledgling recognition by baywings has proven to be difficult so far, but some advances have been made in understanding the function of begging call similarity in host manipulation. Playback experiments conducted at baywing nests during the nestling stage demonstrated that begging calls of screaming cowbird and host chicks were equally effective in eliciting parental provisioning, and more effective than non-mimetic shiny cowbird calls (Ursino et al., 2018). Indeed, shiny cowbird calls did not elicit any increase in provisioning rates from baywings compared to a silent control, despite being more exaggerated than those of baywing and screaming cowbird chicks (Gloag and Kacelnik, 2013; Ursino et al., 2018). Begging call similarity to host fledglings could play a key role in attracting the attention of baywing parents during the post-fledgling stage. This could be tested by using playback experiments to compare the response of adult baywings toward begging calls of conspecific, screaming cowbird (mimetic) and shiny cowbird (non-mimetic) fledglings. If baywings cue on acoustic signals to discriminate against alien fledglings, then they should be less responsive to non-mimetic begging calls than to own-species calls. And, if vocal similarity between screaming cowbird and baywing fledglings serve to avoid host discrimination, then baywings should respond similarly to conspecific and screaming cowbird begging calls. Cross-fostering experiments showed that baywing-like begging calls develop innately in screaming cowbirds. Despite slight variation in call structure with the host environment, screaming cowbird chicks reared in nests of another species retain the acoustic features that serve as recognition signals for baywings (Rojas Ripari et al., 2019b). These observations agree with the prediction of genetically fixed call similarity in host-specialist parasites for which modulating their calls in response to environmental cues could be maladaptive (Jamie and Kilner, 2017).

Screaming cowbirds and baywings have provided an excellent model to study visual and vocal mimicry at the last stage of the nesting cycle, but many questions are still unanswered. For example, it is yet to be determined how baywings integrate visual and vocal signals in fledgling recognition and what acoustic features of screaming cowbird begging calls are key to trick hosts during the post-fledgling stage. Also, the cognitive decision rules involved in fledgling discrimination by hosts are not well understood. Future studies that investigate the species-specific signals and cognitive mechanisms involved in fledgling recognition by baywings would help better illuminate the function and evolution of visual and acoustic manipulative signals in this parasitic cowbird.

TUNING MECHANISMS TO EXPLOIT HOST PARENTAL BEHAVIOR THROUGH BEGGING DISPLAYS

Begging Exaggeration

According to signaling models, begging behavior is modulated by the balance between the benefits of gaining extra resources through more vigorous displays and the potential costs that maintain signal honesty (Godfray, 1995; Kilner and Johnstone, 1997). The latter comprise physiological costs (Kilner, 2001; Soler et al., 2014), increased risk of nest predation (Haskell, 2002), or indirect costs due to competition with closely related nest mates (Trivers, 1974; Briskie et al., 1994; Caro et al., 2016, but see Bebbington and Kingma, 2017). Since obligate brood parasites are unrelated to their hosts, they are generally unconstrained by the inclusive fitness costs of begging (but see

Rivers and Peer, 2016). Therefore, all things being equal, parasitic chicks are expected to beg more selfishly than those of non-parasitic species. Consistently with this, exaggerated begging displays are ubiquitous among evictor and non-evictor parasites (Redondo, 1993). Depending on the taxa, the exaggeration can manifest in traits such as rapid call rates (Davies et al., 1998; Kilner et al., 1999), long begging bouts (Redondo, 1993), tremulous or repetitive begging call structure (Gloag and Kacelnik, 2013), more vigorous displays (Redondo, 1993; Soler et al., 1995; Dearborn and Lichtenstein, 2002; Grim, 2008a) or brightly colored gapes (Álvarez, 2004; Tanaka et al., 2011).

The general view of parasites exhibiting increased levels of begging relative to host chicks is supported by quantitative studies conducted in cowbirds (*Molothrus* spp.; Lichtenstein and Sealy, 1998; Lichtenstein, 2001b; Bortolato et al., 2019; **Figure 2A**), great spotted cuckoo (*Clamator glandarius*; Redondo, 1993; Soler et al., 2012; Bolopo et al., 2015) and common cuckoo (Kilner and Davies, 1999; Kilner et al., 1999). Despite its exaggeration, however, empirical evidence suggests that begging in brood parasitic chicks is still informative regarding their level of need. Begging honesty in parasites has been tested experimentally by manipulating short-term need of parasitic chicks using food deprivation and hand-feeding treatments. In general, these experiments show that begging intensity increases with deprivation time and decreases after satiation, as predicted by honest signaling theory (Kilner and Davies, 1999; Lichtenstein, 2001b; Hauber and Ramsey, 2003; Lichtenstein and Dearborn, 2004; Soler et al., 2012; but see Rivers, 2007). In addition, begging levels can increase with age, as older chicks demand more food (Kilner and Davies, 1999; Butchart et al., 2003; Tuero et al., 2016). The observed effects of short-term need on begging behavior suggest that direct costs of begging could set a limit to begging exaggeration in brood parasites. However, data supporting this hypothesis are scarce. There is some experimental evidence that begging calls of parasitic chicks can increase nest predation risk (Dearborn, 1999; Ibáñez-Álamo et al., 2012), but the detection of physiological costs remains elusive (e.g., Martín-Gálvez et al., 2012).

Exaggerated begging signals of parasitic chicks likely serve to gain resources from their hosts. However, few studies have clearly demonstrated a function of begging exaggeration in manipulating host parental behavior (Soler, 2017). The strongest evidence comes from the very rapid call rates of common cuckoo chicks that stimulate adult reed warblers to provision them at the same rate as an entire host brood (Davies et al., 1998; Kilner et al., 1999). This is because this host integrates visual (i.e., displayed gape area) and vocal (i.e., call rate) signals in a similar manner when provisioning unparasitized and parasitized nests, and cuckoo chicks exploit this rule in their favor by calling at a rate that compensates for the deficient visual stimuli provided by its single gape (Kilner et al., 1999). More recently, a study in the non-evictor shiny cowbird suggests that this species' long and tremulous begging calls could act like a rapid call rate, stimulating higher provisioning rates from both common hosts and non-host species with shorter, monosyllabic begging calls (Gloag and Kacelnik, 2013). However, more studies are needed to better understand how cowbird hosts integrate visual and vocal begging signals, and the function of tremulous calls in host manipulation (Gloag and Kacelnik, 2013). In the closely related brown-headed cowbird (*M. ater*), parasitic chicks reared alone in nests of Bell's vireo (*Vireo bellii*) were fed less than a host's modal brood, suggesting that their faster and more repetitive calls did not fully compensate for deficient visual stimulation (Rivers et al., 2014).

Other studies that compared host provisioning rates between parasitized and unparasitized nests (e.g., Soler et al., 1995; Dearborn et al., 1998; Glassey and Forbes, 2003; Rivers et al., 2010; Ursino et al., 2011; Precioso et al., 2020), or food acquisition by parasitic and host chicks in mixed broods (e.g., Lichtenstein, 2001a; Lichtenstein and Dearborn, 2004; Rivers et al., 2010; Gloag et al., 2012; Bolopo et al., 2015; Bortolato et al., 2019) show conflicting results about the effect of exaggerated begging displays on host parental behavior. Accumulated data from non-evictor parasites indicate that begging exaggeration in these species would not be a key factor *per se* for securing sufficient provisioning (see Soler, 2017 for a recent review); rather, the success of parasitic chicks in mixed broods appears to be more dependent on their size relative to that of host nest mates and the ability to modulate begging effort according to the host environment (Lichtenstein and Sealy, 1998; Soler, 2002; Rivers, 2007; Rivers et al., 2010; Tuero et al., 2016; Bortolato et al., 2019). Disentangling how the multiple attributes of parasitic chicks (e.g., larger size relative to hosts, earlier hatching, and begging behavior) determine their competitive ability in mixed broods (Hauber, 2003) is important to better understand the function of begging exaggeration. Furthermore, it remains an open question whether begging exaggeration in parasitic chicks itself has evolved as an adaptation to parasitism. Two experimental studies have failed to find differences in the begging intensity and the effectiveness to stimulate parental feedings between brown-headed cowbird chicks and those of a related non-parasitic blackbird (Rivers et al., 2013; Li and Hauber, 2021). The lack of comparative studies represents a major gap in the study of begging evolution in brood parasites. Phylogenetic analyses or, at least, further comparisons between parasites and closely related non-parasitic species would be of great help to understand if exaggerated signals evolved specifically for the parasitic lifestyle.

The challenge of stimulating parental care may continue for several days or weeks after parasites fledge from host nests, until they attain nutritional independence. However, begging behavior in parasitic fledglings is poorly known (Hauber and Ramsey, 2003; Grim, 2008a; Tyller et al., 2018). It is possible that begging exaggeration is more relevant for attracting parental care and competing for parental feedings during the post-fledgling stage, but this idea needs to be examined.

Host-Attuned Begging Calls

Parasites that are host-generalists may benefit from varying their begging calls depending on the rearing host species if such fine-tuning allows them to better exploit the provisioning effort of any given host (McLean and Waas, 1987; Butchart et al., 2003; Jamie and Kilner, 2017). Plasticity in begging call development provides a way for parasitic chicks to rapidly attune call rate and/or call structure to different parent-offspring communication systems (Butchart et al., 2003;

Jamie and Kilner, 2017). Evidence supporting this mechanism comes from cross-fostering experiments in the Horsfield's bronze cuckoo, a parasite species that exhibit host-specific begging calls (Langmore et al., 2008). Parasitic females are host-generalist at individual level (Joseph et al., 2002). As mentioned in the previous section, they primarily parasitize fairy-wrens (*Malurus* sp.), but can use a variety of secondary hosts, including thornbills (*Acanthiza* spp.; Brooker and Brooker, 1989; Joseph et al., 2002). Langmore et al. (2008) cross-fostered cuckoo eggs from nests of superb fairy-wrens (*M. cyaneus*) to nests of buff-rumped thornbills (*A. reguloides*) to study begging call development in parasitic chicks. Their results revealed that cross-fostered chicks initially mimic the acoustic structure of fairy-wren calls, indicating that this vocal trickery is innate; however, within a few days after hatching, the chicks modified their call structure to match that of thornbill's begging calls (Langmore et al., 2008). A plausible explanation is that changes in call structure were shaped by adult thornbills if, through the adjustment of food delivery rates, they reinforced the begging calls that more accurately matched their own species' calls (Langmore et al., 2008). Experimental tests to see how thornbills respond toward mimetic and non-mimetic begging calls has not been conducted yet, and more studies are needed to better understand how Horsfield's bronze cuckoo chicks learn to refine call structure in nests of thornbills and other secondary hosts. Nonetheless, these results suggest that both trickery and tuning adaptations can occur through chick development in host-generalist parasites.

The idea that parasitic chicks could learn to modify their begging calls to make them more profitable was first experimentally tested by Madden and Davies (2006) in common cuckoos. This species has distinct host-races each specializing in a single host (Gibbs et al., 2000). Cuckoo chicks do not mimic the begging calls of their respective hosts, but some differences in begging call features between host-races suggest that they could tune their calls in a host-specific manner to better stimulate provisioning (Butchart et al., 2003). Madden and Davies (2006) transferred cuckoo eggs or newly hatched chicks from reed warbler nests to nests of dunnocks (*Prunella modularis*) and robins (*Erithacus rubecula*). Cuckoo chicks cross-fostered to dunnock nests developed begging calls that were acoustically different from those of cuckoos reared by reed warblers, but similar to the begging calls of cuckoo chicks naturally reared in dunnock nests (Madden and Davies, 2006). The authors conducted an additional experiment in which they broadcast begging calls of 6–9 days old "dunnock-cuckoos" and "reed warbler-cuckoos" at nests of reed warblers, dunnocks and robins containing either a single blackbird (*Turdus merula*) or song thrush (*T. philomelos*) chick, similar in size to the cuckoo chick (Madden and Davies, 2006). Hosts responded differentially to each playback type, with dunnocks provisioning at higher rates in response to "dunnock-cuckoo" calls and the other host species showing the opposite trend (Madden and Davies, 2006). These results are consistent with a scenario in which begging call structure is not genetically fixed and parasitic chicks can modify their begging calls through their provisioning experience with a particular host (Madden and Davies, 2006). Jamie and Kilner (2017) termed this mode of begging call development as genetically polymorphic reaction norms in their proposed theoretical framework. According to it, parasitic chicks of distinct host-races attune their begging calls to the rearing host, while retaining certain call signatures of their own host-race (Madden and Davies, 2006).

The above-mentioned studies have provided important insights regarding the role of learning in begging call development and the ways in which parasitic cuckoos can tune their begging calls into different communication systems. However, two studies cast some doubts about the extent of polymorphism in begging call structure across common cuckoo host-races, and the ubiquity of host-attuned begging calls as a mechanism for host manipulation in common cuckoos. On the one hand, Samaš et al. (2020) failed to find differences in begging call rate and structure, after accounting for chick age and sex, between cuckoo chicks from nests of reed warblers and great reed warblers (*A. arundinaceus*), in contrast with a previous study that included these host-races (Butchart et al., 2003). The authors argued, based on these results, that begging development in common cuckoo chicks would better fit a genetically fixed bet-hedging strategy, rather than the proposed genetically polymorphic reaction norm (Jamie and Kilner, 2017). On the other hand, Soler (2017) reported original data on begging calls of cuckoo chicks recorded at nests of rufous-tailed scrub robin (*Cercotrichas galactotes*), robins and great reed warblers. Contrary to the expectation of host-attuned begging calls, begging call rates did not differ among host-races, despite substantial differences in this parameter between the respective hosts' broods (Soler, 2017). Moreover, call rate of cuckoo chicks was more variable within than among host-races, which can be attributed to cuckoo chicks in the sample exhibiting three different call types, none of them exclusive to any particular host (Soler, 2017). Although sample sizes for these analyses were rather small, the results are consistent with the idea that common cuckoos could be more reliant on a bet-hedging strategy (based, for example, on call rate exaggeration) to elicit sufficient provisioning (Soler, 2017).

The role of experience in begging call development has also been examined in the great-spotted cuckoo. Roldán et al. (2013) quantified the begging calls of great-spotted cuckoo chicks from a reciprocal cross-fostering experiment between nests of its primary host, the magpie (*Pica pica*), and nests of carrion crows (*Corvus corone corone*). Contrary to earlier suggestions (Redondo et al., 1988), begging calls of parasitic chicks did not resemble those of host young, neither in magpie nor carrion crow nests (Roldán et al., 2013). Calls were acoustically similar between host species, but the number of notes per call was higher for chicks reared in magpie nests, consistent with the hypothesis that great-spotted cuckoo chicks modified their calls after hatching according to the rearing environment (Roldán et al., 2013). However, there are two important caveats to this conclusion. First, as the authors themselves point out, host-specific variation in call structure was largely restricted to the number of notes per call, which suggests that chicks could have been adjusting their begging effort rather than the acoustic properties of the begging calls (Roldán et al., 2013). Second, whether the observed variation in begging calls is adaptive and socially shaped by the foster parents cannot be established without playback experiments that test host response toward begging calls of cuckoo chicks from magpie and carrion crow nests.

Sensory Exploitation

Tuning through sensory exploitation, as considered here, involves the use of signals that are not part of the host's parent-offspring communication system but serve parasites to effectively exploit pre-existing host's sensory biases (Tanaka and Ueda, 2005b). An interesting example is found in Horsfield's hawk-cuckoo (*Hierococcyx hyperythrus*). The chicks of this species pose a conspicuous yellow skin patch on the underside of each wing that is displayed during begging (Tanaka and Ueda, 2005a). By dying the wing-patch black, Tanaka and Ueda (2005a) demonstrated that it plays a role in stimulating provisioning from its host, the red-flanked bush robin (*Tarsiger cyanurus*). The authors proposed that wing-patches would serve to simulate additional gapes, based on the observation that hosts occasionally attempted to place food onto them when parasitic chicks flapped their wings (Tanaka and Ueda, 2005a). A similar wing-patch begging strategy has been recently reported in a closely related species, the whistling hawk-cuckoo (*H. nisicolor*), and it is possible that it occurs in two other species of the same clade (Luo et al., 2019; **Figure 2B**). The authors hypothesized that the evolution of exuberant begging calls in hawk-cuckoos like those of common cuckoo chicks might be constrained by high predation pressure on host nests (Tanaka and Ueda, 2005a; Luo et al., 2019). Under this scenario, wing-patch begging may pose an alternative evolutionary solution to the problem of having to compensate for a deficient gape area without incurring extra predation costs (Tanaka and Ueda, 2005a; Tanaka et al., 2011; Luo et al., 2019). Additional comparisons of visual signals using avian vision models suggest that the gape and colored wing-patches of cuckoos are more conspicuous than the gape of host chick from the host's perspective (Tanaka et al., 2011), further supporting the idea that Horsfield's hawk cuckoos would have evolved traits that act as supernormal stimuli (Dawkins, 1976; Noble et al., 1999). However, the hypothesis that gape-like wing-patches would play a role analogous to common cuckoo's rapid call rates needs experimental testing. Wing-shaking, as performed by hawk-cuckoo chicks, is a widespread component of begging displays among parasitic and non-parasitic birds (Grim, 2008b). It would be useful in the future to examine if wing-shaking begging is already present in hosts of hawk-cuckoos and how it influences host provisioning behavior (Grim, 2008b). This would help better understand if wing-shaking could have served as a pre-adaptation for the evolution of colored wing-patches in these parasites (Grim, 2008b).

The bright red gape of common cuckoo chicks was formerly considered an irresistible stimulus acting upon the host's nervous system (Dawkins, 1976). This idea received little support because, although there is some evidence that cuckoo chicks have redder gapes than host chicks (Kilner, 1999), experimental tests involving artificial dying of chick gapes in three host species failed to find the expected host preference for redder gapes (Noble et al., 1999). Nevertheless, since these studies were based on human color perception, it would be useful to re-evaluate the function of gape color in parasite chicks from an avian perspective. It is possible that colorful gapes in cuckoo chicks play at least some role in stimulating provisioning under certain situations, such as in host species that rely more on visual than auditory begging cues (Kilner and Davies, 1999; Álvarez, 2004) or in dark nests, where redder gapes may serve to increase chick detectability (Kilner, 1999). Alternatively, the red gape color in parasitic cuckoos could be maintained by phylogenetic constraints given that this trait is also found in some non-parasitic species within the Cuculidae family and there is no evidence that gape color in cuckoos had changed as a result of evolutionary interactions with their hosts (Kilner, 1999).

Sensory exploitation may play a role in host manipulation by great spotted cuckoos (Tanaka and Ueda, 2005b). The chicks of this species exhibit white palatal papillae, a trait that is absent in magpie chicks and influences food allocation within parasitized broods (Soler et al., 1995; **Figure 2C**). Using a repeated-measures design, Soler et al. (1995) showed that parasitic chicks were fed at lower rates when they had their papillae masked with red paint (i.e., the gape color of magpie chicks) than when they were left unpainted. Nest provisioning also decreased after masking the chick's papillae, and painted chicks lost their competitive advantage relative to magpie chicks (Soler et al., 1995). These results suggest that palatal papillae in great spotted cuckoos would serve as a tuning adaptation that exploits preexisting host's sensory biases (Soler et al., 1995). Future studies that disentangle the effects of this trait and other visual and acoustic begging features in stimulating parental provisioning are necessary to corroborate this idea.

CONCLUDING REMARKS AND FUTURE DIRECTIONS OF RESEARCH

Brood-parasitic young possess many morphological and behavioral traits that allow them to exploit the parental behavior of their hosts to their own benefit. However, the study of trickery and tuning adaptations during the nestling and fledgling stages have historically received less attention than those deployed during the egg stage. The discovery of chick rejection behaviors in hosts of bronze cuckoos nearly 20 years ago has led to renewed interest about co-evolved adaptations between parasitic chicks and their hosts. In recent years, new evidence has accumulated on host chick mimicry in evictor and non-evictor parasites driven by host discrimination against alien young. These studies highlight two aspects of this trickery adaptation that are important to consider in future research. First, host chick mimicry can occur in more than one sensory modality within a single parasite lineage. This observation begs for more research into how hosts integrate visual and acoustic signals in chick recognition to better understand the adaptive value of multimodal resemblance to host young in parasitic chicks. Second, even clearly mimetic parasites may show discrepancies with respect to the phenotype of host chicks. Such imperfect mimicry may be owing to additional adaptations in parasitic chicks for better tuning into the sensory preferences of host species (e.g., exaggerated begging traits), which could serve to compete for food with host nestmates or extract additional resources from their hosts. Alternatively, the discrepancies could be neutral or reflect evolutionary constraints on parasites to match more precisely the begging signals of host chicks. To tackle these questions, it is crucial to combine quantitative analyses of similarity that take into account the host's perspective (e.g., avian vision models) with experimental manipulations (e.g., cross-fostering, playback experiments, and phenotype manipulation). Indeed, the application of objective methods for assessing the extent of visual or vocal resemblance to host chicks across more parasite species could certainly help unravel new cases of coevolved host chick mimicry. Phylogenetic studies are also necessary to disentangle the evolutionary pathways that gave rise to host chick mimicry across parasite lineages.

The study of begging behavior in the context of brood parasitism has received considerable attention over the past 30 years, from both empirical and theoretical perspectives. A pattern that has emerged from this body of knowledge is that begging in brood parasites is often exaggerated but informative about chick need, although the costs of maintaining signal honesty are not well understood. Exaggerated begging displays are widespread across parasite species and likely adaptive as a mechanism to exploit host's response toward non-specific begging traits that signal offspring need. However, it has become increasingly clear that its role in securing sufficient provisioning from hosts depends on many other factors including the parasitism strategy (evictor or non-evictor), the relative size of parasitic chicks to their hosts, and how hosts integrate begging signals in making decisions about provisioning effort and food allocation within broods. More experimental and comparative studies on a broader range of parasite species are necessary to better understand the function and evolution of signal exaggeration in parasitic birds. This is especially true if we consider that research on this subject comprises only a minority (∼13%) of the parasite species and, even within those more extensively studied, data are limited to a narrow range of host-parasite associations. Likewise, the study of begging behavior and its role in host manipulation during the fledgling stage represents another major gap, often neglected in the literature on brood parasitism. For instance, little is known yet about host-parasite interactions beyond the nestling stage and the extent to which trickery and tuning adaptations similar to those observed in parasitic nestlings play a role after the young have left the nest.

In this review, we differentiate between begging exaggeration and sensory exploitation mechanisms based on whether parasitic manipulation is based on signals already used in host-parent offspring communication or not. This categorization becomes somewhat diffuse since parasites may exhibit traits that are actually absent in host chicks but imitate host begging signals, as it is the case of the colored wing-patches resembling yellow gapes in hawk cuckoos. Yet, we found this distinction useful to highlight alternative routes to the evolution of manipulative traits in parasitic chicks.

The role of learning in attuning the begging signals to the host environment provides another interesting venue for future research. The ability to modify call structure according to the host environment is a flexible mechanism for tuning into host' acoustic communication in parasite species that are host-generalists at the population level, like the common cuckoo. However, more work is needed to solve discrepancies between studies and see if generalizations can be made regarding how begging calls develop across common cuckoo host-races. Varying levels of plasticity in begging call features have been observed in other parasite species, but in most cases, the specific function of begging call structure in host manipulation has not been assessed. Hence, it is difficult to say if the observed variation reflects an underlying tuning adaptation that makes begging signals more effective to stimulate provisioning in any given host. As it happens with the study of host chick mimicry, sound-spectrogram analyses must be combined with playback experiments to answer these questions. It is interesting to point out that the studies on begging call development have also revealed that parasitic chicks can use different mechanisms of host manipulation throughout their early life. This is clearly illustrated by Horsfield's bronze cuckoos, which innately develop vocal mimicry of its primary host, but if reared by another host species, they can attune their begging calls to this new host within a few days of hatching.

How parasitic chicks tune into host communication channels to obtain sufficient food is a long-standing question that has promoted fruitful research. This review provides an overview of the advances in the study of how parasitic young evade host defenses and attune their begging signals to tap into host provisioning rules. It also outlines some unanswered questions and emphasizes the need that take into account the host's perspective when assessing the existence of mimicry or sensory exploitation in parasitic chicks. In the future, an integrative approach that take into account the function, ecology, evolution and ontogeny of the manipulative signals displayed by parasitic chicks will increase our knowledge about the ways in which parasites are adapted to exploit the parental care of their hosts.

AUTHOR CONTRIBUTIONS

JRR and MDM conducted the literature survey, organized the information, and wrote the first draft of the manuscript. All authors contributed to the conception and design of the review, and manuscript revision, read, and approved the submitted version.

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Preferential Begging Responses of Shiny Cowbirds to the Conspecific Chatter Call

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Avian brood parasites lay their eggs into the nests of other species, which incubate them and raise the chicks until their independence. Despite living their early weeks of life surrounded by heterospecifics, young brood parasites have the ability to recognize and associate to conspecifics after independence. It has been proposed that the initial conspecific recognition develops when a young parasite encounters a unique species-specific signal that triggers the learning of other aspects of the producer of the signal. For cowbirds (*Molothrus* spp.), this species-specific signal is hypothesized to be the chatter call. Young birds also could express auditory biases, which in some cases lead to discrimination in favor of conspecific songs. Therefore, the perceptual selectivity for chatters might be also present in nestlings. Our aim was to assess if nestlings of the shiny cowbird (*M. bonariensis*) present a preferential begging response to conspecific chatter calls. We evaluated if they respond more to the parasitic vocalization than host chicks and if they respond more to the chatter than to heterospecific nonhost calls. We tested shiny cowbird chicks reared by chalk-browed mockingbirds (*Mimus saturninus*) or house wrens (*Troglodytes aedon*) and host chicks, as control species. We randomly presented to 6-day-old chicks the following playback treatments: (1) conspecific chatter calls, (2) host calls, used as positive controls, and (3) nonhost calls, used as negative control. We measured if chicks begged during the playback treatments and the begging intensity. When responding to the playback of chatter calls, shiny cowbird chicks begged at a higher frequency and more intensively than host chicks. Shiny cowbird chicks reared by mockingbirds begged more intensively to playbacks of conspecific chatter calls than to host calls, while those reared by wrens begged with a similar intensity to playbacks of conspecific chatter and host calls. On the contrary, wren nestlings begged more intensively to playbacks of the wren call than to chatter calls. Mockingbird nestlings did not beg during any treatment. None of the three species begged during the playback of nonhost calls. Our results show that the chatter call produced a preferential begging response in cowbird nestlings, which may be the beginning of a process of conspecific recognition.

Keywords: ***Molothrus bonariensis*, password hypothesis, brood parasitism, vocal cues, begging**

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INTRODUCTION

Conspecific recognition is essential for animals, as it allows individuals to identify members of their own species, search for pairs, and mate successfully, avoiding fitness cost through misdirected social and reproductive effort. In species whose juveniles are reared by their parents, as mammals and most birds, after an early experience with conspecific adults, individuals learn the characteristics of their own species forming an internal representation of salient phenotypic attributes or recognition template and restrict their social preferences as adults to them (Bateson, 1966; Immelmann, 1975). This mechanism called imprinting (Lorenz, 1937; Bateson, 1966; Salzen, 1998; Ten Cate and Vos, 1999) allows individuals to recognize and prefer conspecifics and is adaptive in different social contexts (Immelmann, 1975).

Evidence in oscine species has indicated an auditory bias for conspecific songs during the nestling phase (reviewed by Wheatcroft and Qvarnström, 2015). For instance, Shizuka (2014) found that nestlings of the golden-crowned sparrow (*Zonotrichia atricapilla*) produce more vocal responses to conspecific than to playback songs of the heterospecific white-crowned sparrow (*Z. leucophrys*). Moreover, Bliard et al. (2021) found that nestlings of collared flycatcher (*Ficedula albicollis*) discriminate song based on conspecific alarm calls. They manipulated song phrases starting with the conspecific alarm call followed by either conspecific or heterospecific song notes and found that nestlings responded similarly to both treatments due to the inclusion of the call. This early song discrimination can develop independently of early social experience and depends largely on a genetic component (Wheatcroft and Qvarnström, 2017). For example, Wheatcroft and Qvarnström (2017), manipulated the early social experience of collared and pied flycatchers by swapping young embryos between the nests of two species and found that chicks discriminated in favor of the song of its own species, even when raised by adults from the other species.

Social recognition in interspecific avian brood parasites imposes a paradox. Parasitic nestlings of altricial species are raised by heterospecifics and thus do not have the opportunity to use cues provided from the adults that raise them to learn a conspecific recognition template (Hauber and Sherman, 2001; Slagsvold and Hansen, 2001). Nevertheless, after independence, they interact and reproduce with birds of their own species, which indicates that they have solved the problem of conspecific recognition.

The understanding of the mechanisms involved in the ontogeny of social preferences in brood parasites provides an important challenge to animal behaviorists. Hauber et al. (2001) proposed the "password hypothesis," which states that conspecific recognition in brood parasites is initiated when young encounter some unique species-specific signal or "password" (e.g., a vocalization, behavior, or other characteristic) that triggers learning of additional aspects of the phenotype of the password giver. This hypothesis has been supported by studies conducted in the brown-headed cowbird, *Molothrus ater*, a generalist brood-parasitic species, in which the chatter call seems to be the password for conspecific recognition (Hauber et al., 2001). In this species, the chatter call is the only vocalization given by females, and it is used infrequently by males (Friedmann, 1929; Rothstein et al., 1988). This call is genetically programmed, it does not present geographical variation, and individuals perform it even if they are not exposed to conspecifics (Burnell and Rothstein, 1994). Hauber et al. (2001) found that free-living fledglings and adults of the brown-headed cowbird and also captive fledglings approached to a source-emitting playbacks of chatters more quickly or often than to one emitting vocalizations of heterospecifics (Hauber et al., 2001). They also found that 6-day-old nestlings begged more frequently to playbacks of chatters than to other avian sounds and stated that cowbird chicks might have an auditory predisposition for this vocalization. In addition, the chatter call is commonly used by female brown-headed cowbirds in response to song displays of male cowbirds (Burnell and Rothstein, 1994), and it has a fundamental role in shaping behavioral differences on how females interact with preferred males, as the maintenance of the pair bonds is associated with the reciprocal exchange of vocal displays (Kohn, 2018). Neurobiological studies also found an indirect evidence supporting the hypothesis of the chatter call as the password for conspecific recognition in brown-headed cowbirds. Lynch et al. (2017) found that auditory forebrain regions of cowbirds express greater densities of a protein product of the immediate-early gene *ZENK* in response to the chatter call relative to control coos of mourning doves (*Zenaida macroura*). This latter result shows that when cowbirds listen to the conspecific song, they exhibit a specific neural response in brain regions, which are key for social recognition. Moreover, Louder et al. (2019) found that, when acoustically naive juvenile male and female cowbirds were exposed to songs paired with chatter calls, this call enhanced the learning of song production in males and induced a neuro-genomic profile of song familiarity in females, even for heterospecific songs.

The shiny cowbird, *M. bonariensis*, is an interspecific brood parasite closely related to the brown-headed cowbird (Lanyon, 1992; Johnson and Lanyon, 1999). As the brown-headed cowbird, the shiny cowbird is an extreme generalist that uses more than 250 different hosts (Lowther, 2018). After fledging, shiny cowbird young remains associated with their foster parents for approximately 30–40 days, and, at that time, they join foraging flocks and start roosting with conspecifics (Crudele et al., unpublished data). There is no information on a potential sensory bias allowing for early song discrimination in shiny cowbird young, and no previous studies have determined if the chatter call is recognized for nestlings of this species. To have this information will improve the understanding of the perception components involved in the ontogenetic development of conspecific preferences in obligate brood parasites. Moreover, the study of this new species provides us with the opportunity to test if a potential mechanism to avoid misimprinting is shared by a close relative of the brown-headed cowbird within this parasitic lineage.

In this study, we experimentally evaluated the begging response to different playback treatments of 6-day-old nestlings of shiny cowbirds and two frequent hosts included as control species, namely, the house wren (*Troglodytes aedon*) and the chalk-browed mockingbird (*Mimus saturninus*). These hosts differ markedly in their behavior during the chick feeding visits. Wrens perform calls when they arrive at the nest to which nestlings seem to respond immediately (Bortolato et al., 2019), whereas mockingbirds do not call upon arrival, and their chicks are stimulated to beg by the movement of the nest (Crudele et al., unpublished data). We determined if shiny cowbird chicks raised by these hosts respond differently to conspecific chatter calls compared with host chicks. We expected that parasitic chicks respond similarly to host chicks when they listen to the adult host playbacks, as they could have learned to respond to this stimulus as their host-mates. For host chicks, we expected that they respond more to conspecific calls than to chatters. Finally, none of the species should respond to nonhost calls as it does not represent a relevant stimulus for them.

MATERIALS AND METHODS

Study Area

The field work was carried out during October–February 2018–2019 and 2019–2020 at Reserve "El Destino" (35°80′80″S, 57°82′30″W), located within the "Parque Costero del Sur" (MAB-UNESCO) in the Province of Buenos Aires, Argentina. The study site comprises patches of native forest dominated by *Celtis ehrenbergiana* and *Scutia buxifolia* within a matrix of marshy grasslands and pastures. In this site, shiny cowbird uses as main hosts the chalk-browed mockingbird (frequency of parasitism 70–80%, Fiorini and Reboreda, 2006; Gloag et al., 2012) and the house wren (frequency of parasitism 50%, Tuero et al., 2007). In our study area (approximately 155 ha), there are 40–50 territories of chalk-browed mockingbirds, and we put 140 nest boxes that are frequently used by house wrens. From mid-October to the end of January, we searched exhaustively for nests under construction and checked them every day, from the start of laying until the chicks fledged or the nest was abandoned or depredated.

Experiment

To evaluate the response of chicks to different acoustic stimuli, we tested 68 shiny cowbird chicks (42 reared by chalk-browed mockingbirds and 26 reared by house wrens) and 49 host chicks (23 house wrens and 26 mockingbirds) as control species. When nestlings were 6 days of age, they were removed from the nest and placed individually in an artificial nest. To avoid disturbing the parents, the experiment was conducted more than 30 m from the nest and concealed by vegetation. The artificial nest measured 10 cm (diameter) × 5 cm (deep) and was located inside a container (27.5 cm × 21.5 cm × 28 cm; height × width × length). To standardize motivation and control for the level of hunger, the nestlings were fed with a wet paste of premium insect food (CéDé) until they were satiated, and then we waited 40 min before starting the playback experiment (Hauber et al., 2001). After that, we walked away 3 m from the container, and following a silence of 1 min, we started the playback session. We conducted the playback with a Zoom Handy Recorder H4n and video recorded the chick response with a Gopro Hero4 camera. As we only removed the experimental chick from the nest, the rest of the brood remained there, and parents continued normally with their feeding visits. After finishing the experiment, the chick was returned to the nest where it continued with its normal behavior. None of the nests was deserted in association with our manipulation.

We performed the following playback treatments: (1) chatter calls of adult shiny cowbird females, (2) calls of adult host species (mockingbird or wren), and (3) calls of adult nonhost species (saffron finch, *Sicalis flaveola*), a common species in the study area. The playbacks were presented sequentially in random order, each playback lasted 1 min, and there was an interval of 5 min between playbacks. To make the playbacks of chatter calls of shiny cowbird and the calls of adult mockingbirds and wrens, we used seven randomly selected vocalizations of six adult individuals of each species recorded in the area during the 2017 breeding season using a Zoom Handy Recorder H4n. Adult wren calls were recorded during their feeding nest visits, when they performed contact calls to nestlings. As mockingbirds do not produce this type of call at the nest, we recorded adult contact calls (Argel de Oliveira, 1989) near the nest. For the playbacks of the nonhost species (saffron finch), we used seven randomly selected vocalizations of seven adult individuals. In this case, the recordings were obtained from https://www.xeno-canto.org/species/Sicalis-flaveola and came from individuals that were singing near their nests. The amplitude of the playbacks was standardized with root mean square within and between samples. **Figure 1** shows representative spectrograms of the playbacks used in the experiments.

The begging is an unambiguous and easily quantified response behavior that reflects the reaction of nestlings to acoustic stimuli (Hauber et al., 2001). Other behavioral responses as a proxy for paying attention such as "looking" or "moving" were not observed during the recordings of host visits to nests. Therefore, we analyzed the video recordings to determine the following response variables: (1) if the chick responded (i.e., begged) to the playback or not. We considered that the chick begged when it opened the beak during the playback treatment and (2) intensity of begging (begging category for each of the seven calls of the playback treatment). To quantify the intensity of begging, we assigned postural scores using the scale of intensity of Leonard et al. (2003) where: 0 = head down, no gaping; 1 = head down, gaping, sitting on tarsi; 2 = head up, gaping, sitting on tarsi; 3 = same as 2, plus neck stretched upward; 4 = same as 3, but body lifted off tarsi; and 5 = same as 4, plus wings waving. The intensity of begging was determined for nestlings that begged at least once for the seven calls of the playback treatment.

Our experimental work followed the ASAB/ABS Guidelines for the use of animals in research. The study was conducted with the permission of the Provincial Organism for Sustainable Development (OPDS, Buenos Aires, Argentina; permit no. 202/12-OPDS) and complies with the current laws of Argentina.

Comparison of the Calls Used in the Experiment

To analyze differences in the acoustic structure of the chatter, host, and nonhost calls, we produced spectrograms

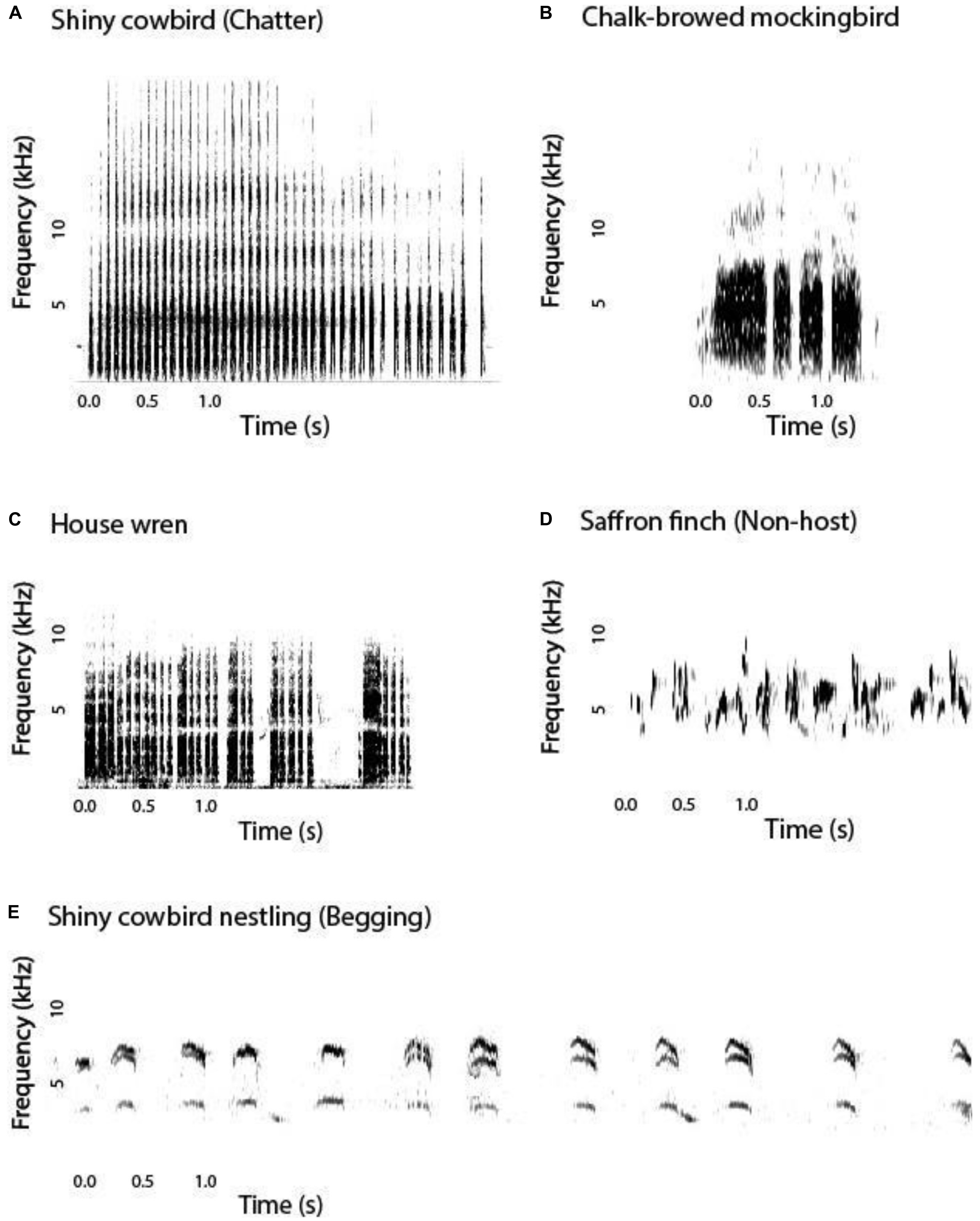

FIGURE 1 | Representative sonograms of the playbacks used in the experiments and of the shiny cowbird begging. **(A)** Chatter call of a female shiny cowbird (*Molothrus bonariensis*), **(B)** call of an adult chalk-browed mockingbird (*Mimus saturninus*), **(C)** call of an adult house wren (*Troglodytes aedon*), **(D)** call of an adult saffron finch (*Sicalis flaveola*, i.e., nonhost species), and **(E)** begging of a shiny cowbird chick.

using RavenPro 1.6.1 (K. Lisa Yang Center for Conservation Bioacoustics, 2019). From the spectrograms, we determined the mean values of five variables (ignoring harmonics): maximum and minimum frequency (Hz), frequency bandwidth (Hz), peak frequency (Hz), and repetition rate (i.e., number of syllables/duration).

Statistical Analyses

We analyzed if the frequency of begging (begging as response variable with two levels, yes-no) was affected by (i) chick category: wren, cowbird reared by wrens, and cowbird reared by mockingbirds (as mockingbird chicks never responded to treatments, they did not provide data variability, and we excluded them from the analysis), (ii) playback treatment (host, chatter, and control), the interaction between "chick category" and "playback treatment," and (iii) date of experiment (as changes across the season might influence begging responses). The model also included two random factors, namely nest (as cowbird and host chicks of the same nest were tested) and chick identity nested within nest (as each chick was tested for the three treatments). For this analysis, we performed a generalized linear model (GLMM) with binomial error distribution and logit link function. We also determined if intensity of begging was related to the same predictor variables used for the previous analysis, through a GLMM with negative binomial error distribution and log link function.

After running the models, we found that the interaction between the chick category and playback treatment was significant. Therefore, we performed analyses for each playback treatment (chatter, host, and control) evaluating differences among chick categories and analyses for each chick category (i.e., cowbird reared by wrens, cowbirds reared by mockingbirds, and wrens) evaluating differences among playback treatments, through GLMMs. For each treatment, we evaluated if there were differences among chick categories in (1) the proportion of chicks that begged, through a GLMM with a binomial error distribution and log link function, and (2) the intensity of begging through a GLMM with negative binomial error distribution and log link function. For each chick category, we evaluated if there were differences among treatments in (1) the proportion of chicks that begged through a GLMM with a binomial error distribution and log link function and (2) intensity of begging for each chick category, with a GLMM with a negative binomial error distribution and log link function. The contrasts among categories of chicks and playback treatments were performed using Tukey multiple comparison tests.

To analyze acoustic differences between the vocalizations used in the playback treatments, we performed a principal component analysis (PCA) with the five acoustic variables (maximum and minimum frequency, frequency bandwidth, peak frequency, and repetition rate).

We used the R software, Version 3.4.0 (R Development Core Team, 2013) and the R Studio, Version 1.0.143 (RStudio Team, 2020) and performed the GLM and GLMM analyses using the lme4 package (Bates et al., 2015) and the glmmTMB (Brooks et al., 2017). The PCA analysis was conducted with the Package vegan version 2.5–7.

RESULTS

Frequency of Begging

We did not detect an effect of the date of the experiment (intercept: estimate ± SE = –19.6 ± 10.9, *df* = 261, $Z = -0.18$, $P = 0.86$; date of experiment: estimate ± SE = –0.004 ± 0.33, $Z = 0.014$, $P = 0.99$), but we detected an effect of the interaction between chick category and playback treatment (interaction: $\chi^2 = 74.3$, $P < 0.0001$; chick category: $\chi^2 = 3.37$, $P = 0.2$; playback treatment: $\chi^2 = 100.8$, $P < 0.0001$). Therefore, we performed the analyses for playback treatments separately comparing among chick categories and for chick categories comparing among playback treatments.

When responding to the playback of chatters, the proportion of begging differed among chick categories (GLMM, intercept: estimate ± SE = –1.6 ± 0.7, *df* = 87, $Z = -2.5$, $P = 0.01$, chick category: $\chi^2 = 14.8$, $P < 0.001$). Cowbird chicks reared by wrens and by mockingbirds begged in a similar proportion (estimate ± SE = –0.7 ± 0.7, $Z = -0.9$, $P = 0.6$) and more than wren chicks (cowbird reared by wren vs. wren: estimate ± SE = 2.3 ± 0.8, $Z = 2.8$, $P = 0.01$ and cowbird reared by mockingbird vs. wren: estimate ± SE = 2.9 ± 0.8, $Z = 3.5$, $P = 0.001$, **Table 1**). When responding to the playback of the host, the proportion of begging differed among chick categories (GLMM, intercept: estimate ± SE = –0.14 ± 0.7, *df* = 87, $Z = 0.2$, $P = 0.8$, chick category: $\chi^2 = 16.3$, $P = 0.0003$). Cowbird chicks reared by mockingbirds begged at a lower proportion than cowbird chicks reared by wrens (estimate ± SE = 3.1 ± 1.0, $Z = 3.0$, $P = 0.008$) and wren chicks (estimate ± SE = –3.3 ± 1.2, $Z = -2.8$, $P = 0.01$), but there were no differences between cowbird reared by wrens and wrens chicks (estimate ± SE = –0.3 ± 0.9, $Z = -0.3$, $P = 0.9$).

Shiny cowbird chicks reared by mockingbirds responded more frequently to conspecific chatter calls (76%) than to calls of mockingbirds (7%, GLMM, intercept: estimate ± SE = 11.3 ± 1.9, *df* = 80, $Z = 6.1$, $P < 0.0001$; playback host: estimate ± SE = –23.8 ± 2.9, $Z = -8.2$, $P < 0.0001$), while shiny cowbird chicks reared by wrens responded similarly to conspecific chatter calls and to calls of wrens (65 vs. 50%, intercept: estimate ± SE = 1.4 ± 0.9, *df* = 48, $Z = 1.4$, $P = 0.2$; playback host: estimate ± SE = –1.3 ± 0.9, $Z = -1.5$, $P = 0.1$). Wren chicks responded more frequently to wren calls than to shiny cowbird chatter calls (48 vs. 17%, GLMM, intercept: estimate ± SE = –3.2 ± 2.0, *df* = 42, $Z = 1.6$, $P = 0.1$; playback host: estimate ± SE = 4.3 ± 2.2, $Z = 1.9$, $P = 0.05$), while mockingbird chicks did not beg during treatments. None of the three species responded to saffron finch calls (**Table 1**).

Intensity of Begging

We did not detect an effect of the date of experiment (intercept: estimate ± SE = –1.1 ± 0.5, *df* = 563, $Z = -2.1$, $P = 0.04$; date of experiment: estimate ± SE = 0.003 ± 0.002, $Z = 1.8$, $P = 0.08$), but we detected an effect of the interaction between chick category and treatment (interaction: $\chi^2 = 48.4$, $P < 0.0001$, chick category: $\chi^2 = 8.1$, $P < 0.02$, treatment: $\chi^2 = 0.1$, $P < 0.7$). Therefore, we performed the analyses for playback treatments separately comparing among chick categories and for chick categories comparing among playback treatments.

When responding to the playback of chatters, the intensity of begging differed among chick categories (GLMM, intercept: estimate ± SE = –0.5 ± 0.3, *df* = 385, $Z = -1.7$, $P = 0.1$, chick category: $\chi^2 = 14.7$, $P = 0.0006$). Cowbird chicks

TABLE 1 | Number of nestlings of each chick category that begged over the total number of nestlings for the different playback treatments.

Playback treatment	Shiny cowbird reared by mockingbird	Shiny cowbird reared by wren	Mockingbird	Wren
Chatter	32/42 (76%)	17/26 (65%)	0/26 (0%)	4/23 (17%)
Mockingbird	3/42 (7%)	–	0/26 (0%)	–
Wren	–	13/26 (50%)	–	11/23 (48%)
Saffron finch	0/42 (0%)	0/26 (0%)	0/26 (0%)	0/23 (0%)

The percentage of responses is shown between parentheses.

reared by mockingbirds begged with a higher intensity than cowbirds reared by wrens (estimate ± SE = –0.8 ± 0.2, Z = –3.0, P = 0.006, **Figure 2**) and more than wren chicks (estimate ± SE = 1.3 ± 0.3, Z = 3.8, P = 0.0001), but there were no differences between cowbirds reared by wrens and wrens chicks (estimate ± SE = 0.5 ± 0.6, Z = 1.5, P = 0.3, **Figure 2**). When listening to the playback of the host, the intensity of begging differed among chick categories (GLMM, intercept: estimate ± SE = 0.5 ± 0.2, df = 176, Z = –2.8, $P < 0.005$, chick category: $\chi^2 = 7.9$, $P < 0.01$). Cowbird chicks reared by mockingbirds begged with a greater intensity than cowbird chicks reared by wrens (estimate ± SE = 1.4 ± 0.6, Z = 2.4, P = 0.04, **Figure 2**) and wren chicks (estimate ± SE = –1.6 ± 0.6, Z = –2.8, $P < 0.01$) and cowbirds reared by wrens begged with a similar intensity than wren chicks (estimate ± SE = –0.2 ± 0.2, Z = –0.9, P = 0.6, **Figure 2**).

Shiny cowbird chicks reared by mockingbirds begged more intensively when they listened a conspecific chatter call than when they listened a mockingbird call (intercept: estimate ± SE = 0.8 ± 0.1, df = 232, Z = 5.9, $P < 0.0001$; playback host: estimate ± SE = –2.2 ± 0.5, Z = –4.4, $P < 0.0001$), while shiny cowbirds chicks reared by wrens showed a similar intensity of begging when they listened a conspecific chatter and a wren call (intercept: estimate ± SE = 0.1 ± 0.2, df = 212, Z = 0.7, P = 0.5; playback host: estimate ± SE = 0.2 ± 0.1, Z = –1.4, P = 0.2). Finally, wren chicks begged more intensively when they responded to a wren call than a shiny cowbird chatter call (intercept: estimate ± SE = –0.5 ± 0.4, df = 114, Z = –1.1, P = 0.3; playback host: estimate ± SE = 0.5 ± 0.2, Z = 2.4, P = 0.01; **Figure 2**).

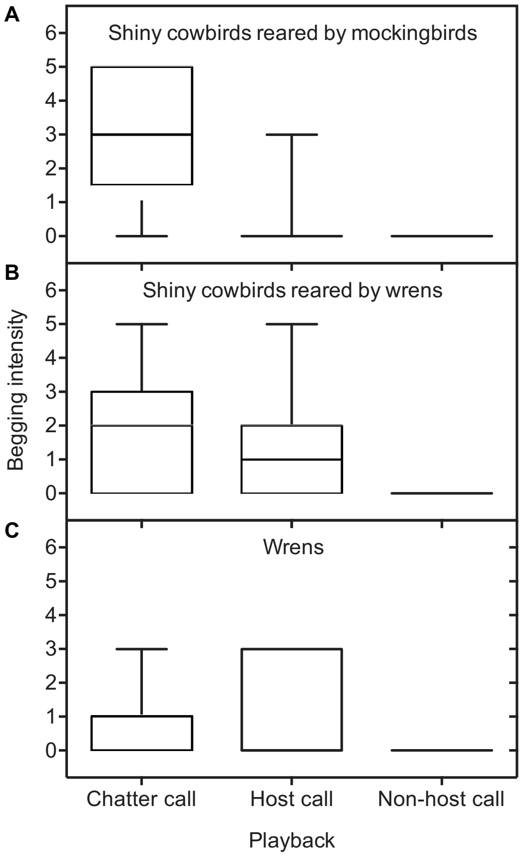

FIGURE 2 | Intensity of the begging response of the three chick categories for different playback treatments (chatter, host, and nonhost playbacks): **(A)** shiny cowbirds reared by mockingbirds, **(B)** shiny cowbirds reared by wrens, and **(C)** wrens. Boxplots show medians, interquartiles, and ranges.

Analysis of the Acoustic Variables

A PCA biplot of the acoustic variables showed differences among shiny cowbird chatter, mockingbird, wren, and saffron finch calls. PC1 and PC2 explained 64.52 and 17.24% of the total variation of the data, respectively. Considering loadings above 0.5, maximum frequency, minimum frequency, peak frequency, and bandwidth were the variables that contributed more to group differentiation along PC1. Shiny cowbird chatter and wren calls were relatively similar and showed a higher bandwidth than the others. Saffron finch calls differ from the other vocalizations in maximum, minimum, and peak frequency (**Figure 3**). The repetition rate was the variable that contributed more to differentiation along PC2, with shiny cowbird chatter call showing more repetition rate than mockingbird call (**Figure 3**).

DISCUSSION

Our results show that shiny cowbird chicks have preferential begging responses to conspecific chatter calls compared with host chicks. When listening to the conspecific vocalizations, parasitic chicks begged at a higher proportion (65–76%) than host chicks (0–17%) and cowbirds reared by wrens also begged more intensively than wren chicks. Although our experiment does not provide direct evidence for the hypothesis of password recognition, as this would require testing cowbirds at an age where they are more likely associated with adult cowbirds (i.e., after fledging), it provides evidence that the chatter call triggers

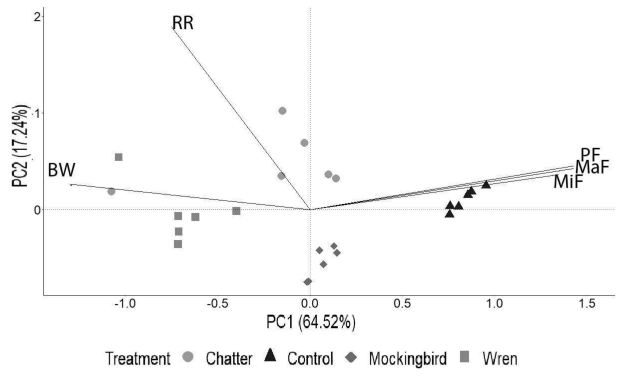

FIGURE 3 | Principal components analysis biplot of acoustic variables used in the playback treatments (shiny cowbird chatter call and wren, mockingbird, and saffron finch adult calls). The first and second principal components were plotted in an *x–y* plane and accounted for 64.52 and 17.24%, respectively, of the total variance. BW, bandwidth; RR, repetition rate; MiF, minimum frequency; MaF, maximum frequency; PF, peak frequency.

preferential begging responses in shiny cowbird nestlings. If this early preference for the conspecific chatter call continues after cowbirds fledge, it may favor the association of cowbird young with individuals of their own species at the time they became independent from their foster parents, allowing them to develop a conspecific recognition template (Dooling and Searcy, 1982; Whaling et al., 1997; Hauber et al., 2001).

We expected that when cowbirds listened to their host vocalization, they beg similarly to host chicks, as they could have learned to recognize adult host calls during the nestling phase (Tuero et al., 2016). In the case of cowbirds reared by wrens, they begged at a high and similar frequency and intensity than wren chicks supporting the prediction for a positive control. In contrast, wren chicks showed a lower frequency and intensity of begging when they listened to the parasitic chatter call than the call of their own species, which would indicate that their response is species-specific. Cowbird chicks reared by mockingbirds also behaved similarly to host chicks, both showing a low frequency and intensity of begging to mockingbird calls. These results were initially surprising because we expected a stronger response for chicks of both species to the mockingbird call treatment. Nevertheless, we observed through video recordings of 60 h in 20 nests that adult mockingbirds approached silently to the nest when feeding nestlings (Crudele et al., unpublished data), and chicks seemed stimulated for the vibrations of branches or the nest when adults perched on them instead of for vocalizations of parents. Moreover, during the first experimental sessions, we noted that mockingbird chicks did not beg when listened to the playbacks. We tested if they responded to a vibration of the nest or to a change of the light passing the hand over them, simulating the arrival of the adult to the nest, and all the chicks begged during this stimulus (N = 11 chicks). The lack of begging of cowbirds when listening the mockingbird playback differed from the results found by Rivers (2007, 2009), in a study in the brown-headed cowbirds parasitizing a taxonomically similar large host, the Brown Thrasher, *Toxostoma rufum*. These authors found that parasitic chicks begged readily to a wide range of stimuli in the presence of adults and even when adults were absent (Rivers, 2007, 2009). This difference between the responses of closely related cowbird species might have relevance to how cowbird species respond to stimuli and thus conspecific recognition.

As regard the playback of the control nonhost species (i.e., negative control), it did not elicit begging responses in either shiny cowbird or host chicks, indicating that nestlings do not respond to sounds from a species that is not relevant to them.

With respect to the mechanism that could explain the response of cowbird nestlings to the chatter call, one possibility is that cowbird chicks have an auditory predisposition for the conspecific chatter call. This perceptual preference or innate sensory bias could develop in young animals without the need of a previous experience with the stimulus (Bolhuis, 1991) and would lead to a preference for conspecific songs (Bliard et al., 2021). Alternatively, the response to the chatter call could be based on a recognition process known as self-referent phenotype matching (Sherman, 1991; Hauber and Sherman, 2001). In this case, young use some phenotype characteristics of their own (in this case its vocalization) as template for discrimination of individuals they will encounter in the future. Hauber et al. (2001) stated that this explanation would be reasonable in the brown-headed cowbirds due to the similarity between the waveforms and peak frequencies of fledgling begging calls and adult chatters.

There are other alternative hypotheses, apart from the existence of a perceptual selectivity for chatters, that could explain the responsiveness to chatter calls of cowbird nestlings. One possibility is that chatters are like the begging solicitation calls of most important host species (Hauber et al., 2001) and cowbirds respond quickly to be fed. A second hypothesis is that chatters are like vocalizations emitted by the hosts when approaching the nest, such as the case of house wrens. Nevertheless, in this case, it would be expected that also wren chicks respond with a similar frequency and intensity to chatters than to wren calls. However, this was not the case, indicating that these calls are not so similar. A third hypothesis could be that if multiple parasitism is common, stronger begging responses of a cowbird toward the chatter call is due to the close resemblance between the chatter call and the cowbird begging call (i.e., phenotype matching). In this case, the higher begging rate toward the playback of chatter calls is possibly due to the competition among conspecific siblings but not for species recognition. This idea may find support as the begging intensity is lower in cowbirds reared by wrens, where the number of cowbird chicks per parasitized nests is one (Tuero et al., 2007) than in mockingbird nests where the number of parasitic chicks is 1.6 (range 1–4, Fiorini, unpublished data). Nevertheless, in nests of this large host, where cowbird chicks are smaller than host chicks, the food competition, instead of the phenotype matching, seems to modulate the begging that chicks emitted not only to chatter but also to host calls (Tuero et al., 2016).

Our experiment showed that shiny cowbird chicks raised in mockingbird nests responded more frequently and intensively to conspecific chatter calls than to host calls, and cowbird chicks reared by both hosts did not respond to nonhost calls when they were in the host nest. This would indicate that the preference for the conspecific chatter call is innate or developed at a very early stage after hatching. Although at that time the parasite chick has no direct contact with adult conspecifics, it could listen the chatter of female cowbirds that are near the nest and eventually see them, which would allow the chick to start learning conspecific characteristics before fledging. In this respect, Soler and Soler (1999) found that adult great spotted cuckoos (*Clamator glandarius*) visited parasitized nests and had contact with old nestlings and suggested that this behavior could promote imprinting in young cuckoos.

Further experiments must be carried out to assess whether the chatter call, in addition to producing a preferred response in nestlings, is the cue used for juveniles to develop the conspecific template. Preliminary results from an experiment, in which we exposed shiny cowbird juveniles with models and calls of conspecifics and heterospecifics, indicate that young cowbirds develop social preferences with the models paired with the chatter call, no matter what is the model species used (Crudele et al., unpublished data). These results would support that an auditory cue—the chatter—mediates subsequent learning of additional morphological characteristics (Louder et al., 2019).

Although evidence showed that 6-day-old cowbird nestlings respond to conspecific chatter (Hauber et al., 2001, this study), the studies conducted so far have not analyzed whether this acoustic cue produces a neural stimulation in the chicks. The studies that analyzed the expression of genes in the auditory forebrain were performed in adults and juveniles of the brown-headed cowbird (Lynch et al., 2017; Louder et al., 2019). It would be interesting to determine if a neurogenomic response to the chatter call also occurs in nestlings and if the exposure to the password influences neuroplasticity. This would allow us to discern between two alternative hypotheses. The first one, as our results suggest, is that cowbirds are sensitive to the password since they are born. In this case, it could be possible that they have a wide sensitive period to imprint (Immelmann, 1975) that starts during the nestling phase but is consolidated after host independence, when juveniles interact with other cowbirds in conspecific flocks (Han and Fleischer, 1995). The second one is that cowbirds have a delayed imprinting, and they are sensitive to the conspecific cue only after they fledge, when the probability to interact with conspecific increases (Bateson, 1979; O'Loghlen and Rothstein, 1993, 2002).

In conclusion, our results indicate that shiny cowbird chicks respond differentially to conspecific chatter calls and are consistent with the password hypothesis, adding new evidence to the idea that parasites have a predisposition for conspecific auditory signals that would initiate species recognition.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Organismo Provincial para el Desarrollo Sostenible of Buenos Aires Province (Permit number 202/12-O.P.D.S.).

AUTHOR CONTRIBUTIONS

IC carried out the field work and analyzed the videos. IC and VF performed the data analysis. IC and JR performed the figures. IC, JR, and VF conceived the initial idea, discussed the results, wrote the manuscript, and approved the submitted version.

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Clutch Abandoning Parasitised Yellow Warblers Have Increased Circulating Corticosterone With No Effect of Past Corticosterone or Differences in Egg Maculation Characteristics

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Parental care can be costly to parents' fitness. As such, abandonment of the current reproductive attempt may benefit potential future opportunities, maximising lifetime reproductive success. Obligate brood parasitism, a reproductive strategy in which parasites lay their eggs in the nests of other species and rely solely on them to raise the parasitic young, is an ideal system to study brood abandonment. Some parasitised host species have evolved anti-parasitic defences, notably clutch abandonment (egg burial and nest desertion), that may mitigate negative consequences of parasitism. Abandonment of clutches due to parasitism is not unlike abandonment of reproduction in times of stress, suggesting that host responses to parasitism could be triggered at least partly by elevated stress hormones that mediate individual decisions. Yet, the mechanistic basis for clutch abandonment remains unclear. Here, we experimentally parasitised clutches of yellow warblers (*Setophaga petechia*), a common host of the brown-headed cowbird (*Molothrus ater*), with model cowbird eggs to examine whether host circulating corticosterone (CORT) differed among females that accepted parasitic eggs or rejected them through clutch abandonment. We also assessed whether feather CORT, a measure of past corticosterone exposure, differed between accepters and abandoners. Finally, we investigated whether egg visual signals, specifically differences in maculation characteristics between model cowbird and host eggs, predicted abandonment of experimentally parasitised clutches. Circulating CORT was higher in females who abandoned their parasitised clutches, but not in those who accepted, relative to controls with no egg addition. Past stress and differences in maculation characteristics did not predict whether individuals accepted or abandoned experimentally parasitised clutches. Moreover, differences in maculation characteristics between the host and model cowbird eggs did not predict CORT levels or nest abandonment. Thus, parasitism with subsequent clutch abandonment may be associated with elevated circulating CORT, but neither past stress nor differences in maculation characteristics influenced abandonment. The combination of these results contributes to our understanding of the roles of corticosterone and egg visual signals in the context of clutch abandonment in brood parasitism specifically, and of parental care more broadly.

Keywords: avian brood parasitism, egg rejection, egg visual signals, clutch abandonment, *Setophaga petechia*, *Molothrus ater*, corticosterone

INTRODUCTION

Brood abandonment (Wiggins et al., 1994) occurs in a variety of taxa from insects (Zink, 2003) to birds (Hosoi and Rothstein, 2000). Parental investment theory suggests that a parent should invest in its current brood to the extent that it maximises lifetime reproductive success, based on a trade-off between current and future reproduction (Klug and Bonsall, 2010; Davies et al., 2012). In some instances, it is beneficial for parents to abandon their current reproductive attempt (i.e., brood abandonment) for the benefit of potential future reproductive opportunities (Davies et al., 2012). In birds, cues that can elicit clutch abandonment vary between and within species, and may include predation (Ackerman et al., 2003; Lima, 2009), harsh weather conditions (Bottitta et al., 2003; Thierry et al., 2013), and poor body condition (Groscolas et al., 2008; Spée et al., 2010, 2011). Under these challenging circumstances, brood abandonment can be adaptive if parents are then able to reproduce under more favourable conditions (Sealy, 1995; Guigueno and Sealy, 2010). Although lifetime reproductive success may be maximised through brood abandonment, abandoners may face extensive costs associated with re-nesting and producing another brood later in the breeding season (Wiggins et al., 1994; Verboven and Tinbergen, 2002; Guigueno and Sealy, 2010). As such, brood abandonment represents a key reproductive decision, where abandoners must consider the trade-offs associated with investing in current versus future clutches (Verboven and Tinbergen, 2002; Johnston, 2011; Fokkema et al., 2016; Griesser et al., 2017).

Brood parasitism, in which parasites rely solely on other species to raise their young (Davies, 2000; Schulze-Hagen et al., 2009; Feeney et al., 2014; Soler, 2017), exploits parental care and imposes severe fitness costs on hosts. A parasitised host may incur fitness costs that decrease reproductive success, such as parasitic hatchlings evicting host eggs and nestlings (Soler et al., 1995; Kilner et al., 2004), outcompeting host offspring for food and space (Lorenzana and Sealy, 1997; Moskát et al., 2017), or causing carry-over effects which delay and decrease future nesting attempts in subsequent breeding seasons (Mark and Rubenstein, 2013). To combat these costs, many host species have evolved antiparasitic defences that reduce the impact of parasitism on lifetime reproductive success (Rothstein, 1990; Davies, 2000; Roldán and Soler, 2011; Medina and Langmore, 2015). For example, some hosts recognise the specific threat posed by brood parasites and strongly defend the nest against them during the egg-stage, when nests are most vulnerable to parasitism, employing front-line defences (Gill and Sealy, 2004; Lawson et al., 2021). If the parasite successfully lays an egg in the host nest, some hosts may eject it through grasp- or puncture-ejection to avoid raising the parasitic young while not having to abandon a clutch entirely (Servedio and Hauber, 2006). However, the ability to eject parasitic eggs is limited by the ability to visually recognise a parasitic egg (Soler et al., 2017) and morphological characteristics (Rasmussen et al., 2009; Guigueno and Sealy, 2011; Soler et al., 2015). Birds must have a bill of sufficient gape-size and strength to either grasp the parasitic egg or puncture it (Spaw and Rohwer, 1987; Picman, 1989), or risk incurring significant costs to their reproductive success by damaging their own eggs in the process (Peer et al., 2018). An alternative antiparasitic defence is clutch abandonment.

In clutch abandonment, hosts abandon clutches in response to brood parasitism. The abandoned clutch may consist of only parasitic eggs or it may include a mix of parasitic eggs and their own (Hosoi and Rothstein, 2000; Soler et al., 2015). Hosts abandon their clutches via either nest desertion followed by renesting at a new site or burial of the parasitised clutch followed by a new clutch laid in a superimposed nest (Guigueno and Sealy, 2010). Abandonment is costlier than egg ejection in terms of energy and time invested, as host eggs may be lost and nests need to be rebuilt (Clark and Robertson, 1981; Sealy, 1995; Moskát and Honza, 2002; Guigueno and Sealy, 2009; Soler et al., 2015). Clutch abandonment varies among species, as well as within and between conspecifics (Hosoi and Rothstein, 2000; Guigueno and Sealy, 2010). Only some host species reject parasitised clutches by abandonment, and within these species, individuals may vary in the forms of egg rejection they employ, using, for example, ejection or desertion (Servedio and Hauber, 2006; Honza et al., 2007) or nest desertion and egg burial (Sealy, 1995). The underlying mechanisms that influence within- and between-species variability in responses and the cues that trigger parents to abandon their nests remain unexplained (Abolins-Abols and Hauber, 2018; Avilés, 2018).

Hormones play a crucial role in modulating behaviour (Tata, 2005) and mediate many behavioural adjustments in birds, including antiparasitic responses (Bonier et al., 2009; Ruiz-Raya et al., 2018; Abolins-Abols and Hauber, 2020). Specifically, the glucocorticoid hormone corticosterone (CORT) is a principal mediator of the behaviours of birds as it increases in response to environmental stressors (Siegel, 1980). CORT is often used as an index of stress, although it is only one part of the integrated stress response and is an approximation of whole animal "stress" (MacDougall-Shackleton et al., 2019). Indeed, CORT has a role in a broad variety of functions unrelated to stress, such as regulating metabolism (Ouyang et al., 2013; MacDougall-Shackleton et al., 2019). Nevertheless, elevated CORT is associated with behavioural changes that promote individual survival (Wingfield, 2003; Angelier et al., 2009; Crossin et al., 2012), such as increasing vigilance while decreasing expression of reproduction-related behaviours (Wingfield et al., 1998; Wingfield, 2003; Schoenle et al., 2017). Therefore, CORT represents a promising candidate to explore as a proximate mediator of host responses to brood parasitism (Bókony et al., 2009). Simply the presence of a parasitic egg in the nest may increase the host's circulating CORT levels (Ibáñez-Álamo et al., 2012; Mark and Rubenstein, 2013; Schoenle et al., 2017) and recent evidence suggests that antiparasitic defences may be partly mediated by CORT. Abolins-Abols and Hauber (2020) experimentally manipulated CORT levels in egg grasp-ejecting American robins (*Turdus migratorius*) and found that parasitised clutches were more likely to be accepted when potential increases in baseline CORT levels were inhibited. As baseline CORT changes in response to experimental parasitism, it thus can provide further insight into the mechanisms mediating a host's response, including clutch abandonment (Landys et al., 2006; Ruiz-Raya et al., 2018; Scharf et al., 2021).

Individual allostatic load (one interpretation of "stress") is related to CORT levels at both short- and long-time scales, with acute (relatively short-term) responses reflected in circulating levels. It is possible that stressful events earlier in the life of an individual influence current behaviours as well. Analyses of CORT in feathers provide a long-term measure of CORT, inferring past stress during feather growth over the moulting period (Bortolotti et al., 2008, 2009; Sherriff et al., 2011). During feather growth when cell differentiation is occurring, circulating CORT continually diffuses in a time-dependent manner through the blood quill into highly vascularised follicles along the entirety of the feather (days-to-weeks, Lattin et al., 2011; Jenni-Eiermann et al., 2015; Freeman and Newman, 2018; Aharon-Rotmann et al., 2021). Feather CORT can thus be used as a biomarker for an integrated measure of CORT secretion representing stress levels over a longer period, rather than to a specific environmental stressor as revealed by measures of circulating CORT from plasma (Fairhurst et al., 2013). Hence, feather CORT, representing general conditions from a different phase of the annual cycle, may be associated with how individuals respond to current reproductive decisions due to cumulative allostatic load over time (Martínez-Padilla et al., 2013; Hansen et al., 2016). This idea is analogous to the developmental stress hypothesis, which predicts that stress early in life can affect behaviour and stress responses later in life, such as a major reproductive decision like abandoning a clutch (Nowicki et al., 2002; Spencer and MacDougall, 2011).

For a CORT response to be initiated, a stressor must appear that causes the hypothalamus to initiate the hypothalamic-pituitary-adrenal cascade leading to CORT secretion. For brood parasite hosts, one such stressor may be the visual recognition of a host egg, as visual differences in colour or maculation between host and parasite eggs often drive host responses (Rothstein, 1974, 1990; Brooke and Davies, 1988; Lyon, 2003; Manna et al., 2017; Gómez et al., 2021). The ability of a parent to successfully recognise their own eggs or a discordant egg within their clutch and reject a parasitic egg or parasitised clutch should be adaptive, as the host would no longer incur the costs associated with brood parasitism (Hauber et al., 2019; Ducay et al., 2021). However, visual signal recognition ability may largely rely on the extent of differences in maculation between parasitic and host eggs (Rothstein, 1982; Lahti, 2006; Cassey et al., 2008; Spottiswoode and Stevens, 2010; Honza and Cherry, 2017; Hanley et al., 2019). Common cuckoo (*Cuculus canorus*) egg rejection increases with differences in appearance, such as extent of maculation and spot size, between cuckoo and host eggs (Davies, 2000). Appearance-based discrimination also occurs in brown-headed cowbird (*Molothrus ater*, cowbird hereafter) hosts (Segura et al., 2016; Dainson et al., 2017). Thus, the ability to recognise a parasitic egg may initiate rejection via egg ejection, but the role of differences in egg visual signals in the context of clutch abandonment is less clear (Davies and Brooke, 1989a; Langmore et al., 2005; Spottiswoode and Stevens, 2010). In addition to egg visual signals stimulating rejection, they may also affect circulating CORT levels. Indeed, Eurasian blackbirds (*Turdus merula*) have higher circulating CORT levels when parasitised with non-mimetic eggs than mimetic ones (Ruiz-Raya et al., 2018). Hence, clutch abandonment may then be mediated by increases in circulating CORT initiated by the extent of maculation characteristic differences between parasitic eggs and their own.

The yellow warbler (*Setophaga petechia*, hereafter "warbler") is a common North American passerine that is frequently parasitised by the brown-headed cowbird. They are the only host of the cowbird known to regularly reject parasitised clutches by two forms of clutch abandonment: nest desertion and egg burial (Clark and Robertson, 1981; Sealy, 1995; Mico, 1998; Hauber et al., 2006; Guigueno and Sealy, 2012). About 35% of female yellow warblers bury cowbird eggs, sometimes with their own eggs, and attempt to re-nest, whereas 12% of females desert the nest entirely and renest at a new site (Sealy, 1995). If parasitism occurs early in the laying period, roughly 50% of females bury (Clark and Robertson, 1981). However, warblers rarely desert when parasitised during incubation, possibly due to significant costs related to time lost and building an entirely new nest (Guigueno and Sealy, 2009). In addition, warblers are more likely to bury or desert parasitised clutches early in the breeding season when there is time to re-nest (Guigueno and Sealy, 2010). Due to high costs of clutch abandonment, warblers may rely on multiple stimuli when making their decision, such as interaction with the adult parasite at the nest (Guigueno and Sealy, 2011). Whereas the colours of the egg background (off-white) and maculation (dark brown) are similar between warbler and cowbird eggs (Guigueno and Sealy, 2009; Guigueno et al., 2014), the extent of differences in maculation characteristics (maculation cover and spot profile) between cowbird and warbler eggs have not been quantitatively measured despite obvious qualitative differences noted in the field.

Here, we experimentally parasitised nests of warblers with model cowbird eggs to investigate the relationship between CORT levels (current circulating via plasma and past via feathers), differences in maculation characteristics, and abandonment of parasitised clutches. As circulating CORT can change rapidly in response to environmental stressors (Wingfield, 2003), we predicted that circulating CORT would be higher in females who accepted (1a) model cowbird eggs, and (1b) those who abandoned experimentally parasitised clutches compared to controls. Given that accepters and clutch abandoners both received model cowbird eggs, we expected that individuals who experienced elevated past CORT would have an amplified current CORT in response to the presence of parasitic eggs (Martínez-Padilla et al., 2013; Hansen et al., 2016). Control individuals and individuals who received eggs each come from a random sample, but within the group of individuals who received eggs, abandoners and accepters would presumably split non-randomly if past stress played a role. Therefore, (2a) we reasoned that control individuals would have intermediate feather CORT levels between accepters and clutch abandoners with a greater variance than each of the two experimentally parasitised groups. We also explored two alternatives that (2b) increased feather CORT would be associated with increased circulating CORT and increase the probability of clutch abandonment and that (2c), if an individual experienced past stress and carry-over effects existed, hosts would not be able to pay the costs of abandonment and thus, individuals with higher feather CORT would be more likely to accept. As such, we predicted significant differences in means and variance among all three groups (control, accepters, and abandoners), with the greatest difference between abandoners and accepters. Finally, because differences in egg visual signals between cowbird and host eggs could potentially influence circulating CORT and the probability of abandoning a parasitised clutch (Ruiz-Raya et al., 2018), we assessed the extent of differences in egg maculation characteristics between warbler and model cowbird eggs added to clutches. We predicted that (3a) circulating CORT would be positively associated with the extent of differences in egg maculation characteristics between host and model cowbird eggs and (3b) accepters would have smaller differences in maculation characteristics compared to clutch abandoners. The combination of these analyses provides us with a fuller understanding of the effects of hormones and egg visual signals in the context of clutch abandonment in brood parasitism specifically, and of parental care more broadly.

MATERIALS AND METHODS

Study Design

The study was conducted within a radius of 35 km of Kalamazoo, MI, United States (42°17′24″N, 85°35′09″W), between May and June in 2010, 2011, and 2019. Nest sites were situated in habitats consisting of marshes and shrubs where yellow warblers were abundant and cowbirds were observed daily.

We found nests by observing singing males and females carrying nest-building materials. We sought to find nests as early in the breeding season as possible, ideally in the preliminary nest building stages, to ensure proper timing of experimental parasitism. We visited nests during the building stage every 2 days until nest-lining was complete. We then monitored the nests daily to identify the day of the first egg laid. Eggs were numbered in their laying order with a non-toxic permanent marker. Because natural parasitism by cowbirds is too infrequent at our field sites to provide a sufficient sample size, we made puncturable artificial eggs out of plaster of Paris, closely mimicking cowbird eggs in shape, colouration, weight and volume (see Guigueno et al., 2014). Two model cowbird eggs were added to each experimental nest, which does occur naturally (Kuehn, 2009), to increase the likelihood the females detected the clutch manipulation. On laying day 1, we added two cowbird eggs to the warbler's clutch between 6:00 and 12:00 (Eastern Daylight Time), to limit the gap to the cowbirds' egg laying window. The nests were monitored daily over the next 6 days to observe laying progression and to record acceptance or clutch abandonment via nest desertion or burial. Egg burial was recognised by the slight pushing down of the eggs and/or the addition of lining material over the eggs, while desertion was recognised when the female was not observed at the nest for 3 days in a row and eggs were cold (Guigueno and Sealy, 2010). One out of every three nests found were randomly chosen to act as controls and did not receive model cowbird eggs. Control nests were monitored at the same frequency as the experimental group.

Circulating Plasma Corticosterone

Female warblers abandon on 2–3 days after parasitism, with burials occurring after 2.3 ± 0.1 days and nest desertion after 2.5 ± 0.3 days (mean ± SE, Guigueno and Sealy, 2010). Therefore, we collected a blood sample on laying day 4 to capture a snapshot of the female's circulating CORT during this critical period. We captured females between 8:00 and 14:00 (Eastern Daylight Time). This time window was chosen to minimise time of day differences among individuals, while giving time to capture hard-to-capture birds and to sample multiple females on the same day. We sampled a small number of females after 12:00 pm to ensure that we collected blood samples on the appropriate day in the female's laying cycle. On laying day 4, mist nets were placed near the focal host nest to passively capture the female. We then aimed to collect a blood sample from the alar vein within 3 min of capture, recording the time from capture to sample acquirement. In nine cases, sampling time exceeded 3 min, which would increase CORT due to acute stress of capture (Romero and Reed, 2005); therefore, we ran a correlation analysis between capture time and CORT level of all individuals and estimated at 3 min the CORT level for those females whose sampling time was prolonged. Each female was banded with a numbered United States Fish and Wildlife Service aluminium band and a unique combination of colour bands before being released to ensure we did not resample females within or between years. Blood samples were kept on ice until they were centrifuged to separate plasma from red blood cells. The separated plasma was pipetted into a separate labelled vial and frozen in a −20°C freezer for long-term storage.

Plasma corticosterone was determined using an enzyme-linked immunosorbent assay (ELISA) kit from ENZO Life Sciences, Inc. (Farmingdale, NY, United States), following kit instructions. This ELISA has been previously validated and used to measure plasma corticosterone in multiple songbirds including sparrows (Wada et al., 2007; Ouyang et al., 2021), wrens (Strange et al., 2016), finches (Kraft et al., 2021) and swallows (Sarpong et al., 2019). Plasma samples were diluted to fall within the range of the standard curve, generally 1:40 or 1:50, incubated with 1% steroid displacement reagent, and run in triplicate or duplicate depending on the sample volume. All samples from a single year were run on the same plate; the intra- and inter-assay coefficient of variation were less than 10%. Assay sensitivity was calculated to be 20.34 pg/ml and all samples read above that value.

Feather Corticosterone

During capture of the female warbler on the fourth laying day, the outermost rectrices (R6) on both sides of the tail were collected. We included both second-year and after second-year individuals within our study. Second-year individuals grew feathers during their juvenal moult in the nest, while after second-year birds grew rectrices feathers as part of their complete moult on the breeding grounds between June and September (Pyle et al., 1997). As such, birds within both age-classes grew sampled feathers within the previous breeding season, but, in non-overlapping timeframes. As such, we tested for differences between second-year and after second-year individuals using a linear model on R Statistical Package (R Core Team, 2020), with age as a predictor and feather CORT as the dependent variable. As there were no significant differences between age and feather CORT ($p = 0.61$), we merged the two groups for subsequent analyses.

Extraction of corticosterone from feathers was conducted following a procedure similar to that previously described in Bortolotti et al. (2008). The calamus was removed and the length and weight of the feather were recorded using a digital caliper and an analytical balance, respectively. The feather (vane and rachis) was cut and minced into small pieces and put into a 50 ml conical tube with 10 ml of methanol. The tubes were sonicated for 30 min in a sonicating bath and then incubated overnight at 50°C in a shaking water bath. The next morning, the methanol was filtered through a 70 μm cell strainer and further filtered through a 0.22 μm cellulose acetate syringe filter into a glass tube. Methanol was evaporated by placing tubes into a 50°C shaking water bath in a fume hood. Once evaporated, samples were reconstituted with 1 ml ELISA assay buffer with gentle rocking for 3 h. Samples were transferred to microcentrifuge tubes and frozen at −20°C until assayed no more than 7 days later.

Feather corticosterone was determined using an ELISA from ENZO Life-Sciences Inc., following the kit instructions with the extension of the standard curve by one value on the low end (total range: 20,000 – 6.4 pg/ml). All samples from a single year/season were run on the same plate in triplicate without dilution. Assay sensitivity was 6.4 pg/ml and all samples read well above this value. Intra- and inter-assay coefficients of variation were less than 10%. Corticosterone values were normalised to feather length.

Egg Visual Signals

On laying day 4, we collected the last laid egg. Some nests were inaccessible because of height or heavy vegetation and as a result, it was only possible to determine clutch size but not possible to either mark or sample the eggs, resulting in smaller sample sizes for eggs when compared to circulating CORT samples collected from females (**Table 1**).

We followed a similar egg image analysis procedure as Hauber et al. (2018). The fourth laid warbler eggs were each compared to one standardised model cowbird egg. Eggs were placed on their side on a colour standard card with a ruler and were photographed with a digital camera mounted on a tripod. The eggs were each photographed four times, with a 90° rotation along the axis between photos. Image analysis on warbler and model cowbird eggs was done using Adobe Photoshop®. We first cropped each egg image into three sections along its long axis: (1) apex-end third, (2) middle-region third, and (3) blunt-end third. We quantified maculation coverage percentage within each third by converting the maculated area to black (RGB = 0,0,0) and the non-maculated area to white (RGB = 255,255,255). We then measured the percentage of black pixels within each third. We obtained the maculation coverage percentage for each third by finding the mean of the percentages between the four images for each egg. We calculated the absolute difference in maculation coverage percentage between the blunt and apex ends for all eggs, thus producing a measure of end-to-end maculation coverage variation. The model cowbird egg's end-to-end maculation coverage value was then subtracted from the warbler egg's maculation coverage, providing a measure of the end-to-end difference in maculation coverage between these two eggs. As such, a positive value signified that the host egg had greater variation than the cowbird model egg and a negative value would indicate that there was less variation.

We obtained an average spot length-to-width ratio of the apex and blunt thirds by randomly selecting five spots within each third of each egg image. The mean spot ratio within each of the apex and blunt thirds of the egg was then averaged across the four images for each egg, which we termed average spot profile. We then calculated the absolute difference in spot profile between the blunt and apex ends for all eggs, to have a consistent measure of within-egg end-to-end variation. We used the absolute spot profile difference for both the cowbird model egg and host egg. Finally, we subtracted the model cowbird egg's absolute difference in within-egg spot profile variation from that of the host egg, producing either a positive or negative value. As such, positive value signified that the host egg had greater spot profile variation

TABLE 1 | Summary of CORT and egg visual signals measurements organised by treatment.

Factor	Egg added		Control
	Accepted	Clutch abandoned (burial or desertion)	
Plasma CORT	19	6	15
Feather CORT	16	5	8
Egg visual signals	12	3	NA

"Accepted" are experimentally parasitised clutches accepted by the warbler, "Clutch abandoned" are those that were either buried or deserted, and "Control" are the nests monitored without being experimentally parasitised.

than the cowbird model egg and negative values indicated there was less variation.

Statistical Analysis

All statistical analyses were conducted on R Studio (2021) with R Core Team (2020). Data were categorised into three treatment groups; controls, accepters, and abandoners, as detailed above. All graphs were constructed using "*ggplot2*" (Wickham, 2016) and "*ggsignif*" (Ahlmann-Eltze and Patil, 2019) packages for R. All data are presented as mean ± SE.

We took a two-analysis approach to explore the effects of experimental parasitism, testing whether egg addition was associated with elevated CORT and then testing whether egg addition and abandonment combined were associated with elevated CORT compared to controls. We reasoned that combining accepters and abandoners in a single experimental group would result in an inflated variance. Similarly, the statistical power for the comparison between accepters and abandoners would be low, and therefore we would expect lower ability to detect a difference if it existed. Before analysis, circulating CORT data were normalised using a log-transformation. There was one unusually small outlier that we corrected to one, which was still the lowest data point, to avoid deformations in the log-transformation. We had *a priori* directional predictions for (1a) and (1b), expecting higher CORT levels in experimental treatments compared to controls. As such, we used one-tailed Student's *t*-test (using the *"t.test"* function) to detect differences between groups. To test the prediction that egg addition alone would result in higher CORT in accepters than in controls (1a), we excluded the outcome of clutch abandonment. To test the prediction that circulating CORT was associated with clutch abandonment (1b), abandoners were compared to controls. Because variance was similar between accepters and controls, but about half for abandoners versus controls, we ran *t*-tests with homogenous versus heterogeneous variances for predictions (1a) and (1b), respectively.

Length-normalised feather CORT data was subsequently log-transformed to meet the assumption of normality. Levene's test was used to test for variance between the treatment groups using *"leveneTest"* function of the *"car"* package (Fox and Weisberg, 2019). To investigate the relationship between accepted, abandoned and controls clutches we used a generalised linear model using the "*glm*" function. If significant, we planned to do *post hoc* tests between all three groups to test predictions 2a and 2b.

For egg visual signals, analyses were conducted using only eggs from experimentally parasitised nests, where the warblers would have been confronted with the sight of the model cowbird eggs. We ran two linear models using the "*lm*" function: one associating corrected log-CORT with difference in within-egg maculation coverage variation, and a second pairing corrected log-CORT and within-egg spot profile variation between the host egg and the model cowbird eggs. Additionally, we used one-tailed Student's two-sample *t*-tests ("*t.test*" function in R) to determine whether maculation coverage and spot profile variation were greater among warblers that abandoned their experimentally parasitised clutch relative to those that accepted.

RESULTS

Our sample consisted of a total of 40 yellow warbler nests, with 25 experimentally parasitised and 15 control nests (**Table 1**). Within the experimentally parasitised group, 19 females accepted the experimental parasitism (76%), and 6 abandoned their parasitised clutch (24%) through either desertion ($n = 4$) or burial ($n = 2$).

Circulating Plasma Corticosterone

A total of 40 circulating CORT samples were collected (**Table 1**). Circulating CORT levels of accepters were not different than controls ($t = 0.45$, df = 33, $p = 0.33$; **Figure 1A**). In contrast, circulating CORT levels of abandoners were significantly higher than controls ($t = 1.96$, df = 15, $p = 0.03$; **Figure 1B**).

Feather Corticosterone

Feather samples from 29 individuals were collected (**Table 1**). Variances were similar across treatments ($F_{2,26} = 0.43$, $p = 0.65$; **Figure 2**). Mean feather CORT concentrations were similar among treatment groups ($F_{2,26} = 0.11$, $p = 0.89$; **Figure 2**); as such, *post hoc* tests were not conducted.

Egg Visual Signals

In total, 22 warbler eggs were analysed (**Table 1**). The model cowbird egg had an end-to-end maculation coverage variation of 36.6% and an end-to-end spot profile variation ratio of 0.04. Contrastingly, the warbler eggs had an average end-to-end maculation coverage variation of 33.3 ± 2.32% and an average end-to-end spot profile variation ratio of 0.16 ± 0.07.

Circulating CORT levels were not associated with differences in within-egg maculation coverage variation between cowbird model and host eggs ($R^2 = -0.05$, $p = 0.44$; **Figure 3A**), nor were they associated with spot profile ($R^2 = 0.09$, $p = 0.27$; **Figure 3B**). Separating the correlation between treatments (accepted versus abandoned) did not change the results. Further, differences in maculation coverage (df = 13, $t = -0.43$, $p = 0.66$) and spot profile (df = 13, $t = -0.01$, $p = 0.50$) variation between eggs were not greater in abandoners compared to accepters (**Figures 4A,B**).

DISCUSSION

In this study, we examined mechanisms associated with clutch abandonment in a frequently parasitised host of the brown-headed cowbird, the yellow warbler, which regularly rejects naturally parasitised clutches via egg burial and nest desertion two forms of clutch abandonment (Sealy, 1995). Circulating CORT levels were higher in females who abandoned their parasitised clutches, either by egg burial or nest desertion, relative to females at control nests in which no model cowbird eggs were added (**Figure 1B**). Simply adding model cowbird eggs to clutches did not increase circulating CORT, as females that accepted cowbird eggs did not have elevated CORT relative to controls (**Figure 1A**). In addition, past stress, as approximated by feather CORT, did not predict whether females accepted or abandoned experimentally parasitised clutches (**Figure 2**). Finally, differences in egg visual signals, as measured by

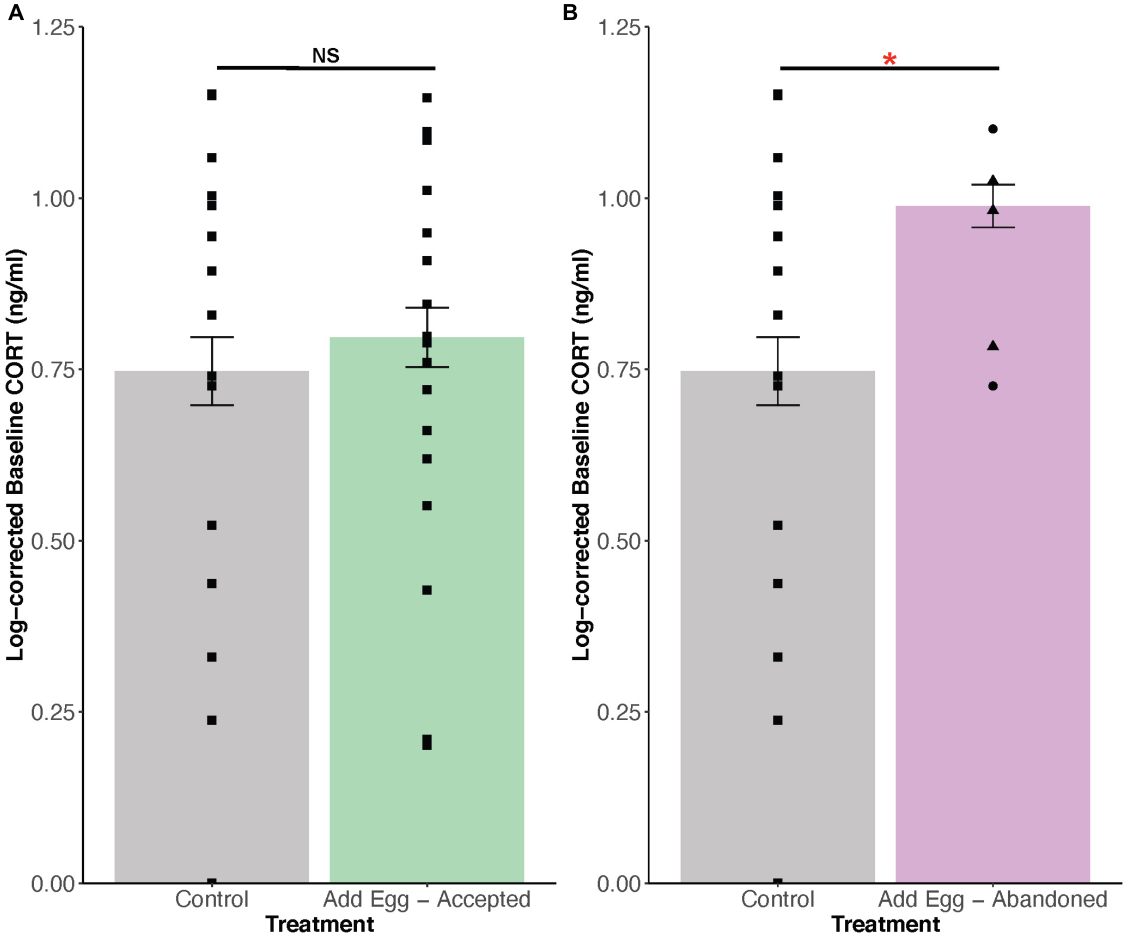

FIGURE 1 | (A,B) Comparisons in log-corrected, circulating corticosterone (CORT) levels in plasma of controls (no model eggs added to clutches) and two experimental treatments (model eggs added). Among individuals with experimentally manipulated clutches that abandoned, abandoners by desertion are indicated with triangles, while burials are indicated with circles. **(A)** Individuals who accepted the cowbird model eggs did not have significantly higher circulating CORT than controls ($t = 0.45$, df = 33, $p = 0.33$). **(B)** In contrast, individuals who abandoned their experimentally parasitised clutches has higher circulating CORT than controls ($t = 1.96$, df = 14.77, $p = 0.03$). NS and * indicate "not significant" and $p < 0.05$, respectively. Mean ± SE.

differences in maculation coverage and spot profile variation between the model cowbird egg and host eggs, did not predict abandonment nor were they correlated with circulating CORT levels of females who abandoned their nests (**Figures 3**, **4**).

Several explanations for the increased CORT in abandoners relative to controls are possible. First, females with naturally higher circulating CORT may be more likely to abandon their nests, such that the presence of a parasitic egg might not alter host CORT levels. That is, individuals with high CORT are more predisposed to abandon for any reason, and the presence of a parasitic egg may tip them past the threshold of abandonment. Second, the presence of a parasitic egg might increase CORT, but only in some females, which then abandon the nest. Third, a parasitic egg might increase CORT in all females, but females whose CORT levels exceed a certain threshold may be more likely to abandon the nest than those whose CORT remains below the threshold. Fully resolving these differences would require a study design in which blood samples were collected before and after parasitism, preferably coupled with experimental manipulations of CORT via injections or implants. These approaches require capturing females multiple times during laying, which may substantially increase abandonment, thereby overwhelming any experimental effect due to experimental parasitism. Our data provides information to target appropriate hormone concentrations for future implant studies for testing these ideas.

Circulating Corticosterone (Plasma)

Circulating glucocorticoids, such as CORT, vary in response to environmental stressors (Siegel, 1980; Wingfield, 2003). In our study, females who abandoned their parasitised clutch had higher circulating CORT, relative to females with control clutches that were not parasitised (**Figure 1B**). However, circulating CORT did not differ between control and accepter females. Females were sampled 3 days (laying day 4) after the clutch manipulation (laying day 1), suggesting that abandoners in the population

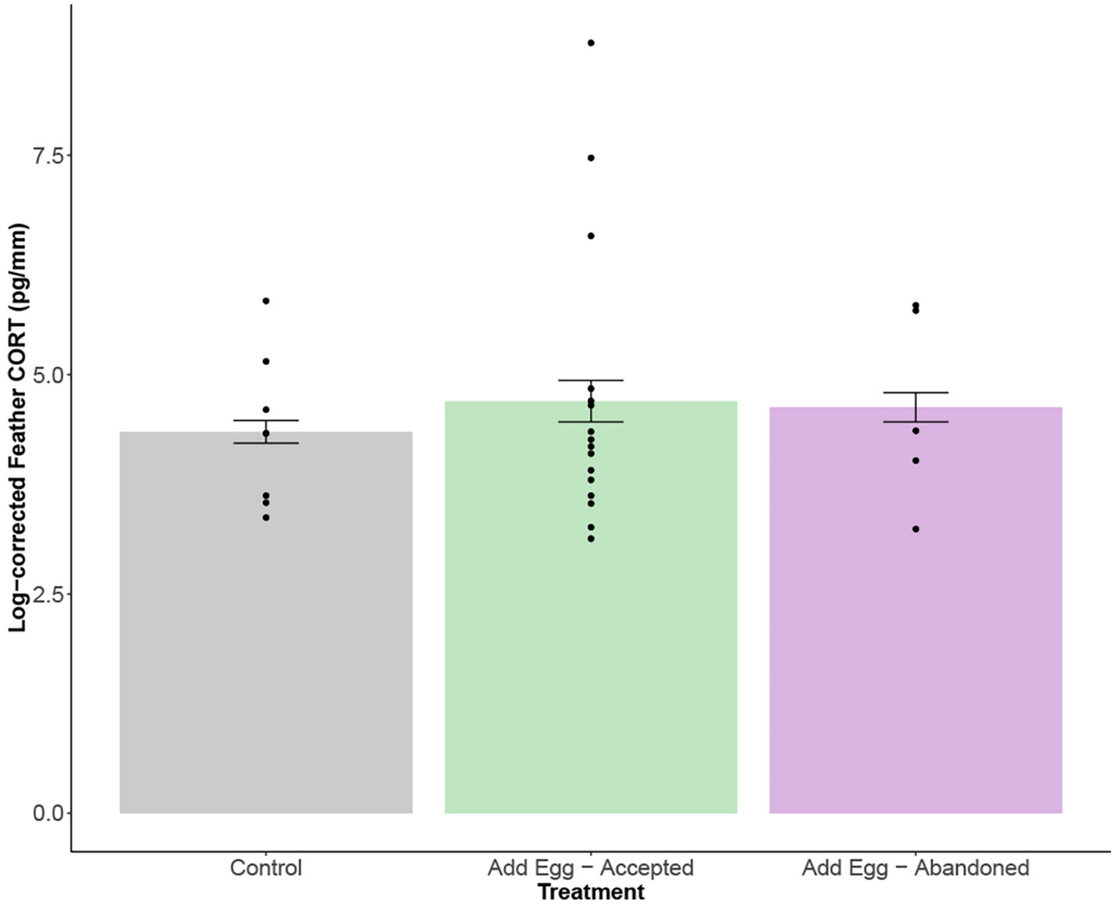

FIGURE 2 | Log-corrected feather corticosterone (CORT) levels (pg/ml) in feathers of females across the three experimental groups. There was no significant difference in the variance (*p* = 0.65) or the mean (*p* = 0.89) between groups. A. $F_{2,26} = 0.43$, $p = 0.65$; B. $F_{2,26} = 0.11$, $p = 0.89$.

may have had a more pronounced endocrine response than accepters (**Figure 1A**). Given that clutch abandonment is a generalised response, similar increases in circulating CORT occur in association with other abandonment-triggering factors, such as inclement weather (Romero et al., 2000; Thierry et al., 2013). Therefore, circulating CORT could provide us with a broader understanding of how environmental stressors, whether biotic or abiotic, affect nest abandonment in birds.

Brood parasitism *per se* does not appear to affect circulating CORT levels in female yellow warblers. Our results show that simply adding model cowbird eggs, without the host abandoning the clutch, was not associated with higher circulating CORT relative to control females. The absence of a difference between controls and accepters, contrary to our prediction, suggests that females who did not abandon experienced a comparable level of stress compared to unparasitised (control) individuals, a finding that differs from previous research. For instance, a common cuckoo host, the Eurasian blackbird, experienced increased circulating CORT following experimental parasitism (Ruiz-Raya et al., 2018). The difference in findings could depend on several factors including parasitism rates, predation rates, breeding season length, and other life history traits (Bókony et al., 2009). The fact that our study focused on a species with a variable abandonment response to parasitism may explain the different pattern of circulating CORT variation that we observed compared to species with more consistent responses such as Eurasian blackbirds (Ruiz-Raya et al., 2018). Our results are in line with the findings of Abolins-Abols and Hauber (2020), who found a causal link between stress-induced CORT and egg ejection rate in American robins, an egg ejecter species. Together, these findings suggest circulating CORT may mediate antiparasitic responses across different parasite-host systems and across rejection methods (Ibáñez-Álamo et al., 2012; Mark and Rubenstein, 2013; Schoenle et al., 2017).

Past Corticosterone (Feathers)

Feather CORT, in contrast to circulating plasma CORT, indicates a long-term past "stress" history (Bortolotti et al., 2008), integrating levels of circulating CORT across the moult period. Our predictions (2a and 2b) that abandoners and accepters would have higher and lower past CORT than controls, respectively, focused on whether an individual's prior state could predict a response to the future stressor of experimental parasitism. As such, these predictions resemble those of the developmental

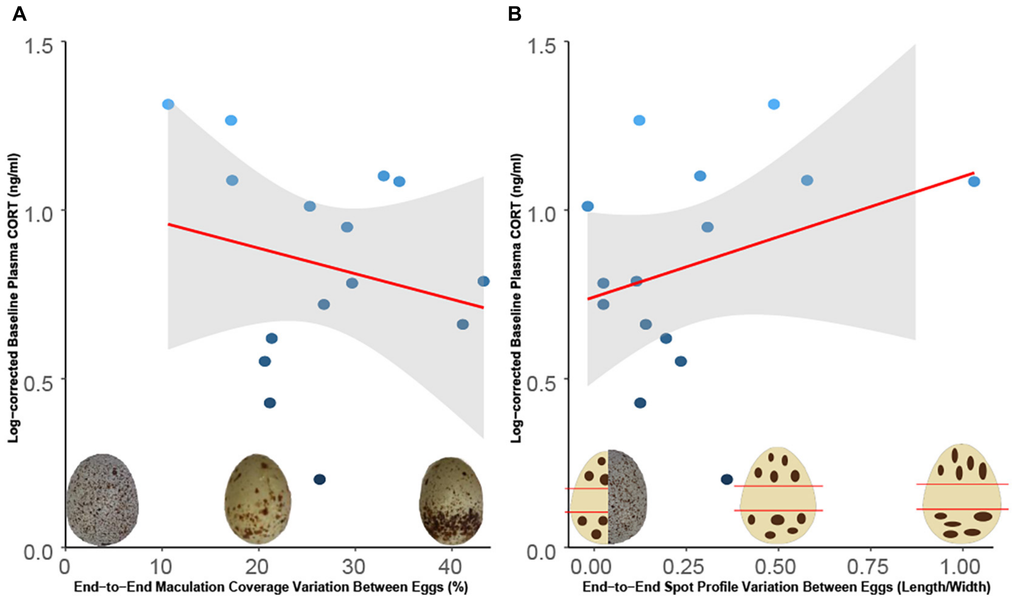

FIGURE 3 | (A,B) Correlation between log-corrected circulating CORT levels from individual female warblers and increasing variation in **(A)** maculation coverage (p = 0.44) and **(B)** spot profile (p = 0.27) of their eggs relative to the model cowbird egg. The shaded area indicates 95% confidence interval of the mean slope. A. R^2 = −0.05, B. R^2 = 0.09

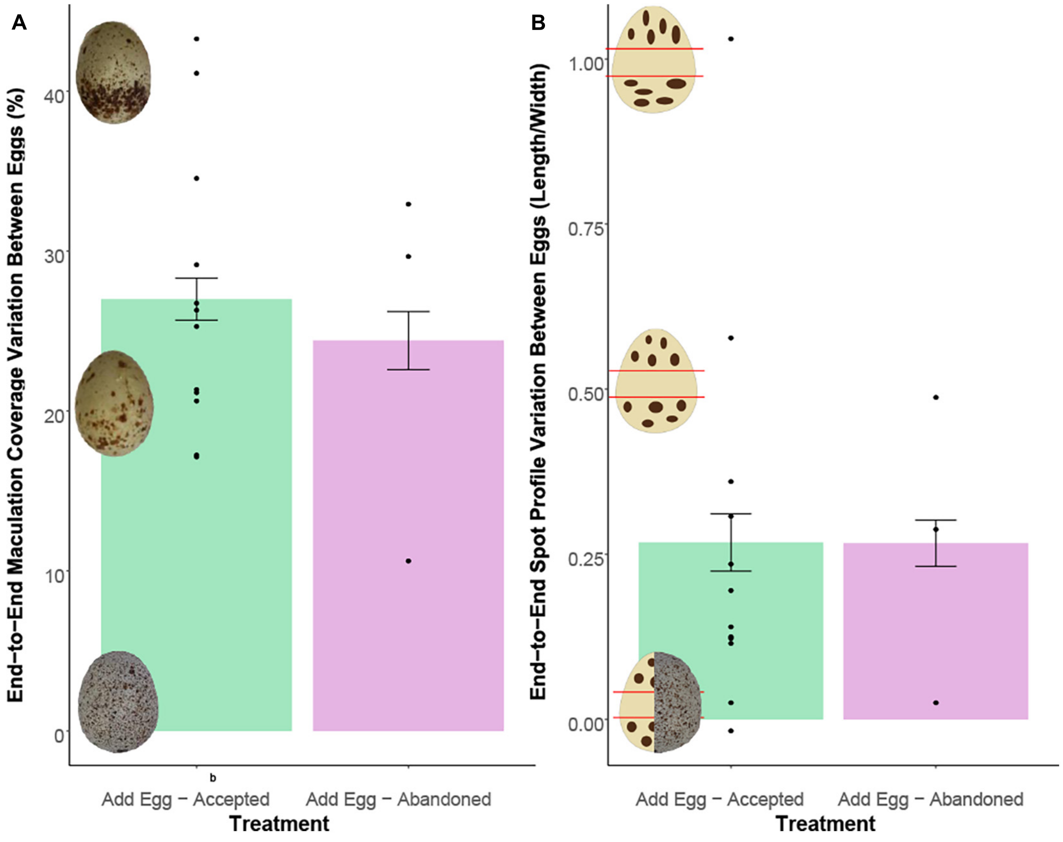

FIGURE 4 | (A,B) Females who abandoned their experimentally parasitised clutch did not have eggs that varied more in **(A)** maculation coverage (p = 0.44) or **(B)** spot profile (p = 0.27) relative to the model cowbird egg.

stress hypothesis, which proposes that stress early in life (e.g., nestling nutritional state) can affect behaviour and cognition later in life (e.g., adult song quality). Although this hypothesis focused primarily on song quality and nutrition (Nowicki et al., 2002), it was later applied to broader contexts including other sexually selected traits (Spencer and MacDougall, 2011). We proposed a similar reasoning to explain why abandoners and accepters co-exist in some host populations, including yellow warblers (Sealy, 1995; prediction 2a). However, feather CORT was not associated with the host's response to the model cowbird eggs (**Figure 2**). In addition, variance in feather CORT among the three treatment groups (control, accepted, abandoned) was similar (**Figure 2**). These results contrast with our predictions that control individuals, who did not experience the stress of experimental parasitism, would display more variable stress histories and that experimental females would respond to parasitism according to their past stress histories (Bortolotti et al., 2008), thereby splitting the experimental parasitism groups into accepters and abandoners. Thus, feather CORT levels of female warblers did not predict responses to parasitism. This lack of an effect could occur if stress during moult was not high enough to induce carry-over effects (O'Connor et al., 2014). Overall, these results suggest that past stress, as measured through feather CORT, does not predict abandonment of parasitised clutches in yellow warblers within this study. Nonetheless, feather CORT only provides a proxy of CORT levels near the time of moult, and warblers may be responding to other time windows, such that an additional study is warranted.

Egg Visual Signals

The role of visual pattern differences in egg recognition has been widely established in other species, primarily in ejecters (Cassey et al., 2008; Spottiswoode and Stevens, 2010; Honza and Cherry, 2017; Hanley et al., 2019). In response to increased egg recognition by hosts, some specialist brood parasites, such as common cuckoos, have evolved eggs that mimic the colour and maculation of their host (Davies and Brooke, 1989a,b; Kilner, 2006; Kilner and Langmore, 2011; Spottiswoode and Stevens, 2011; Thorogood et al., 2019). However, in generalist brood parasites such as brown-headed cowbirds, egg visual signals differ from hosts to a greater extent compared to specialist parasites, and thus, the signals may be more noticeable and reduce the opportunity for the co-evolution of egg mimicry (Davies, 2000; Krüger, 2007). For instance, many cowbird hosts lay eggs that differ to varying degrees in colour and/or maculation from cowbird eggs, like those of yellow warblers (Abernathy and Peer, 2014). Warblers in our study had greater intra-egg maculation coverage and spot profile variation than the model cowbird egg (**Figures 3**, **4**), and females could have potentially used this egg visual signal as a cue to abandon their parasitised clutch. Indeed, yellow warblers can determine their clutch has been manipulated using both tactile and visual cues (Guigueno and Sealy, 2012), but, prior to this study, the extent of differences in maculation were not quantitatively analysed.

Visual recognition of parasitism via differences in maculation could modulate CORT changes. As such, we examined whether differences in maculation and spot profile variation between warbler eggs and our model cowbird eggs correlated with circulating CORT (prediction 3a, **Figure 3**). Increased differences in visual signals could be associated with increases in circulating CORT and a decrease in parental care (Angelier et al., 2009). However, circulating CORT was not correlated with differences in maculation characteristics (**Figure 3**). Perhaps differences between cowbird and yellow warbler egg maculation were simply not large enough to stimulate a response. Indeed, some yellow warbler eggs look nearly identical to brown-headed cowbird eggs in maculation characteristics, and anti-parasitic behaviours in yellow warblers may be mediated primarily through tactile, rather than visual, cues (Guigueno and Sealy, 2009, 2012; Guigueno et al., 2014). Yet another possibility is that individuals that rejected experimental parasitism had higher baseline CORT, although we might still expect that abandonment would be associated with an interaction between CORT levels and visual signals.

Differences in egg maculation characteristics could be an important cue in eliciting egg rejection in brood-parasitic hosts, as has been shown in ejecters, but the same may hold true for clutch abandoners. Thus, we also examined whether differences in maculation characteristic variation predicted whether hosts accepted or abandoned their experimentally parasitised clutches (prediction 3b, **Figure 4**). However, abandoners did not show greater variation in maculation coverage or spot profile between their eggs and the model cowbird eggs, relative to accepters (**Figure 4**). Although yellow warblers from a previous study gazed longer at manipulated clutches, this increased gaze time only occurred between eggs that differed in both background colour and maculation (i.e., blue vs. off-white; brown maculation vs. none; Guigueno and Sealy, 2012). In addition, tactile cues, as measured by the warblers' egg probing and body shifting during incubation, had a stronger effect than visual cues (Guigueno and Sealy, 2012). To abandon a clutch, females simply need to recognise they have been parasitised and use other cues such as tactile recognition of volumetric differences between the larger parasitic egg and the smaller host eggs (Guigueno and Sealy, 2012) and interaction with the adult parasite (Guigueno and Sealy, 2011). In sum, yellow warblers, as with other hosts that use clutch abandonment as an antiparasitic defence, seem to rely less on visual signals than egg-ejecting hosts to distinguish between their own eggs and the parasitic egg.

CONCLUSION AND FUTURE RESEARCH

We explored the effects of current (plasma) CORT, past (feathers) CORT, and differences in egg maculation variation in the context of clutch abandonment in parasitised yellow warblers. We found evidence that current stress, as approximated by circulating CORT in plasma, was associated with clutch abandonment, but we did not find that maculation or past stress played a role. To further explore the action of CORT, future studies could experimentally increase CORT to determine whether this manipulation elicits abandonment by female warblers. This approach would be opposite of that of Abolins-Abols and Hauber (2020)'s, in which authors decreased ejection in an American robin (*Turdus migratorius*) by experimentally decreasing CORT synthesis with mitotane injections. Robins normally eject at a high rate (i.e., ∼ 90%; Croston and Hauber, 2014), so decreasing CORT would result in more individuals accepting cowbird eggs. In contrast, yellow warblers normally abandon 20–30% of experimentally parasitised clutches, but up to about 44% of naturally parasitised clutches (Sealy, 1995; Guigueno and Sealy, 2011), possibly because females interact with the adult parasite (Guigueno and Sealy, 2011). As such, in yellow warblers, increasing circulating corticosterone, via time-release pellets or silastic implants (Torres-Medina et al., 2018), for example, would potentially result in a larger number of abandoners than observed in our current study (i.e., a larger sample size) and would allow for causal, as opposed to correlational, conclusions. Experimentally increasing rates of abandonment through hormone manipulations would still reflect rates seen naturally, as cowbird parasitism and associated abandonment rates vary greatly geographically in yellow warblers (Kuehn, 2009). In sum, we conclude that circulating CORT was associated with abandonment of experimentally parasitised clutches and advocate for follow-up studies that manipulate hormone levels to explore causal mechanisms.

In addition to manipulative studies, more research is needed to understand the role of other hormones and life history in mediating responses to brood parasitism. Hormones other than or in addition to CORT could influence clutch abandonment. Testosterone, progesterone, prolactin, and mesotocin may all play roles in abandonment of parasitised clutches, although this has primarily been examined in hosts that eject cowbird or cuckoo eggs, such as American robins and Eurasian blackbirds, respectively (Abolins-Abols and Hauber, 2018; Ruiz-Raya et al., 2020). In addition, stress is a mediator of life-history trade-offs (Bókony et al., 2009; Bonier et al., 2009; Abolins-Abols and Hauber, 2018), and it would be valuable to incorporate parasitism status and sympatry with cowbirds in a large-scale analysis to examine interactions between hormones, life history traits, and brood parasitism. There is a great opportunity for future research to take a comprehensive endocrine approach in examining the costliest form of rejection in brood-parasitic hosts, clutch abandonment.

This study provides initial findings pertaining to the endocrine correlates of clutch abandonment in brood-parasitic hosts and confirms that egg visual signals play a less important role in eliciting abandonment than tactile cues. The yellow warbler-cowbird system provides a strong model to address mechanistic questions of clutch abandonment, and our research invites future experimental studies incorporating additional species that abandon clutches from diverse host-parasite systems. Our study, along with the proposed follow-up studies, will contribute to a comprehensive understanding of the intricate links between brood parasitism, hormones, and clutch abandonment, and the fitness-related trade-offs between incurring versus avoiding costs associated with brood parasitism and clutch abandonment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by this project was approved and conducted under the Banding Permit no. 23665, United States Fishes and Wildlife Service permit MB72878C-0 and Institutional Animal Care and Use Committee protocol # 19-05-02, all issued to SG.

AUTHOR CONTRIBUTIONS

MG, SG, and AT-VR developed the study design. MG, AT-VR, CP, SG, and JS conducted the data collection and field work. CP, CAP, SG, and JS completed the sample analysis. AT-VR and MG did statistical analysis. AT-VR, CP, and MG wrote the first draft of the manuscript. SG and CP did additional writing, with revisions provided by all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.711732/full#supplementary-material

Supplementary Figure 1 | (A–F) The stages of the experiment conducted on yellow warbler nests near Kalamazoo, Michigan, from initial completion of building of the nest to laying day 4. **(A)** A completed nest in the final building stage; **(B)** Laying day 1, when we artificially parasitised the clutch; **(C)** A nest after we added two model cowbird eggs to the clutch on laying day 1; **(D)** Laying day 2; **(E)** Laying day 3; **(F)** Laying day 4, days of host blood and feather sampling.

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