THE DEVELOPMENT AND FITNESS CONSEQUENCES OF SEX ROLES

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PUBLISHED IN: Frontiers in Ecology and Evolution

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-016-9
DOI 10.3389/978-2-88976-016-9

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THE DEVELOPMENT AND FITNESS CONSEQUENCES OF SEX ROLES

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Citation: Pogány, Á., Krause, E. T., Bókony, V., Roth, O., eds. (2022). The Development and Fitness Consequences of Sex Roles. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-016-9

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EDITORIAL
published: 05 May 2022
doi: 10.3389/fevo.2022.912520

Editorial: The Development and Fitness Consequences of Sex Roles

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Keywords: early development, sex ratio, sex reversal, cognitive mate choice, breeding system, parental behaviour

Editorial on the Research Topic

The Development and Fitness Consequences of Sex Roles

INTRODUCTION

In most sexually reproducing species, males and females are characterised by differences in reproductive behaviours and life-history strategy (Kokko et al., 2006; Fairbairn, 2013). Exploring the origin and consequences of sex roles are key questions in evolutionary biology, yet we are far from understanding their developmental mechanisms and adaptive significance. We aim to highlight this research field and broaden the range of taxa and phenomena investigated in relation to sex roles.

SEX-SPECIFIC LIFE HISTORIES, SEX RATIOS, AND SEX REVERSAL

A major driver of the evolution of sex roles is adult sex ratio (Liker et al., 2013; Kappeler, 2017). Avian studies suggested sex-ratio variation to stem from sex differences in juvenile and adult mortality, and maturation times (Székely et al., 2014; Ancona et al., 2020). Recently, however, a more complex picture developed, emphasising early-life processes and environmental effects, which proved to be a popular subject in the Research Topic with three articles. Furthermore, sex-specific life-history strategies can influence the time and probability of dispersal from the natal territory (Emlen, 1982; Li and Kokko, 2019), or the adjustment of parental investment in response to the partner's effort in biparental species (Houston et al., 2005); two further research papers focus on these strategic questions in our collection.

Nusbaumer et al. investigate expression of sex-specific life-history in early life stages. In a stressful, polluted environment, female lake char (*Salvelinus umbla*) hatch later and are more likely to die than male embryos, indicating faster growth rates and higher resistance to pollution in males during embryo development. This implies that sex-specific life-history strategies, population sex ratios and divergent sex roles can be a result of environmental challenges impacting the earliest life stages.

Lambert et al. investigate the relative importance of sex-specific larval mortality and sex reversal in green frogs (*Rana clamitans*). With advancing stages, the genetic sex ratios shift towards females, implying male-biassed mortality. Sex reversal in both directions also contributes to the phenotypic

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *04 April 2022*
Accepted: *26 April 2022*
Published: *05 May 2022*

Citation:
Pogány Á, Krause ET, Roth O and Bókony V (2022) Editorial: The Development and Fitness Consequences of Sex Roles. Front. Ecol. Evol. 10:912520. doi: 10.3389/fevo.2022.912520

sex ratios at metamorphosis. Female-to-male sex reversal is the highest in the pond with the most acidic pH and lowest dissolved oxygen levels. Although the effects are small, they suggest that population sex ratios may be influenced by early-life sex differences in survival and sex reversal due to environmental stressors.

In agile frogs (*Rana dalmatina*), another species with sex reversal, Bókony et al. investigate whether sex reversal is associated with life-history and early behavioural differences. Of 16 variables related to growth, development, larval foraging, and risk-taking behaviours, they find only a few differences between spontaneously sex-reversed and sex-concordant individuals, with no consistent support for either higher or lower fitness prospects to sex-reversed individuals. In light of their earlier findings on reduced fitness when sex reversal was triggered by heat stress, they formulate a new hypothesis that the fitness consequences of sex reversal may depend on its aetiology.

Josi et al. investigate the costs and benefits associated with sex- and life-history- specific dispersal in the cooperatively breeding cichlid *Neolamprologus savoryi*. Focusing on growth rates and survival, they identify distinct sex-specific routes to independent breeding. The study concludes that differences in dispersal decisions between and within the sexes are tightly linked to divergent life-history trajectories, including different growth rates, the age at obtaining breeder status, and survival.

Wang et al. investigate in burying beetles (*Nicrophorus vespilloides*) how parents change their effort and trade-off current and future reproductive investments when parental effort by their mate and brood reproductive value change simultaneously. They show that males compensate for loss of female care regardless of brood size, and this negatively affects their subsequent parental investment. Female compensation, however, depends on brood size, with compensation only at small broods. These findings suggest sex differences in the adjustment of reproductive allocation and sex-dependent reproductive trade-offs between current and future reproduction.

BREEDING SYSTEM AND PARENTAL SEX ROLES

Providing care, especially food provisioning, is among the most important and costly contributions of parents to realise and enhance fitness (Clutton-Brock, 1991; Kokko and Jennions, 2008; Royle et al., 2012). Three articles in our collection investigate the origin and consequences of parental sex role differences and their potential influence on breeding system evolution.

Fresneau et al. report their observations on courtship behaviour, mating competition, polygamy, and male-only care for offspring in pheasant-tailed jacanas (*Hydrophasianus chirurgus*), a species with sex-role reversal. While polyandry in females is close to the expected level (ca. 82%), males (with ca. 5% polygyny) perform more courtship displays and females participate partly in brood care. The study of Fresneau et al. is a useful contribution to understand the gradual evolution of sex-role reversal in this species and in general.

Ratz et al. using burying beetles (*Nicrophorus vespilloides*) as models, manipulate resource availability to investigate its potential role in the emergence of biparental care. They find that duration of male parental care is increased with more access to resources, whereas female care is independent of the manipulation. Thus, sex differences in parenting and the level of parental cooperation depends on environmental conditions.

Applying a full cross-fostering experimental design, Morvai et al. disentangle genetic and social parental effects in the ontogeny of parental sex roles in zebra finches (*Taeniopygia guttata*). By comparing incubation and offspring provisioning between the fostered second generation and the genetic/social parents, the authors report weak context-dependent genetic, social, and non-social environmental effects. They conclude the strongest and most consistent effect is the social effect by the current mate and highlight the importance of parental negotiation in explaining individual variation of parental sex roles in biparental species.

COGNITIVE MATE CHOICE IN VERTEBRATES

Selection on cognitive abilities might be different in the two sexes. Sex-specific behaviours may rely on cognitive skills to varying degrees, and mate choice and courtship behaviour may also involve cognition, contributing to sexual dimorphism in various cognitive abilities (Jacobs, 1996; Lindenfors et al., 2007). In a review paper, Fuss (a) provides support for the existence of cognitive sex differences, and the potential influence of cognition on mate choice decisions. The paper overviews evidence for context-dependent cognitive sex differences in mammals, birds, and fish. There is also strong evidence for cognitive abilities and learned elements in male display behaviours to influence individual mate choice decisions. Answering the question of how and to what extent individuals use their own cognitive skills to assess those of their prospective partners proves to be challenging. The author suggests methodological improvements for future test paradigms.

In another paper Fuss (b) reviews the neuronal prerequisites that support the triad of mate choice, sex roles and sexual cognition. Empirical research based on different methods reveal convincingly that the sex and the sex roles within the prevalent mating system are mirrored at neuronal levels in individual brain regions. Our knowledge so far relies on a confined set of networks and selection of brain regions. The author suggests behavioural studies should be combined with state-of-the-art neuroanatomical techniques to establish causal relationships and interactions between observed behavioural patterns and underlying mechanisms.

CONCLUSIONS

Contributions to this Research Topic reflect renewed interest and diversifying approaches to understand the ontogeny and evolution of sex roles. Sex differences in life history and mortality in early ontogenetic stages are increasingly identified as important drivers of population sex ratios and thereby of sex roles, especially in taxa where developing young are directly exposed to the environment. Focusing on environmentally induced sex reversal offers a powerful approach for studying the genetic and developmental determinants of sex roles. Ample empirical evidence supports the role of cognition in mate choice, however, understanding the interplay of cognitive sex differences, sex roles, cognitive mate choice and its neuronal prerequisites needs further research. We encourage further work on diverse taxa to facilitate a comprehensive understanding of proximate and ultimate drivers of various sex roles manifesting in different breeding systems.

AUTHOR CONTRIBUTIONS

All authors contributed to writing and editing the article, approved the submitted version, and administered the Research Topic collection.

FUNDING

ÁP was supported by the Hungarian Ethology Foundation. VB was supported by the National Research, Development and Innovation Office of Hungary (K135016), the János Bolyai Research Scholarship of the Hungarian Academy of Sciences, and by the New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund (ÚNKP-21-5). OR was supported by the European Research Council (ERC) under the European Union's Horizon Research and Innovation Program (MALEPREG: eu-repo/grantAgree-ment/EC/H2020/755659) and by the German Research Foundation (RO-4628/4-2).

ACKNOWLEDGMENTS

We thank all authors and reviewers for their contribution to this Research Topic, and the staff at Frontiers in Ecology and Evolution for their professional administrative support throughout the process.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 20 July 2021
doi: 10.3389/fevo.2021.712425

Access to Resources Shapes Sex Differences Between Caring Parents

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In species where both parents cooperate to care for their joint offspring, one sex often provides more care than the other. The magnitude of such sex differences often varies both between and within species and may depend on environmental conditions, such as access to resources, predation risk and interspecific competition. Here we investigated the impact of one such environmental variable – access to resources for breeding – on the magnitude of sex differences in parental care in the burying beetle *Nicrophorus vespilloides*. This species breeds on the carcasses of small vertebrates, which are the sole food source for parents and offspring during breeding. We manipulated access to resources by providing pairs with mouse carcasses from a broad mass range (3.65–26.15 g). We then monitored subsequent effects on the duration and amount of care provided by males and females, male and female food consumption and weight change during breeding, and larval traits related to offspring performance. We found that males increased their duration of care as carcass mass increased, while females remained with the brood until it had completed its development irrespective of carcass mass. There were thus more pronounced sex differences in parental care when parents had access to fewer resources for breeding. Overall, our findings show that sex differences between caring parents vary depending on access to resources during breeding. The finding that males extended their duration of care on larger carcasses suggests that access to more resources leads to a shift toward more cooperation between caring parents.

Keywords: behavioural plasticity, biparental cooperation, parental care, environmental variation, *Nicrophorus vespilloides*

Edited by:
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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 20 May 2021
Accepted: 29 June 2021
Published: 20 July 2021

Citation:
Ratz T, Kremi K, Leissle L,
Richardson J and Smiseth PT (2021)
Access to Resources Shapes Sex
Differences Between Caring Parents.
Front. Ecol. Evol. 9:712425.
doi: 10.3389/fevo.2021.712425

INTRODUCTION

Biparental care occurs when male and female parents cooperate to care for their joint offspring. It is the predominant pattern of care in birds (Cockburn, 2006) but has also evolved in a small number of mammals, amphibians, fishes, and arthropods (Balshine, 2012; Trumbo, 2012). Biparental care is often associated with sex differences in the amount or duration of care with females usually making greater contributions than males (Kokko and Jennions, 2012; West and Capellini, 2016). For example, females provide more care than males in red-winged blackbirds (Whittingham, 1989), house sparrows (Schwagmeyer et al., 2008), oldfield mice (Margulis, 1998), convict cichlids (Lavery and Keenleyside, 1990), and burying beetles of the genus *Nicrophorus*

(Smiseth and Moore, 2004; Trumbo, 2007). Such sex differences in care reflect differences between males and females in the benefits and/or costs of care. For example, in the cichlid fish *Herotilapia multispinosa*, where males desert the brood earlier than females, males presumably incur higher costs of care because they can mate with a new partner quicker, and thus lose more mating opportunities than females when continuing to provide care (Keenleyside, 1983). The magnitude of such sex differences varies both between and within species, and this variation would depend on environmental conditions that have a differential impact on the costs and/or benefits of care to males and females. For example, prior work shows that the magnitude of sex differences in parental care varies with ambient temperatures (e.g., Vincze et al., 2013) or the intensity of interspecific competition (e.g., Hopwood et al., 2015). Variation in access to resources is likely to be a key environmental condition in this respect given that such variation may have a differential impact on the benefits and/or costs of care to males and females (e.g., Eldegard and Sonerud, 2010). In order to advance our understanding of variation in the magnitude of sex differences in parental care, there is now a need for experiments that manipulate access to resources and then monitor effects on male and female care.

Access to resources may also impact on sexual conflict between parents over parental care (Lessells, 2012). Sexual conflict arises because the benefits in terms of enhanced offspring fitness result from the combined effort of the two parents, whilst the costs in terms of reduced future survival and reproduction depend on each parent's personal effort (Trivers, 1972; Chase, 1980). As such, biparental care involves a balance between cooperation and conflict, and any shift in this balance could be detected as a change in the frequency and/or duration of biparental care (Westneat and Sargent, 1996; Lessells and McNamara, 2012; Johnstone and Savage, 2019). Greater access to resources may reduce the benefits of biparental cooperation in species where parents provision food to the offspring. When food is abundant, females can provision more food to the brood on their own, thereby reducing the benefits to males from assisting females (Crook, 1963; Leisler et al., 2002; Barve and La Sorte, 2016). Yet, on the other hand, greater access to food may increase the benefits of biparental cooperation in species where parents protect the offspring from predators or conspecific intruders. For example, if greater access to food increases the risk of nest predation or infanticide by conspecific intruders (e.g., Wilson and Fudge, 1984; Robertson, 1993), there may be an increase in the benefits to the male from assisting the female when food is more abundant. Thus, experiments that manipulate access to resources should also monitor effects on the frequency and/or duration of biparental care relative to uniparental care.

We used the burying beetle *Nicrophorus vespilloides* to investigate how availability of resources alters the magnitude of sex differences in care and shifts the balance between cooperation and conflict. Burying beetles of the genus *Nicrophorus* are ideal to address these issues because they breed on carcasses of small vertebrates that vary considerably in mass (Müller et al., 1990; Smiseth and Moore, 2002). The vertebrate carcass used for breeding is the sole source of food for both developing larvae and caring parents (Scott and Traniello, 1990; Scott, 1998; Pilakouta et al., 2016). Thus, it is straightforward to manipulate the availability of resources by simply providing parents with carcasses of variable masses (Smiseth et al., 2014). Unlike birds where two parents can supply more food to the brood than a single parent, the supply of food in burying beetles is limited by the size of the carcass and should not be dependent on the number of parents attending the brood. These species show facultative biparental care, whereby male and female parents cooperate to varying degrees by caring for the developing larvae (Eggert et al., 1998; Scott, 1998). Thus, a shift in the balance between cooperation and conflict could be detected as a change in the duration of biparental care. Both female and male parents provide care by provisioning pre-digested carrion to the larvae and defending the carcass and the brood from conspecific intruders (Eggert et al., 1998; Scott, 1998). Females spend more time on parental care (e.g., Smiseth et al., 2005; Georgiou-Shippi et al., 2018) and care for longer than males (Bartlett, 1988; Ford and Smiseth, 2016), yet it is unclear what impact variation in carcass mass would have on the magnitude of such sex differences in care. Prior work also shows that there are synergistic effects of biparental cooperation, and that that these often outweigh the detrimental effects of sexual conflict (Pilakouta et al., 2018). However, it is unclear how variation in carcass mass would impact on the balance between cooperation and conflict.

Our aim was to test for effects of variation in carcass mass on sex differences in care and the balance between cooperation and conflict. We provided breeding pairs with mouse carcasses of variable mass (3.65–26.15 g). We then monitored subsequent effects on the duration of biparental care, sex differences in the duration of male and female care and the amount of time spent providing care by males and females, resource consumption and weight change by males and females during breeding, and brood size and mean larval mass at the time of larval dispersal. We predicted that sex differences in parental care would be more pronounced as carcass mass decreased. The reason is that the benefits of male care should be lower as carcass size decreases given that smaller carcasses are less valuable to conspecific intruders. We also predicted that females would respond less to an increase in carcass mass than males in terms of carrion consumption and weight gain given that caring parents have greater access to the carcass as a food source for themselves (Pilakouta et al., 2016). This is because females are predicted to remain at the carcass for a similar amount of time regardless of carcass size, whereas males are predicted to provide care for longer on larger carcasses, thereby giving them more opportunities to consume from the carcass (Keppner et al., 2018). As argued above, an increase in carcass mass may lead to a shift toward either more conflict or more cooperation between parents. The latter prediction seems more likely in *N. vespilloides* given that larger carcasses are more valuable to conspecific intruders, and that two parents are more efficient at protecting their brood against intruders than single ones (Trumbo, 1991). In the wild, breeding success relies greatly on the attendance of both parents (e.g., Scott and Traniello, 1990;

Trumbo, 1991, 2006, 2007; Eggert and Sakaluk, 2000; Hopwood et al., 2015), and we therefore used the duration of biparental care as a proxy for the level of cooperation between the male and female parents.

MATERIALS AND METHODS

Origin and Rearing of Experimental Beetles

We used virgin beetles from an outbred laboratory population maintained for at least four generations at the University of Edinburgh. The laboratory population descended from beetles that originally were collected in Hermitage of Braid and Blackford Hill Local Nature Reserve, Edinburgh, United Kingdom. We maintained non-breeding adult beetles in individual transparent plastic containers (12 cm × 8 cm × 2 cm) filled with moist soil, under a constant temperature (20°C) and a 16:8 h light:dark photoperiod. We fed non-breeding adult beetles a small piece of organic beef twice a week.

Experimental Design and Procedures

We designed a laboratory experiment where we tested for effects of variation in carcass mass on the magnitude of sex differences in care and the balance between cooperation and conflict by manipulating the mass of the carcass that pairs were provided with at the start of breeding. We started the experiment by pairing virgin females with a randomly assigned, unrelated, virgin male partner. To ensure that all beetles were sexually mature and to avoid any confounding effect of age on parental traits, we used males and females aged between 10 and 28 days following eclosion. We weighed all males and females at this stage to record their pre-breeding mass. To initiate breeding, each pair was moved to a larger, transparent container (17 cm × 12 cm × 6 cm) filled with 1 cm of moist soil and provided with a previously frozen mouse carcass (Livefoods Direct, Sheffield). We randomly assigned each pair with a mouse carcass that weighed between 3.65 and 26.15 g (mean ± SE = 13.41 ± 0.396 g). This mass range matches that used by our study species under natural (2–30 g; Müller et al., 1990) and laboratory conditions (2–40 g; Smiseth and Moore, 2002). Varying the size of the carcass is a well-established protocol in burying beetle species allowing us to manipulate access to the breeding resource (e.g., Bartlett, 1988; Eggert and Müller, 1992; Trumbo, 1992; Xu and Suzuki, 2001; Smiseth and Moore, 2002; Creighton et al., 2009; De Gasperin and Kilner, 2015; Magneville et al., 2018).

From the day of mating onwards, we checked each container daily for the presence of eggs. We did this to record the day on which the first eggs were laid. Females lay their eggs in the soil surrounding the carcass, and most eggs are visible from the bottom of the transparent container in a thin layer of soil (Monteith et al., 2012), as used in our experiment. We counted the eggs 2 days after the onset of egg-laying (i.e., the day preceding the time of hatching of the first eggs in the clutch) and used the number of eggs as a measure of clutch size. On the following day, when the eggs started to hatch, we counted the number of newly hatched larvae, using this as a measure of brood size on the day of hatching. Given that females lay their eggs asynchronously over a mean period of 27 h (Müller, 1987; Smiseth et al., 2006), the final brood size may be larger than brood size on the day of hatching.

We recorded shifts in the balance between cooperation and conflict by monitoring the duration of biparental care. We checked the containers daily from the time of mating until the time of dispersal, recording whether the male and the female were still present on the carcass or whether either of them had deserted the brood. We scored the male or the female as having deserted the brood if the male or the female was absent from the crypt (i.e., the depression in the soil surrounding the carcass) on two consecutive days. We removed any parent that had deserted the brood from the breeding container to prevent the deserting parent from posing a risk to the brood. Note that we refrained from removing any deserting parent before we conducted the behavioural observations 24 h after hatching (see details below). Removing a deserting parent matches what would happen under natural conditions given that deserting parents leave the carcass permanently (Scott and Traniello, 1990). We removed deserting parents because it may kill larvae when maintained with the brood beyond the time of desertion (Authors' personal observation). We weighed any deserting parent to record information on weight change during breeding (see below). We recorded the duration of biparental care as the number of days from mating until one of the parents deserted the brood. If both parents cared for the brood until the larvae dispersed from the carcass, we recorded the duration of biparental care as the number of days from mating until the larvae dispersed from the carcass (normally 7 days; Scott, 1998; Grew et al., 2019).

We monitored the behaviour of parents on the day after the first eggs had hatched to estimate the amount of time that each parent spent providing care and consuming resources. This time point corresponds to the peak of parental food provisioning to larvae in this species (Smiseth et al., 2003). We conducted behavioural observations for 30 min under red light, recording the behaviour of both parents at 60 s intervals in line with established protocols (e.g., Smiseth and Moore, 2002, 2004; Pilakouta et al., 2018). Note that, apart from the light, laboratory conditions were identical during behavioural observations (i.e., constant 20°C temperature). We recorded whether each parent was provisioning food, defined as any mouth-to-mouth contact between a parent and at least one larva, maintaining the carcass, defined as excavation of the soil around the carcass or coating the carcass with exudates, or in near proximity to the brood, defined as whenever a parent was at a distance from larvae that was approximately equal to or shorter than its pronotum length (e.g., Smiseth and Moore, 2002, 2004). We recorded time spent consuming carrion as any instances where a parent was feeding within the crater (i.e., the opening on the top of the carcass; e.g., Pilakouta et al., 2016). Feeding from the crater generally reflects that parents consume carrion for their own use or to regurgitate to the larvae (Pilakouta et al., 2016), although it can sometimes reflect that parents are enlarging the crater (e.g., Shukla et al., 2018). At each scan, we also recorded the number of larvae that were begging to a parent. We then calculated the average proportion of time spent begging per larva in the brood as

$B = (\sum b/n)/p$, where $\sum b$ is the cumulative number of begging events during the 30-min observation period, n is the brood size at the time of observation, and p is the number of scans during which a parent was in close proximity to the brood.

We left experimental broods undisturbed until the larvae dispersed from the carcass. At the time of dispersal, we counted the number of larvae to gain information on brood size and we weighed the whole brood to calculate mean larval mass as total brood mass divided by brood size. We also weighed each parent again at dispersal and calculated relative weight change during breeding as the difference in body mass measured at dispersal (or removal) and pre-breeding mass, divided by pre-breeding mass. In this species, parents feed from the carcass during breeding (Pilakouta et al., 2016), and parental weight change is used as a proxy for investment in future reproduction (Creighton et al., 2009; Billman et al., 2014; Gray et al., 2018).

Statistical Analysis

All statistical analyses were conducted using R version 3.6.0 (R Core Team, 2019) loaded with the packages *car* (Fox et al., 2017), *MASS* (Ripley et al., 2017), and *glmmTMB* (Brooks et al., 2017). We analysed data on the shift between cooperation and conflict between the two parents as a number of days of biparental care using a generalised linear model (GLM) assuming a Poisson error structure and including carcass mass as the only fixed effect. We analysed data on sex differences in the duration of care using GLMs assuming Poisson error structures. We verified the absence of over-dispersion and the good fit of the models by plotting the residuals using the "simulateResiduals" function of the *DHARMa* package in R (Hartig, 2017). To analyse data on sex differences in parental behaviour on the day after hatching (i.e., the amount of time spent provisioning food to the brood, maintaining the carcass, and consuming carrion), we used GLMs with zero-adjusted binomial distributions to account for zero-inflation and over-dispersion. We used linear models to analyse data on parental weight change over breeding. In all other models, we included carcass mass, the sex of the focal parent and, to test for potential sex-specific responses to resource availability, the interaction between carcass mass and sex. We also tested whether potential effects of carcass mass on parental behaviours on the day of hatching were fully or partially driven by clutch size or brood sizeat the time of observation or brood size. The reason for this is that parents adjust the amount of care that they provide to the number of offspring in the brood (Smiseth et al., 2007; Ratz and Smiseth, 2018), and that brood size covaries with carcass size (Bartlett and Ashworth, 1988; Smiseth et al., 2014). To determine whether any overall effect of carcass mass was causally linked to variation in clutch size or brood size, we first ran each model excluding clutch size or brood size at the time of observation and then compared this model to a full model that included clutch size or brood size at the time of observation as a fixed effect. We used the "Anova" function of the R package *car* (Fox et al., 2017) to obtain χ^2 and p-values provided in tables and the "summary" function to obtain the estimates, z-values and p-values provided in the text.

For our analyses on offspring behaviour and performance, we used a GLM assuming a binomial error structure to analyse data on the average time spent begging by individual larvae, a GLM assuming a negative binomial error structure to analyse data on brood size at dispersal, and a linear model to analyse data on mean larval mass at dispersal. All models included carcass mass as a fixed effect. We also examined the effect of biparental cooperation on offspring performance by including the duration of biparental care as a covariate in models on brood size and mean larval mass at dispersal. As described above, we first excluded clutch size or brood size at the time of observation from the models and then ran each model again including clutch size or brood size at the time of observation as an additional fixed effect. As described above, χ^2 and p-values were obtained using the "Anova" function and estimates, z-values and p-values were obtained using the "summary" function in R.

TABLE 1 | Effects of the interaction between sex of the focal parent and carcass mass on the duration of uniparental care when clutch size excluded (a) and included (b). Effects of the interaction between sex of the focal parent and carcass mass on time spent provisioning food to the brood, maintaining the carcass and consuming carrion when brood size at the time of observation was excluded (a) and included (b).

	Sex: carcass mass			Sex			Carcass mass			Clutch/brood size		
	χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*
Duration of care												
(a)	8.12	1	**0.004**	48.8	1	**<0.001**	0.340	1	0.562			
(b)	6.70	1	**0.010**	38.6	1	**<0.001**	0.005	1	0.943	5.56	1	**0.018**
Food provisioning												
(a)	2.40	1	0.121	39.1	1	**<0.001**	0.021	1	0.884			
(b)	2.53	1	0.111	39.4	1	**<0.001**	0.286	1	0.592	4.61	1	**0.032**
Carcass maintenance												
(a)	9.56	1	**0.001**	48.2	1	**<0.001**	0.176	1	0.674			
(b)	10.0	1	**0.001**	48.7	1	**<0.001**	1.18	1	0.275	14.3	1	**<0.001**
Carrion consumption												
(a)	**<0.001**	1	0.998	15.9	1	**<0.001**	3.78	1	0.051			
(b)	0.194	1	0.659	20.0	1	**<0.001**	3.42	1	0.064	0.177	1	0.673

Values are obtained from GLMMs using the "Anova" function in R (Fox et al., 2017). Statistically significant P-values (<0.05) are shown in boldface.

RESULTS

Sex Differences in Duration of Care

There was a significant effect of the interaction between the sex of the focal parent and carcass mass on the duration of care (**Table 1**). This interaction effect reflected that males provided care for longer as carcass mass increased, whilst females tended to provide care until the time of larval dispersal regardless of carcass mass (**Figure 1A**; sex × carcass mass: estimate = 0.016, SE = 0.006, $z = 2.59$, $P = 0.010$). Thus, as predicted, sex differences in parental care became more pronounced as carcass mass decreased. There was no significant main effect of carcass mass on the duration of female care (**Table 1**). However, males deserted the brood earlier, and thus provided care for a shorter period of time, than females as carcass mass decreased [**Table 1**; mean ± SE duration of care from the day of mating: male = 4 ± 0.15 days, female = 7 ± 0.13 days; estimate (male versus female) = −0.64, SE = 0.103, $z = -6.21$, $P < 0.001$].

Sex Differences in Amount of Care

There was no effect of the interaction between the sex of the focal parent and carcass mass on the amount of time parents spent provisioning food to the brood on the day after hatching (**Table 1**). There was no significant main effect of carcass mass on the amount of time spent provisioning food to the brood (**Table 1**). Males spent significantly less time, on average, provisioning food to the larvae than females [mean ± SE time spent provisioning food out of 30 min: male = 0.74 ± 0.18 min, Female = 4.4 ± 0.3 min; estimate (male versus female) = −4.59, SE = 0.732, $z = -6.27$, $P < 0.001$].

The interaction between the sex of the focal parent and carcass mass had a significant effect on the time spent maintaining the carcass (**Table 1** and **Figure 1B**), reflecting that males spent more time maintaining the carcass as carcass mass increased whereas carcass mass had no noticeable effect on the amount of time spent maintaining the carcass by females (sex × carcass mass: estimate = 0.148, SE = 0.046, $z = 3.17$, $P = 0.001$). There was no main effect of carcass mass on time spent maintaining the carcass (estimate = −0.031, SE = 0.028, $z = -1.09$, $P = 0.275$). However, females spent significantly more time maintaining the carcass than males [mean ± SE time spent on carcass maintenance out of 30 min: male = 1.4 ± 0.23 min, female = 5.6 ± 0.38 min; estimate (male versus female) = −5.34, SE = 0.764, $z = -6.98$, $P < 0.001$].

Given that the number of offspring in the brood is positively correlated with carcass mass ($r = 0.20$, $t = 3.0365$, df = 204, $P = 0.002$), we compared models where we excluded and included clutch size or brood size at the time of observation as fixed effects to analyse the duration and the amount of parental care, respectively. We did this to disentangle the causal effects of carcass mass and the number of offspring in the brood on parental behaviour. Excluding or including clutch size or brood size at the time of observation did not change the effect of carcass mass (**Table 1**), suggesting that the effects of carcass mass on the behaviour of the parents were independent of any potential effects due to the number of offspring in the brood.

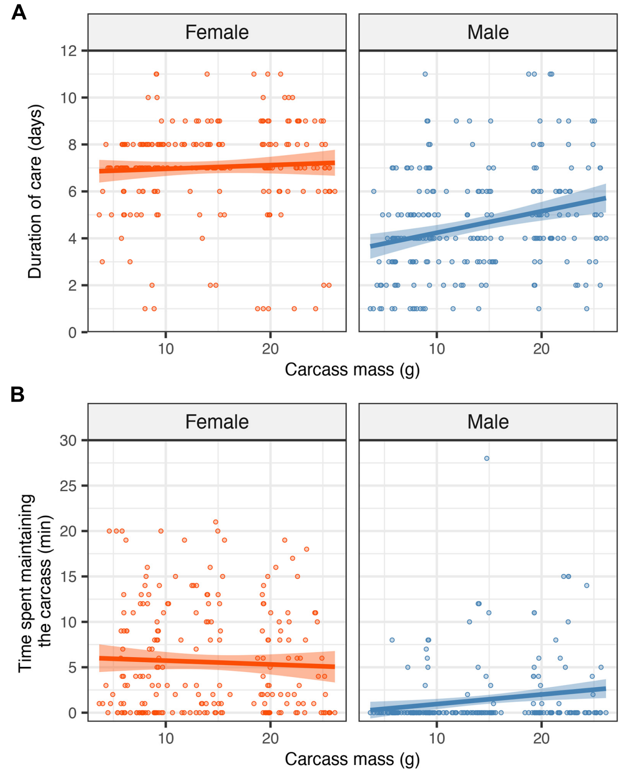

FIGURE 1 | Effects of carcass mass on the duration of female and male parental care **(A)** and on the time each parent spent on carcass maintenance **(B)**. Filled circles represent individual data points, lines represent linear regression lines and shaded ribbons the 95% confidence intervals.

Sex Differences in Carrion Consumption and Weight Change

There were no significant effects of the interaction between the sex of the focal parent and carcass mass and no significant main effects of carcass mass on the amount of time spent consuming carrion by the female or male parent measured on the day after hatching (**Table 1**). However, females spent significantly more time consuming carrion than males [mean ± SE time spent consuming out of 30 min: male = 0.87 ± 0.21 min, female = 3.6 ± 0.33 min; estimate (male versus female) = −3.69, SE = 0.825, $z = -4.47$, $P < 0.001$].

There was a significant effect of the interaction between the sex of the focal parent and carcass mass on weight change over the breeding attempt ($F_{1,368} = 0.046$, $P = 0.027$), reflecting that carcass mass had a stronger positive effect on female weight change than on male weight change (**Figure 2**; mean ± SE weight change: male = 0.027 ± 0.006 g, female = 0.068 ± 0.007 g). Parents gained more mass as carcass mass increased (estimate = 0.005, SE = 0.001, $t = 4.52$, $P < 0.001$). There was no significant difference between male and female parents in the average weight change ($F_{1,368} = 0.0009$, $P = 0.754$). Excluding or including clutch size at the time of observation did not change the effect of carcass mass, suggesting that any effect of

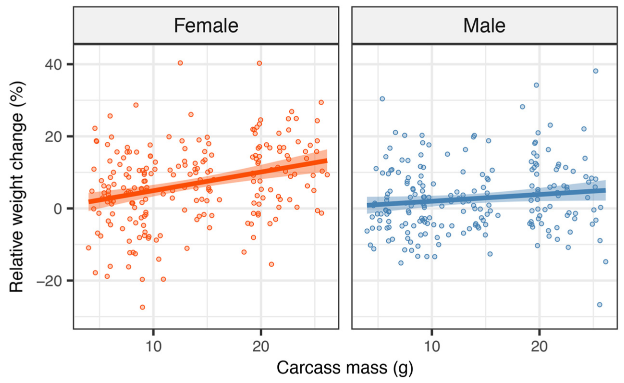

FIGURE 2 | Effects of carcass mass on the weight change of each parent. Filled circles represent individual data points; lines represent linear regression lines and shaded ribbons the 95% confidence intervals.

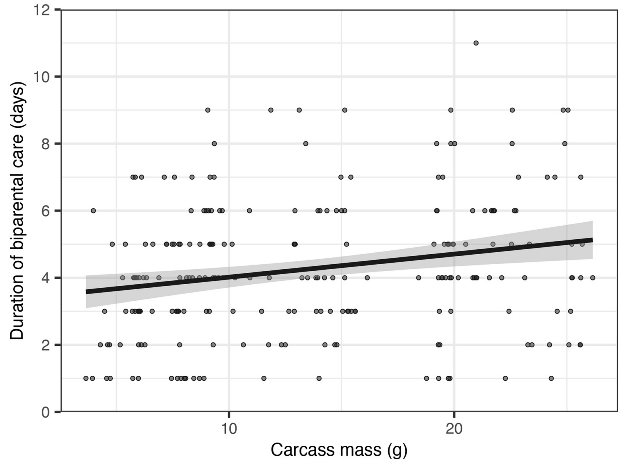

FIGURE 3 | Effects of carcass mass on the duration of biparental care. Filled circles represent individual data point, the line represents linear regression lines and shaded ribbons the 95% confidence intervals.

carcass mass on the weight gain of parents was independent of any potential effects due to the number of offspring in the brood.

Balance Between Cooperation and Conflict

The duration of biparental care increased by approximately 0.6 days for each additional 10 g of carcass (**Figure 3**; estimate = 0.012, SE = 0.005, z = 2.28, P = 0.022), supporting the prediction that an increase in carcass mass was associated with a shift toward more cooperation between parents. Clutch size had a significant positive effect on the duration of biparental care (estimate = 0.007, SE = 0.003, z = 2.09, P = 0.037). Including clutch size in the model, however, did not change the direction or the significance of the effect of carcass mass on the duration of biparental care.

TABLE 2 | Effects of carcass mass on larval begging, brood size at dispersal, and mean larval mass at dispersal when clutch size is excluded (a) and included (b) in the model.

	Carcass mass			Clutch size		
	χ^2	df	P	χ^2	df	P
Begging						
(a)	0.082	1	0.774			
(b)	0.187	1	0.665	0.666	1	0.414
Brood size at dispersal						
(a)	6.08	1	**0.014**			
(b)	3.42	1	0.064	5.07	1	**0.024**
Mean larval mass						
(a)	16.0	1	**<0.001**			
(b)	14.65	1	**<0.001**	16.4	1	**<0.001**

Values are obtained from GLMs using the "Anova" function in R (Fox et al., 2017). Statistically significant P-values (<0.05) are shown in boldface.

Offspring Behaviour and Performance

There was no significant effect of carcass mass on the average time spent begging by individual larvae (**Table 2**). However, brood size at dispersal increased by approximately 2 larvae for each additional 10 g of carcass (**Table 2** and **Figure 4A**; estimate = 0.016, SE = 0.006, z = 2.51, P = 0.012) and, for smaller carcasses (i.e., below 10 g), mean larval mass at dispersal increased by approximately 0.026 g for each additional 10 g of carcass (**Table 2** and **Figure 4B**; estimate = 0.025, SE = 0.006, t = 4.00, $P < 0.001$). There were significant effects of both the quadratic (χ^2 = 8.89, df = 1, P = 0.0028) and the cubic (χ^2 = 5.52, df = 1, P = 0.018) terms of carcass mass on mean larval mass at dispersal. Thus, mean larval mass increased with carcass mass when carcasses were relatively small and plateaued as carcass mass approached the upper end of the range of carcasses used in our experiment (**Figure 4B**). In addition, the duration of biparental care had a positive effect on brood size at dispersal (χ^2 = 5.91, df = 1, P = 0.015), increasing by approximately 0.8 larvae for each additional day of biparental care. The duration of biparental care had no effect on mean larval mass at dispersal (χ^2 = 0.324, df = 1, P = 0.568). Including clutch size in the model of brood size at dispersal removed the significant effect of carcass mass (**Table 2**), suggesting that the effect of carcass mass on brood size at dispersal was driven by differences in the number of eggs laid on carcasses of different masses. Including or excluding clutch size in the model on mean larval mass did not change the effect of carcass mass (**Table 2**), suggesting that the effects of carcass mass on mean larval mass was independent of any potential effects due to the number of offspring in the brood.

DISCUSSION

Here we show that a decrease in carcass mass was associated with more pronounced sex differences in both the duration of care and the time spent providing care, reflecting that males deserted the brood earlier and spent less time maintaining the carcass as carcass mass decreased. In contrast, females nearly always provided care until the larvae dispersed and spent a similar

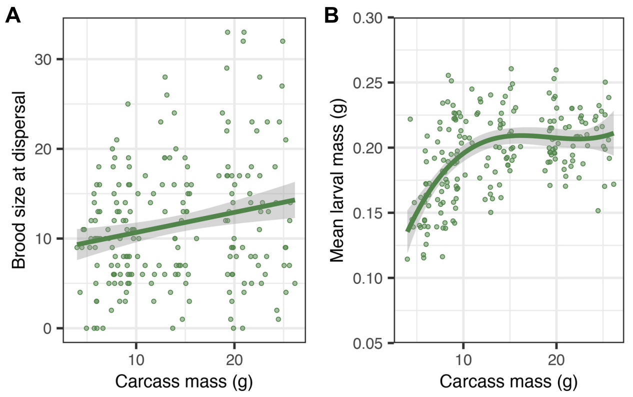

FIGURE 4 | Effects of carcass mass on brood size at dispersal **(A)** and mean larval mass at dispersal **(B)**. Filled circles represent individual data points; lines represent a linear regression line in panel **(A)** and a polynomial regression line in panel **(B)**, and shaded ribbons the 95% confidence intervals.

amount of time maintaining the carcass regardless of carcass mass. Furthermore, an increase in carcass mass was associated with a greater increase in weight gain by females than by males. Thus, variation in access to resources altered the magnitude of sex differences in parental care and parental weight change during breeding. We also found that an increase in carcass mass was associated with an increase in the duration of biparental care and in the benefits of care in terms of offspring survival, indicating a shift toward more cooperation between male and female parents as access to resources increased. Below we discuss the wider implications of our results for our understanding of how environmental conditions may drive the origin and maintenance of biparental care.

Our first main result was that there was a significant effect of the interaction between the sex of the focal parent and carcass mass on the duration of care and the amount of time spent maintaining the carcass on the day after hatching. These interaction effects reflected that males provided care for longer and spent more time maintaining the carcass as carcass mass increased, while carcass mass had no effect on the duration of care or time spent maintaining the carcass by females. These results are consistent with the findings on a related species of burying beetle (Kishida and Suzuki, 2010) and support our prediction that sex differences in parental care would be more pronounced as carcass mass decreased. Our results are consistent with prior work on *N. vespilloides* showing that females spend more time provisioning food to the brood (e.g., Smiseth et al., 2005; Georgiou-Shippi et al., 2018) and care for longer than males (Bartlett, 1988; Ford and Smiseth, 2016), and that males often adjust the amount of care they provide in response to variation in environmental conditions, whilst females tend to provide a similar amount of care regardless of such variation (Walling et al., 2008; Royle et al., 2014; Smiseth et al., 2005). These sex differences in parental care are thought to reflect that males can gain some reproductive success by mating away from a carcass whilst female require access to a carcass in order to reproduce (Müller et al., 2007). Thus, variation in access to resources may have a greater impact on the duration of male care because it increases their benefits of providing care relative to their benefits of deserting to mate with females away from a carcass (Ward et al., 2009).

We found that carcass mass had a greater positive effect on female weight gain than on male weight gain. This finding contrasts with our prediction that carcass mass would have a stronger impact on male weight change. Our prediction was based on the assumption that, if males provided care for longer on larger carcasses, this would give them more opportunities to consume from the carcass. Thus, our results contradict our initial assumption that sex differences in weight change would be linked to sex differences in parental care. This assumption is also contradicted by the finding that females gained more weight as carcass mass increased, even though females nearly always provided care until the larvae dispersed. Females gaining more weight as carcass mass increased suggests that females balance the personal benefits of consuming food from the carcass in terms of enhancing their own condition at the end of breeding against the costs of consuming food to the detriment of the larvae (Gray et al., 2018; Keppner et al., 2020). In this species, both the parents and the larvae feed from the carcass, and any increase in food consumption by a parent would therefore reduce the amount of food available to the other parent and the brood. Thus, females might restrict their own food consumption when breeding on smaller carcasses to avoid inflicting a cost to the larvae. On larger carcasses, where food is more plentiful, females may consume more food and put on more weight without inflicting such a cost to the larvae. However, it is unclear why this argument would only apply to female weight change. One potential explanation for why males seem to gain a similar amount of weight regardless of carcass mass is that males have a lower optimal body weight compared to females. Females may have a higher optimal body weight than males given that females must secure a carcass to reproduce, which means that they must fly in search of a carcass and compete with rival females. Gaining more weight might be beneficial given that flight is energetically costly and that heavier females tend to win more fights than lighter ones (Richardson et al., 2020). In contrast, males can attract and mate with females away from a carcass by emitting pheromones (Pukowski, 1933) and emitting pheromones is presumably less energetically costly than flying. Although carrion consumption might have a positive effect on male pheromone production and attractiveness (Chemnitz et al., 2017), a potential interpretation of results from the present study and others reporting greater body weight in females relative to males (e.g., Pilakouta et al., 2016; Paquet and Smiseth, 2017) is that males benefit less from putting on more weight than females. Alternatively, it could reflect greater energy expenditure by males increasing their effort in maintaining larger carcasses compared to smaller ones. This is because larger carcasses, which are heavier and have a greater surface area, potentially require greater effort to bury, prepare, and suppress bacterial and fungal growth from its surface (Xu and Suzuki, 2001). This is, however, unlikely to explain our results given that we found no evidence that males on larger carcasses consume more food. Nevertheless, we encourage future research to investigate this issue and examine the potential causes for sex differences in optimal body mass.

Our second main result was that the duration of biparental care increased with carcass mass. This result, together with the fact that males gained a similar amount of weight on smaller and larger carcasses, support our prediction that there was a shift toward more cooperation when parents had access to more resources. The rationale for our prediction was that the benefits of biparental cooperation would be greater on larger carcasses given that such carcasses are more valuable as a breeding resource to conspecific intruders, which may attempt to take over the carcass from the resident parents (Trumbo, 1991). If successful, such intruders would eliminate the original brood and use what is left of the carcass to rear their own brood. Furthermore, a study on the closely related *N. orbicollis* found that two parents are better able to protect the brood against conspecific intruders than single parents (Trumbo, 1991). Given that larger carcasses are subject to more intense competition than smaller ones (Wilson and Fudge, 1984; Robertson, 1993), it seems likely that the benefits to the male from assisting the female (and to the female from accepting assistance from the male) in terms of enhanced offspring survival would be greater as carcass mass increases. Our results contrast with comparative studies on birds, which have found that biparental cooperation was less common in species that breed in environments where there is greater availability of resources (Crook, 1963; Leisler et al., 2002; Barve and La Sorte, 2016). In altricial birds, greater access to food may reduce the benefits of biparental cooperation given that the female is more likely to be able to provision sufficient food for the brood on her own when food is plentiful as compared to when it is scarce. Biparental cooperation over food provisioning may be particularly important in altricial birds because parents must provide a constant supply of food from the surrounding environment. Thus, in altricial birds, the benefits of the male assisting the female may be greater when food is scarce. In contrast, biparental cooperation over food provisioning may be less important in burying beetles of the genus *Nicrophorus*. The reason for this is that these beetles breed on a fixed resource (i.e., a vertebrate carcass), which means that the supply of food will be limited by the size of the carcass rather than by the number of caring parents.

Our final results were that parents produced larvae with a greater mean mass when breeding on larger carcasses, whilst carcass mass had no effect on larval begging or brood size when controlling for clutch size. In contrast, the duration of biparental care had a positive effect on brood size only. The positive influence of carcass mass is consistent with previous findings reporting positive effects of carcass size on offspring growth and mass at dispersal (e.g., Xu and Suzuki, 2001; Andrews et al., 2017; Gray et al., 2018) but no effect on larval begging (Smiseth and Moore, 2002; Sieber et al., 2017). Such positive effects on offspring performance are likely to reflect that larvae simply have access to more food when self-feeding from the carcass, rather than an increase in the amount of care provided by the male. This is because the carcass represents the sole source of food for the larvae, and larvae may run out of food earlier on a smaller carcass than on a larger one. Moreover, prior work suggests that male care has no detectable effects on offspring growth and survival under laboratory conditions (Smiseth et al., 2005; Ratz et al., 2018), and may even have detrimental effects on females (Boncoraglio and Kilner, 2012). Our finding that the duration of biparental care had a positive effect on brood size, even when accounting for potential initial differences in clutch size, suggests that larvae cared for by two parents had a higher survival than larvae cared for by a single parent (Pilakouta et al., 2018). Taken together, these findings reveal that greater carcass mass can have positive effects on offspring performance through multiple mechanisms: (1) increasing the amount of food available to larvae, which enhances larval growth; and (2) increasing the duration of biparental care, which enhances larval survival.

In summary, we found that greater access to food reduced sex differences in parental care and shifted the balance toward more cooperation between parents. Overall, our findings stress the importance that environmental conditions, such as access to resources, play in determining the magnitude of any sex differences in parental behaviour, as well as determining the balance between cooperation and conflict over care. This is perhaps not surprising given that resource availability has long been recognised as a crucial environmental condition driving the emergence and maintenance of parental care in general (Tallamy and Wood, 1986; Klug et al., 2012). However, less consideration has been given to the role that resource availability plays as an environmental driver of the evolution of biparental care. Our findings also highlight the link between the magnitude of sex differences in care and shifts in the balance between cooperation and conflict. Such a link seems likely to emerge whenever variation in environmental conditions is associated with a greater reduction in the duration of care by parents of one sex as we report in our study.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

TR, JR, and PS conceived and designed the experiments. KK, LL, and TR collected the data. TR analysed the data. TR and PS wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

TR was supported by the Darwin Trust of Edinburgh.

ACKNOWLEDGMENTS

We thank the City of Edinburgh Natural Heritage Service for permission to collect beetles in their reserve at the Blackford Hill Local Nature Reserve. We thank Lilia Galvez for assistance with maintaining the laboratory population. We are grateful to Jarrod Hadfield and Trine Bilde for thoughtful input on an earlier version of this manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 05 October 2021
doi: 10.3389/fevo.2021.739396

Parental Care System and Brood Size Drive Sex Difference in Reproductive Allocation: An Experimental Study on Burying Beetles

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Life-history theory predicts that increased resource allocation in current reproduction comes at the cost of survival and future reproductive fitness. In taxa with biparental care, each parent can adjust investment on current reproduction according to changes in their partner's effort, but these adjustments may be different for males and females as they may have different reproductive strategies. Numerous theoretical and empirical studies have proposed the mechanism underlying such adjustments. In addition, the value of the brood or litter (brood size) has also been suggested to affect the amount of care through manipulation of brood size. While the two conditions have been studied independently, the impact of their interplay on potential sex-dependent future reproductive performance remains largely unknown. In this study, we simultaneously manipulated both care system (removal of either parent vs. no removal) and brood size in a burying beetle (*Nicrophorus vespilloides*) to understand their joint effect on reproductive allocation and trade-off between current and future reproduction. Our results show that males compensated for mate loss by significantly increasing the level of care regardless of brood size, while females exhibited such compensation only for small brood size. Additionally, with an increase in allocation to current reproduction, males showed decreased parental investment during the subsequent breeding event as a pair. These findings imply a dual influence of parental care system and brood size on allocation in current reproduction. Moreover, the impact of such adjustments on sex-dependent differences in future reproduction (parental care, larvae number, and average larval mass at dispersal) is also demonstrated. Our findings enhance the understanding of sex roles in parental investment and highlight their importance as drivers of reproductive allocation.

Keywords: ***Nicrophorus vespilloides*, parental care system, brood size, sex difference, reproductive trade-offs**

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *10 July 2021*
Accepted: *14 September 2021*
Published: *05 October 2021*

Citation:
Wang W, Ma L, Versteegh MA, Wu H and Komdeur J (2021) Parental Care System and Brood Size Drive Sex Difference in Reproductive Allocation: An Experimental Study on Burying Beetles. Front. Ecol. Evol. 9:739396. doi: 10.3389/fevo.2021.739396

INTRODUCTION

Life-history theory predicts that individuals trade off the reproductive investment between current and future reproduction, because an increased allocation to the current breeding attempts means fewer resources available to future breeding attempts (Williams, 1966; Reznick, 1985; Stearns, 1989; Clutton-Brock, 1991; Wolf et al., 2007). Under the constraints of time and energy, an increase in current parental effort can incur future fitness costs, such as reduced survival or reproductive performance. To maximize lifetime reproductive success, each parent should therefore adjust its investment allocation between current and future reproduction (Rivalan et al., 2005; Creighton et al., 2009; Bleu et al., 2013). In species with biparental care, the net result of this reproductive trade-off is sexual conflict over parental effort between parents as the fitness benefits depend on the combined effort of both parents, whereas the fitness costs are determined by individual effort (Parker, 2006; Szentirmai et al., 2007; Harrison et al., 2009; Royle et al., 2010; Lessells, 2012; Bebbington and Hatchwell, 2016). Each parent is expected to minimize its own current investment by shifting as much of the workload as possible over to its partner (Trivers, 1972; Lessells, 1999; Houston et al., 2005; Szentirmai et al., 2007; Harrison et al., 2009; Royle et al., 2010). The amount of contribution a parent provides to current reproduction is influenced by their partner's effort (Markman et al., 1996; Sanz et al., 2000; Houston et al., 2005; Johnstone and Hinde, 2006; Harris and Uller, 2009; David et al., 2015). These adjustments may be different for males and females as they may have different reproductive strategies which include different resource allocation, mate selection, and parenting patterns due to sex-dependent pay-offs of certain reproductive behaviors (Gross, 1996; Sanz et al., 2000; Kotiaho and Simmons, 2003; Hoffman et al., 2008). Theoretically, how parents respond to changes in their partner's effort depends on different evolutionarily stable strategies which include sealed-bid, negotiation (partial and full compensation) and matching strategy (Houston and Davies, 1985; McNamara et al., 1999; Johnstone and Hinde, 2006). Sealed-bid strategy assumes that parents do not respond to their partner's investment changes, as they make an initial fixed decision about how much investment to provide (Houston and Davies, 1985). In contrast, negotiation and matching strategies propose that parents adjust their investment to their partners' contribution (Johnstone and Hinde, 2006). Negotiation occurs when parents partially or fully compensate for the reduced care provided by their partners by increasing their effort (McNamara et al., 1999), whereas matching predicts that parents adjust their care to their partners' contributions by matching any increase or reduction in the same direction (Johnstone and Hinde, 2006). Several studies have tested how parents adjust their contribution based on changes of their partner's effort by handicapping or removing one parent and then assessing the response of the other parent. These studies showed mixed results: sealed-bid [house sparrow (*Passer domesticus*), Schwagmeyer et al., 2002; Nakagawa et al., 2007], negotiation [partial compensation: burying beetle (*Nicrophorus vespilloides*), Smiseth et al., 2005; burying beetle (*N. orbicollis*), Rauter and Moore, 2004; full compensation: great tit (*Parus major*), Sanz et al., 2000; magnificent frigatebird (*Fregata magnificens*), Osorno and Székely, 2004], and matching strategy [great tit, Hinde, 2006; zebra finch (*Taeniopygia guttata*), Mariette and Griffith, 2012, 2015; blue tits (*Cyanistes caeruleus*), Iserbyt et al., 2019] have all been found. Different strategies have also been found within the same species (great tit (*P. major*), full compensation and matching strategy) (Sanz et al., 2000; Hinde, 2006). Meanwhile, males and females respond differently to variation in their partner's effort because of sex differences in reproductive strategies. Some studies show that males increase their investment in offspring provisioning after female removal, whereas females show no response to partner removal (Rauter and Moore, 2004; Suzuki and Nagano, 2009; Creighton et al., 2015; Cantarero et al., 2019). It is because females typically provide more care than males, and they may work to their physical limitations and there is no room to intensify the provisioning rates [e.g., burying beetle (*Nicrophorus* spp.), Rauter and Moore, 2004; Suzuki and Nagano, 2009; Creighton et al., 2015; rock sparrow (*Petronia petronia*), Cantarero et al., 2019]. However, other studies suggest that females compensate for their partner's decrease in feeding rate, while males tend to do less because males focus more on the territory defense and lack confidence in paternity (great tit, Sanz et al., 2000).

In addition, brood or litter size may also affect the amount of care, as has been shown through manipulations of brood size. Parents adjust their effort by increasing their investment with increased brood sizes and decreasing it with decreased brood sizes (Wright and Cuthill, 1990; Sanz, 2001; Parejo and Danchin, 2006; Komdeur et al., 2007; Kokko and Jennions, 2008; Low et al., 2012; David et al., 2015; Griffioen et al., 2019). Although some parents can fully compensate for the effect of enlarged brood size by providing food at higher rates (Neuenschwander et al., 2003; García-Navas and Sanz, 2010), they may do so at a cost. It has been suggested that brood size manipulation affects future reproduction as enlarged broods decrease the survival of parents and the probability to produce subsequent broods (Tinbergen and Boerlijst, 1990; Parejo and Danchin, 2006). Several theoretical studies have been developed to explain the causes of parent-offspring conflict for parental care (Trivers, 1974; Godfray, 1995; Kilner and Hinde, 2012). These studies show that brood size has the potential to affect the allocation of resources between current and future reproduction, and the relative contribution of each sex to parental effort. Previous work has reported that there can be a sex difference in response to brood manipulation. In some studies, males increase their parental investment when brood size increases, whereas females do not seem as responsive as males to increases in brood size (MacGregor and Cockburn, 2002; Mock et al., 2005; Nakagawa et al., 2007; Low et al., 2012). However, others suggest that males show less increase in feeding when raising enlarged broods or more decrease in feeding when raising reduced broods than females (Ardia, 2007). That is, the allocation to current reproduction may be influenced both by partner's effort and by brood size, and these adjustments in parental effort may be different for males and females as they have different reproductive strategies. Additionally, the evidence for the trade-off between current and future reproduction is mixed. Previous work on reproductive trade-offs have manipulated traits that affect levels of parental effort to current reproduction (such as brood size or clutch size) and then assessing the induced changes in future survival, life span, or reproductive performance. Some studies have found that increased allocation to current reproduction leads to a future cost, such as lower body condition (Ratz and Smiseth, 2018), lower fecundity (Billman et al., 2014), shorter life span (Daan et al., 1996), or reduced competitive ability over food resources (Richardson et al., 2020), whereas other studies failed to demonstrate the predicted trade-off relationships in future breeding attempts (Roff and Fairbairn, 2007; Santos and Nakagawa, 2012; Ratz et al., 2020). Several explanations have been proposed for whether the trade-off between current and future reproduction are predictable or not. One such explanation is that this results from sex differences in allocation strategies. Considering that males and females of most species have different reproductive strategies and differ significantly in their cost of reproduction (Trivers, 1972; Santos and Nakagawa, 2012), because, for example, females provide more care than males while males invest further in obtaining additional mating opportunities (Queller, 1997), males and females may thus evolve distinct parental allocation strategies.

Although we have a good understanding of the impacts of partner's effort and brood size manipulations on the parental effort of each sex separately, little is known about their joint effects. In addition, males and females respond to partner's effort and brood size differently and thus may exhibit different allocation strategies. In order to test the combined effects of partner's effort and brood size on reproductive allocation between current and future reproduction, we use the burying beetle (*N. vespilloides*) in which we experimentally manipulated brood size and parental care system (experimental removal of a parent of either sex or no removal). To our knowledge, this is the first study to provide experimental evidence for the combined effects of parental care system and brood size on parental investment and reproductive trade-off between current and future reproduction.

Burying beetles (*Nicrophorus* spp.) are excellent model for studying the combined effects of flexible parental care system and parental investment due to brood size on reproductive trade-offs, because both uniparental and biparental care occurs and brood size is highly variable (Eggert et al., 1998; Scott, 1998; Ratz and Smiseth, 2018; Woelber et al., 2018). In these species, extended parental care is provided, consisting of the carcass maintenance stage and the larvae provisioning stage (Fetherston et al., 1990; Scott, 1990, 1998; Müller et al., 1998). Both parents prepare the carcass by burying it, removing any hair or feather from it, rounding it into a ball, and preserving it with anti-microbial secretions (Rozen et al., 2008; Trumbo, 2017). Typically, females spend more time provisioning larvae and care for longer than males, whereas males are more involved in carcass maintenance and defense, and desert the brood earlier than females because they have greater residual reproductive value than females (Bartlett, 1988; Smiseth and Moore, 2004; Ward et al., 2009; Ratz and Smiseth, 2018). The carcass serves as the sole source of food for both parents and larvae during breeding (Scott, 1998; Trumbo and Xhihani, 2015; Pilakouta et al., 2016), thus female burying beetles benefit from male desertion by feeding more from the carcass (Boncoraglio and Kilner, 2012). Both parents gain weight during the entire breeding, and the weight gained can serve as a proxy for investment in future reproduction (Creighton et al., 2009; Billman et al., 2014; Pilakouta et al., 2016; Richardson et al., 2020). Likewise, the weight loss during the larvae provisioning is an indicator of the reproductive investment to current reproduction (Trumbo and Xhihani, 2015). Prior work shows that both male and female beetles respond to brood size manipulations by increasing their parental care toward enlarged broods (Rauter and Moore, 2004). However, male but not female provisioning increased with brood size when caring for very large broods (Rauter and Moore, 2004; Smiseth and Moore, 2004). The difference in how males and females respond to brood size manipulation is in line with the difference in how males and females respond to the partner removal. Studies on several species of burying beetles have also found that males and females respond differently to partner removal. Males adjust their efforts to partner removal, while females generally show no response because they typically provide more care than males and their degree of compensation behavior is limited (Rauter and Moore, 2004; Smiseth and Moore, 2004; Smiseth et al., 2005; Suzuki and Nagano, 2009; Creighton et al., 2015). These findings suggest that there might be interaction between the effects of partner's presence and brood size on the amount of parental investment provided by each sex, yet previous studies did not allow us to tease apart these effects.

In this study, we aim to examine (i) how each sex adjusts its parental investment based on its partner's presence, brood size and their combined effects, (ii) whether the adjustments incur a sex difference in reproductive trade-offs. For the first question, we predicted that uniparental parents provide more care to the brood than biparental parents and that parents provide more care to larger broods than to smaller ones. We also expected an effect of the interaction between partner's presence and brood size on the amount of parental care provided by each sex. We predicted that males may compensate for mate removal regardless of brood size, whereas females show no response to mate loss when raising a large brood compared to raising smaller broods, because females typically provide more care than males and their ability of compensation may be restricted by physical limitations than males. In addition, we predicted that uniparental parents may gain the same weight from the carcass as biparental parents, and that parents gain more weight when caring for small broods than larger broods. Because uniparental parents have chance to feed more from the carcass but provide more care than biparental parents, whereas parents caring for large broods share resource with more offspring and provide more care than parents caring for small broods. We predicted that males may gain more weight than females as they typically provide less care than females. For the second question, we predicted that parents that allocated more resources in current reproduction reduce their parental effort and reproductive outcome in future reproduction. We expected that uniparental parents and parents that had cared for large broods may suffer a future cost in subsequent parental care and reproductive outcome (i.e., larvae number and average larval mass). We also predicted that the carry-over effects of increased allocation to current reproduction may diminish with time as parents gain benefits from the carcass. The carry-over effects of parental care system and brood size may be more obvious during the carcass maintenance stage than during the larvae provisioning stage.

MATERIALS AND METHODS

Study Animals

All burying beetles (*N. vespilloides*) used for this study were first-generation laboratory-reared offspring of adults collected at the field station of the University of Groningen in estate "de Vosbergen," Eelde, The Netherlands. Up to six same-sex adult beetles that descended from the same broods were kept in plastic boxes (length: 15 cm; width: 10 cm; height: 8.5 cm) filled with 2 cm of moist soil under a 16:8 h light: dark cycle and a temperature of 21°C. All adult beetles were fed with mealworms (*Tenebrio molitor*) twice a week with 2–3 mealworms per beetle each time.

Experimental Design

In order to test the combined impact of partner's presence and brood size on reproductive allocation of each sex and sex-dependent reproductive trade-offs, we performed an experiment which consisted of two parts (**Supplementary Figure 1**).

Experiment 1: Effects of Parental Care System and Brood Size and Their Interaction on the Amount of Parental Care and Weight Change of Parents

In the first experiment, we investigated how each sex adjusts its investment based on (1) partner's presence by manipulating the parental care system through experimental removal of a parent of either sex (uniparental male, uniparental female) or no removal (biparental parents) after all the eggs were laid, and (2) brood size (5 vs. 15 larvae), and (3) their interaction during the larvae provisioning stage. We allowed a pair of beetles to establish and breed on a carcass. Unrelated (i.e., non-sibling), virgin adult beetles, aged approx. 2 weeks old at posteclosion, were randomly selected for use in our experiments. Each pair was placed in a breeding box (length: 19 cm; width: 23 cm; height: 12.5 cm) filled with 2 cm of soil and provided with a previously frozen mouse (15 g).

In *N. vespilloides*, females start egg-laying about 21 h and the eggs hatch about 81 ∼ 111 h at 20 ± 1℃ after parents are given access to a carcass (Smiseth et al., 2006). After 3–4 days, shortly before the hatching of the larvae, we moved the parents and the carcass to a new breeding box with fresh soil, and at this point, randomly removed one parent to generate three treatment groups: uniparental (male care, $N = 40$; female care, $N = 40$), or biparental beetles (male and female care, $N = 40$). To make sure that the parents did not have eggs in the new breeding boxes, we checked each box three times daily (06:30–08:00 am, 14:00–15:30 pm, 21:30–23:00 pm, 6-h intervals) from the bottom of the boxes and renewed the soil when new eggs were found (biparental parents: 3 pairs). Because the larvae would crawl to the carcass after hatching, we kept the eggs in the soil of the old boxes to avoid the hatched larvae entering the carcass before we manipulated brood size. After egg laying, we started checking each old box three times daily for larvae hatching. When the eggs started to hatch (ca 3 days after egg-laying), we transferred 5 or 15 newly hatched larvae to the new boxes with parents to generate experimental broods (20 repetitions of both 5 and 15 larvae groups). We chose brood size of 5 and 15 larvae as treatment levels because they are within the natural range for this species when providing a 15 g mouse carcass under laboratory conditions (range larvae produced: 1–32, mean ± SE = 16.1 ± 6.3, $N = 271$; range larvae surviving to adult stage: 1–28, mean ± SE = 13.9 ± 5.2, $N = 245$, W. Wang, unpublished data). We manipulated brood size to 15 larvae which is very close to the average as a large treatment level to avoid filial cannibalism (Bartlett, 1987), and 5 larvae as a small treatment level to ensure that not all offspring died during breeding. As parents cannot directly recognize their offspring as long as the larvae are at the same developmental stage (Müller and Eggert, 1990; Oldekop et al., 2007), we added larvae that were produced by other females when needed. When the larvae dispersed (ca 6 days after larvae-hatching), we moved larvae from the same nest together into new boxes for pupation. We transferred all surviving parents into new boxes for 1 week, which were used for the second experiment.

Once larvae were added to the carcass, we recorded parental care activity of female and male parents three times daily (instant scanning) until larvae dispersal (the larvae provisioning stage) by visual inspection. In burying beetles, presence on the carcass is a strong indicator of parental care (Smiseth and Moore, 2004; Smiseth et al., 2005; Walling et al., 2008; Head et al., 2014). We checked presence or absence of parents by carefully removing the surface soil of the carcass. We recorded no parental care as when a parent was invisible (when in the soil), and parental care as when it was present on or inside the carcass. We estimated the amount of parental care as the proportion of times that parents spent on the carcass during the entire observation period, and then we calculated the parental care per larvae by dividing this proportion by brood size. In addition, we recorded combined biparental care as either or both of the parents being present on or inside the carcass. We defined the day of terminating care when the parents were absent from the carcass for three consecutive observations (Benowitz et al., 2013; Head et al., 2014). We then calculated the caring days as the duration from the day of larvae hatching until the time of terminating care (Parker et al., 2015). At the start of the experiment, the body size of each parent was recorded by measuring the pronotum width (accuracy: 0.01 mm), because body size may influence the amount of parental care (Pilakouta et al., 2015). Each beetle was weighed (accuracy: 0.0001 g) three times during breeding: at the start of the experiment (w_0), at the time of egg hatching (w_i) and at the time of larval dispersal (w_{ii}). For each parent, we recorded the weight change during the carcass maintenance stage (w_i-w_0, **Table 1**) to examine any effects on the amount of parental care during the larvae provisioning stage, because the resource acquisition at the onset of breeding and the nutritional status of parents affects their reproductive performances (Trumbo and Robinson, 2004; Richardson and Smiseth, 2019). We recorded the weight change during the larvae provisioning stage (w_{ii}-w_i, **Table 1**), because it is a good indicator of the reproductive investment to current reproduction (Trumbo and Xhihani, 2015). We recorded the weight change during the entire reproductive period (w_{ii}-w_0, **Table 1**). This is important because parents can

TABLE 1 | Measurements of weight and the calculation of weight change of parents during the first brood.

Weight measurement	Parameter
At the start of the experiment	w_0
At the time of egg hatching	w_i
At the time of larval dispersal	w_{ii}
Weight change of parents	
Carcass maintenance stage	w_i-w_0
Larvae provisioning stage	w_{ii}-w_i
Entire reproductive period	w_{ii}-w_0

increase in weight by feeding from the carcass during breeding and the weight gain can benefit future reproduction (Creighton et al., 2009; Billman et al., 2014; Pilakouta et al., 2016; Richardson et al., 2020). We excluded trials from our analyses in which females failed to produce eggs ($N = 2$; biparental female, brood 5: $N = 1$, brood 15: $N = 1$), or either of the parents died before larvae dispersal, which yielded the following final sample sizes of our first experiment: uniparental female care (brood 5: $N = 19$, brood 15: $N = 18$), uniparental male care (brood 5: $N = 18$, brood 15: $N = 16$), biparental care (brood 5: $N = 15$, brood 15: $N = 16$).

Experiment 2: Sex-Dependent Reproductive Trade-Offs Between Current and Future Reproduction: Effects of Parental Care System and Brood Size

In the second experiment we experimentally investigated the sex-dependent reproductive trade-offs between current and future reproduction. We paired the surviving parents with the same partners as during the first experiment to exclude any potential effects due to changes in partner's body size on parental care, because burying beetle parents adjust their contribution toward care based on both their own and their partner's size (Pilakouta et al., 2015). All parents that were removed from the boxes in the first experiment for the uniparental care treatment, were kept in isolation and fed with mealworms twice a week until they were used in this experiment. We provided each pair of beetles with a new carcass to initiate their second breeding event. We recorded their parental care from the onset of breeding until larvae dispersal (the carcass maintenance stage and the larvae provisioning stage) three times daily as described above. The observations were performed blindly from the experimental conditions in the first breeding attempt. We also recorded their reproductive outcome (the number of larvae and the total mass of larvae at dispersal) in the second brood. We calculated the average larval mass by dividing the brood mass by the number of larvae. We excluded all trials in which females failed to produce eggs ($N = 5$; uniparental female, brood 5: $N = 1$; uniparental male, brood 5: $N = 2$; biparental female, brood 15 = 2), or either or both of the parents died from our further analyses, which yielded the following final sample sizes of our second experiment: uniparental female care (brood 5: $N = 17$, brood 15: $N = 17$), uniparental male care (brood 5: $N = 14$, brood 15: $N = 15$), biparental care (brood 5: $N = 13$, brood 15: $N = 12$). There was no difference in body size (pronotum width) of parents among the different parental groups both during the initial and subsequent breeding (**Supplementary Table 1**). We thus excluded any potential effects due to variation in body size of parents on the amount of parental care given that body size influences the amount of care (Pilakouta et al., 2015). There was no difference in weight change of parents during the carcass maintenance stage and thus its potential effect on differences in the amount of larvae provisioning were excluded (**Supplementary Table 1**).

Statistical Analyses

All statistical tests were performed using R version 4.0.3 (R Core Team, 2020) loaded with packages *car* (Fox and Weisberg, 2019), *lme4* (Bates et al., 2015), *multcomp* (Hothorn et al., 2008), *emmeans* (Lenth, 2021), *DHARMa* (Hartig, 2021), and *pwr* (Champely, 2020). Body size (pronotum width) of parents did not differ among treatments and was therefore excluded from our analysis. We excluded the effect of mixing or non-mixing offspring because parents cannot recognize their offspring when the larvae are at the same stage (Müller and Eggert, 1990; Oldekop et al., 2007). Larvae number was significantly correlated with average larval mass and brood mass (larvae number vs. average larval mass: Pearson's correlation, $t = -6.178$, $r = -0.554$, $P < 0.001$; larvae number vs. brood mass: Pearson's correlation, $t = 9.544$, $r = 0.717$, $P < 0.001$; average larval mass vs. brood mass: Pearson's correlation, $t = 1.544$, $r = 0.164$, $P = 0.126$). Therefore, larvae number and mean larval mass at the larval dispersal time were used to assess the reproductive benefits of parents. We used linear (mixed) models for traits that had a normal error structure (LMs: average larval mass at dispersal; LMMs: weight change of parents), and generalized (mixed) linear models for traits that had a binomial error distribution (GLMMs: the amount of parental care) and Poisson error structure (GLMs: larvae number; GLMMs: number of caring days, *lme4* package). All models included the fixed factors parental care system (uniparental males, uniparental females, and biparental males, biparental females for parents' traits or combined biparental care for larvae traits) and brood size (small vs. large) and the interaction between them. Group identity was included as a random factor in (G)LMMs. Biparental males and females of the same pair share the same group identity, whereas uniparental males and females all have different identity. We used *post hoc* Tukey contrasts to test for differences whenever parental care system or the interaction had a significant effect on the variable of interest (*multcomp* and *emmeans* package). The over/under-dispersion of the models was estimated using the '*testDispersion*' function and the good fit of the models was verified by plotting the residuals using the '*simulateResiduals*' function (*DHARMa* package). Some models were over- or underdispersed or did not have good fits (i.e., weight change during the larvae provisioning stage, larvae number, caring days, the amount of parental care during the carcass maintenance stage). For weight change analyses during the larvae provisioning stage, we compared models that included or excluded four outliers (four biparental males gained weight during the larvae provisioning stage, weight change > 0). Additional analyses of larvae number, caring days and the amount of parental care during the carcass maintenance stage were conducted by running L(M)Ms. Although excluding the four individuals and fitting alternative models solved problems with dispersion and improved the model fits, the results of the models were qualitatively similar to those obtained when using all data or GL(M)Ms, except for the interaction between parental care system and brood size on the amount of parental care during the carcass maintenance stage (see below). Therefore, we present the results from the analysis including all data, and using GLMMs because these allowed us to account for number of observations in parental care measures. Finally, we calculated the statistical power by using '*pwr.chisq.test*' in *pwr* package. We used a desired power of 0.8, a significance level of 0.05 and chi-square test with four groups to determine the effect size (*w*). The *w* values of 0.3 are commonly considered to represent a medium effect size (Cohen, 1977). The complete dataset and R code used for the analyses are provided in **Supplementary Material**.

We performed two sets of analyses. In the first set of analysis, we used data from the first experiment (initial breeding) to examine the combined effects of parental care system and brood size on reproductive investment: the amount of parental care, weight change of parents during the larvae provisioning stage and during the entire reproductive period. We also examined the effect of the amount of parental care on weight change of parents during the larvae provisioning stage and during the entire reproductive period. To this end, we included the amount of parental care as an additional fixed factor in the model for weight change of parents. We then compared this model to a model that excluded the amount of parental care. We also compared the amount of parental care provided per larva and the number of caring days among different treatments.

In the second set of analysis, we used the data of the second experiment (subsequent breeding) to examine whether the increased allocation to current reproduction carried-over into reduced investment in the subsequent breeding: the amount of parental care, larvae number and average larval mass at dispersal. In the model for parental care, we included partner's effort as a covariate, because whether partner had experienced larvae provisioning or not in the first breeding may affect their effort in subsequent breeding (**Supplementary Table 1**), and parents may adjust their investment based on their partner's contribution. These covariates were included in the final models when they significantly improved the fit and the variance inflation factor (VIF, *anova*' and '*vif*' function in *car* package) was smaller than 2, indicating no problems with collinearity (Zuur et al., 2010). We then examined the impact of prior care and weight change of parents during the entire reproductive period in the first breeding on the amount of parental care in the second breeding. We did this by comparing models in which the amount of prior care and weight change of parents during the entire reproductive period in the first breeding were included or excluded as additional effects.

RESULTS

Experiment 1: Effects of Parental Care System and Brood Size and Their Interaction on the Amount of Parental Care and Weight Change of Parents

We found that parents adjusted their care based on their partner's presence. Both males and females spent significantly more time providing care when their partner was removed. The parental care system had a significant effect on the amount of parental care (**Table 2**). Uniparental parents compensated for mate removal by providing significantly more care than when they shared care with a partner (**Figure 1** and **Table 3**). Within sexes, uniparental males spent a higher amount of care than biparental males, but less than biparental parents combined (**Figure 1A** and **Table 3**). Likewise, uniparental females provided more care than biparental females, but less than biparental parents combined (**Figure 1B** and **Table 3**). Across sexes, females spent significantly more time than males provisioning larvae in both uni- and biparental care groups (**Table 3**), and terminated caring for offspring later than males in both uni- (females vs. males: Estimate ± SE = 0.315 ± 0.117, $z = 2.686$, $P = 0.026$) and biparental care groups (females vs. males: Estimate ± SE = 0.366 ± 0.128, $z = 2.870$, $P = 0.015$).

In addition, we found that brood size had a significant positive effect on the amount of time spent providing care (**Figure 1** and **Table 2**). Parents that cared for large broods provided more care than parents that cared for small broods (**Figure 1**, **Table 4**, and **Supplementary Table 2**). However, the amount of parental care provided per larva was less in large broods than in small broods (brood 5, mean ± SE = 0.108 ± 0.029; brood 15, mean ± SE = 0.046 ± 0.009; Estimate ± SE = 4.423 ± 0.185, $t = 23.974$, $P < 0.001$). Furthermore, there was a significant effect of the interaction between parental care system and brood size on the amount of parental care (**Table 2**). The *post hoc* pairwise comparisons showed that males and females differed significantly in response to mate removal when brood size increased from 5 to l5 larvae. Specifically, uniparental males partially compensate for mate removal by spending more time providing care than biparental males regardless of brood size (**Figure 1A** and **Table 4**). However, uniparental females fully compensated for mate removal by providing more care than biparental females when caring for small broods, but provided the same amount of care as biparental females when caring for large broods (**Figure 1B** and **Table 4**).

We found that parental care system had a significant effect on weight change of parents during the larvae provisioning stage and the entire reproductive period (**Table 2**). Males lost less weight during the larvae provisioning stage (**Figures 2A,B**) and gained more weight during the entire reproductive period (**Figures 2C,D**) than females in both uniparental and biparental care groups (**Table 3**). However, there were no significant differences between uni-and biparental parents (**Figure 2** and **Table 3**). In addition, brood size had a significant effect on weight change of parents during the larvae provisioning stage and the entire reproductive period (**Table 2**). Parents that cared for large

TABLE 2 | Effects of parental care system (uniparental male, uniparental female, biparental male, biparental female) and brood size (5 vs. 15), and their interaction on the amount of parental care and weight change of burying beetle parents during the larvae provisioning stage and the entire reproductive period in the first breeding.

Factor	***N***	**Amount of parental care (The larvae provisioning stage)**			**Weight change (The larvae provisioning stage)**			**Weight change (The entire reproductive period)**		
		χ^2	***df***	***P***	χ^2	***df***	***P***	χ^2	***df***	***P***
Brood size	133	58.096	1	**<0.001**	56.497	1	**<0.001**	32.117	1	**<0.001**
Parental care system	133	82.466	3	**<0.001**	24.648	3	**<0.001**	26.060	3	**<0.001**
Interaction	133	8.806	3	**0.032**	0.536	3	0.91	0.888	3	0.83

df, degree of freedom; N, sample sizes; Significant P-values are indicated in bold.

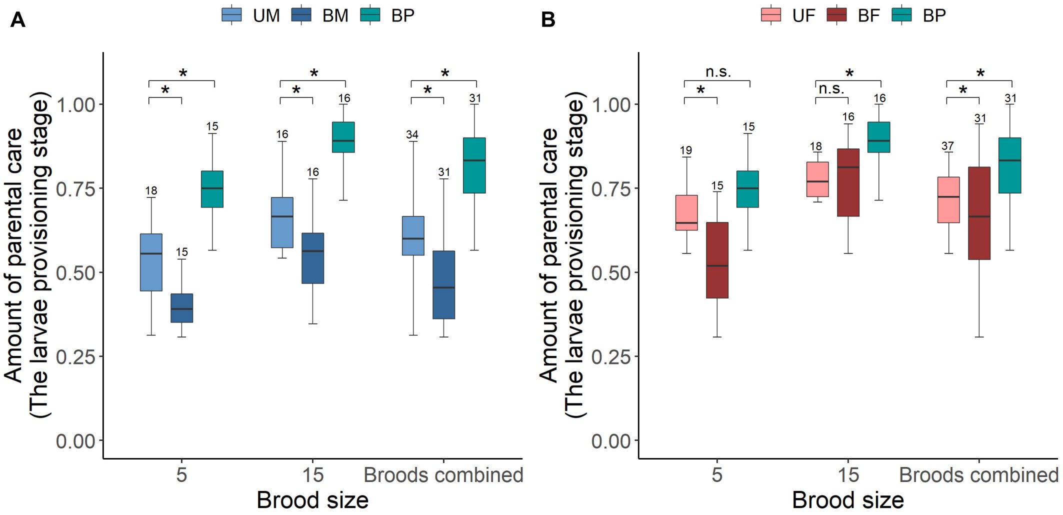

FIGURE 1 | The effect of parental care system (uniparental male, uniparental female, biparental male, and biparental female) and brood size (5 vs. 15 larvae) on the amount of parental care provided by **(A)** male and **(B)** female burying beetles in the first breeding. Boxplots show median, interquartile range, and minimum/maximum range. Numbers above error bars are sample sizes. For statistical analyses see **Tables 2–4**. Asterisks, significant ($P < 0.05$); n.s., not significant; UM, uniparental male; UF, uniparental female; BM, biparental male; BF, biparental female; BP, the total amount of parental care for biparental male and biparental female combined.

TABLE 3 | Pairwise comparison (Tukey *post hoc* test) for the effect of parental care system (uniparental male, uniparental female, biparental male, and biparental female) on the amount of parental care and weight change of burying beetle parents during the larvae provisioning stage and the entire reproductive period in the first breeding.

Comparison	**Amount of parental care (The larvae provisioning stage)**				**Weight change (The larvae provisioning stage)**				**Weight change (The entire reproductive period)**			
	Est	**SE**	***z***	***P***	**Est**	**SE**	***t***	***P***	**Est**	**SE**	***t***	***P***
UM- BM	0.538	0.118	4.580	**<0.001**	−0.002	0.002	−0.825	0.81	0.005	0.003	1.604	0.32
UF-BF	0.299	0.118	2.540	**0.040**	0.001	0.002	0.190	0.99	0.001	0.003	0.170	0.99
UM-UF	−0.579	0.115	−5.025	**<0.001**	0.007	0.002	3.946	**<0.001**	0.013	0.003	4.816	**<0.001**
BM- BF	−0.818	0.120	−6.815	**<0.001**	0.009	0.002	4.681	**<0.001**	0.010	0.003	3.099	**0.007**
BP- UM	1.063	0.134	7.944	**<0.001**								
BP- UF	0.484	0.131	3.682	**0.001**								

Est, estimate; SE, standard error; UM, uniparental male, N = 34; UF, uniparental female, N = 37; BM, biparental male, N = 31; BF, biparental female, N = 31; BP, the total amount of parental care for biparental male and biparental female combined, N = 31; Significant P-values are indicated in bold.

broods lost more weight during the larvae provisioning stage and gained less weight during the entire reproductive period than parents that cared for small broods (**Figure 2** and **Supplementary Table 2**). Finally, we found that the amount of parental care had a significant effect on weight change of parents during the larvae provisioning stage ($\chi^2 = 8.812$, $P = 0.003$), and the entire reproductive period ($\chi^2 = 13.194$, $P < 0.001$). Parents that provided more care for their offspring lost more weight during the larvae provisioning stage (**Figures 3A,B**) and gained less weight during the entire reproductive period than parents that provided less care (**Figures 3C,D**). No significant effects of interactions were observed on the weight change of parents during the larvae provisioning stage and the entire reproductive period (**Table 2**).

TABLE 4 | Pairwise comparisons (Tukey *post hoc* test) for the interaction of parental care system (A: uniparental male, uniparental female, biparental male, biparental female; B: uniparental male, uniparental female, biparental male and female combined) and brood size (5 vs. 15) on the amount of parental care during the larvae provisioning stage in the first breeding.

Comparison A/B		Amount of parental care (the larvae provisioning stage)			
		Est	SE	*z*	*P*
Brood 5	UM- BM	0.516	0.168	3.074	**0.025**
	UF- BF	0.602	0.164	3.666	**0.003**
	UM- UF	−0.617	0.159	−3.874	**0.001**
	BM- BF	−0.531	0.173	−3.073	**0.025**
	BP- UM	0.877	0.178	4.939	**<0.001**
	BP- UF	0.260	0.176	1.482	0.53
Brood 15	UM- BM	0.539	0.164	3.295	**0.012**
	UF- BF	−0.030	0.175	−0.175	0.99
	UM- UF	−0.542	0.166	−3.274	**0.013**
	BM- BF	−1.111	0.171	−6.484	**<0.001**
	BP- UM	1.310	0.208	6.308	**<0.001**
	BP- UF	0.768	0.203	3.790	**0.001**
UM	Brood 5- 15	−0.571	0.167	−3.428	**0.007**
UF	Brood 5- 15	−0.496	0.158	−3.138	**0.020**
BM	Brood 5- 15	−0.549	0.165	−3.329	**0.010**
BF	Brood 5- 15	−1.129	0.179	−6.310	**<0.001**
BP	Brood 5- 15	−1.004	0.217	−4.635	**<0.001**

Est, estimate; SE, standard error; UM, uniparental male (Brood 5, N = 18; Brood 15, N = 16); UF, uniparental female (Brood 5, N = 19; Brood 15, N = 18); BM, biparental male (Brood 5, N = 15; Brood 15, N = 16); BF, biparental female (Brood 5, N = 15; Brood 15, N = 16); BP, the total amount of parental care for biparental male and biparental female combined (Brood 5, N = 15; Brood 15, N = 16). Significant P-values are indicated in bold.

Experiment 2: Sex-Dependent Reproductive Trade-Offs Between Current and Future Reproduction: Effects of Parental Care System and Brood Size

We found that increased allocation to current reproduction carried-over into reduced future investment for males but not for females. Brood size manipulations in the first breeding had a significant effect on the amount of parental care during the carcass maintenance stage in the second breeding (**Table 5**). Parents that had cared for small broods in their first breeding provided more care during the carcass maintenance stage in subsequent breeding than parents that had cared for large broods (**Figure 4A**). Brood size had no effect on the amount of parental care during the larvae provisioning stage (**Table 5**). In addition, parental care system treatments in the first breeding had a significant effect on the amount of parental care during the carcass maintenance stage and the larvae provisioning stage in the second breeding (**Table 5**). Males provided less care than females during the carcass maintenance stage in the second breeding for both beetles that had uniparental and biparental care in the first breeding (**Figure 4B** and **Table 6**). However, there was no significant difference between males and females during the larvae provisioning stage (**Figure 4C** and **Table 6**). There were no significant differences in the amount of parental care between uni- and biparental parents during the carcass maintenance stage and the larvae provisioning stage (**Figures 4B,C** and **Table 6**). No significant effect of the interaction was observed, although for parental care during the carcass maintenance stage it approached significance (**Table 5**). The interaction was significant ($\chi^2 = 40.443$, $P < 0.001$) when we ran a LMM on parental care during carcass maintenance stage. However, pairwise comparisons on the interaction were qualitatively similar and suggested that only males exhibit a significant difference between small and large broods in the second breeding (uniparental male, 5 vs. 15: Estimate ± SE = 0.859 ± 0.276, $z = 3.106$, $P = 0.023$; biparental male, 5 vs. 15: Estimate ± SE = 1.025 ± 0.330, $z = 3.101$, $P = 0.023$). In contrast, there was no significant difference in subsequent parental care between females that had cared for small and large broods (uniparental female, 5 vs. 15: Estimate ± SE = 0.098 ± 0.307, $z = 0.320$, $P = 0.99$; biparental female, Estimate ± SE = 0.055 ± 0.351, $z = 0.156$, $P = 0.99$). Finally, we found that weight gain of parents during the entire reproductive period in the first breeding period had a significant positive effect on the amount of parental care during the carcass maintenance stage ($\chi^2 = 5.886$, $P = 0.015$) but not during the larvae provisioning stage ($\chi^2 = 1.818$, $P = 0.18$) in the subsequent breeding. However, prior care in the first breeding did not affect the amount of parental care during the carcass maintenance stage ($\chi^2 = 1.148$, $P = 0.28$) and the larvae provisioning stage ($\chi^2 = 0.081$, $P = 0.78$) in the subsequent reproduction.

We also found that brood size and parental care system and their interaction had no effects on subsequent larvae number and average larval mass at dispersal (**Table 5** and **Supplementary Table 3**). Increased allocation to current reproduction did not affect the performance of offspring produced during subsequent reproduction event as there was no significant difference in

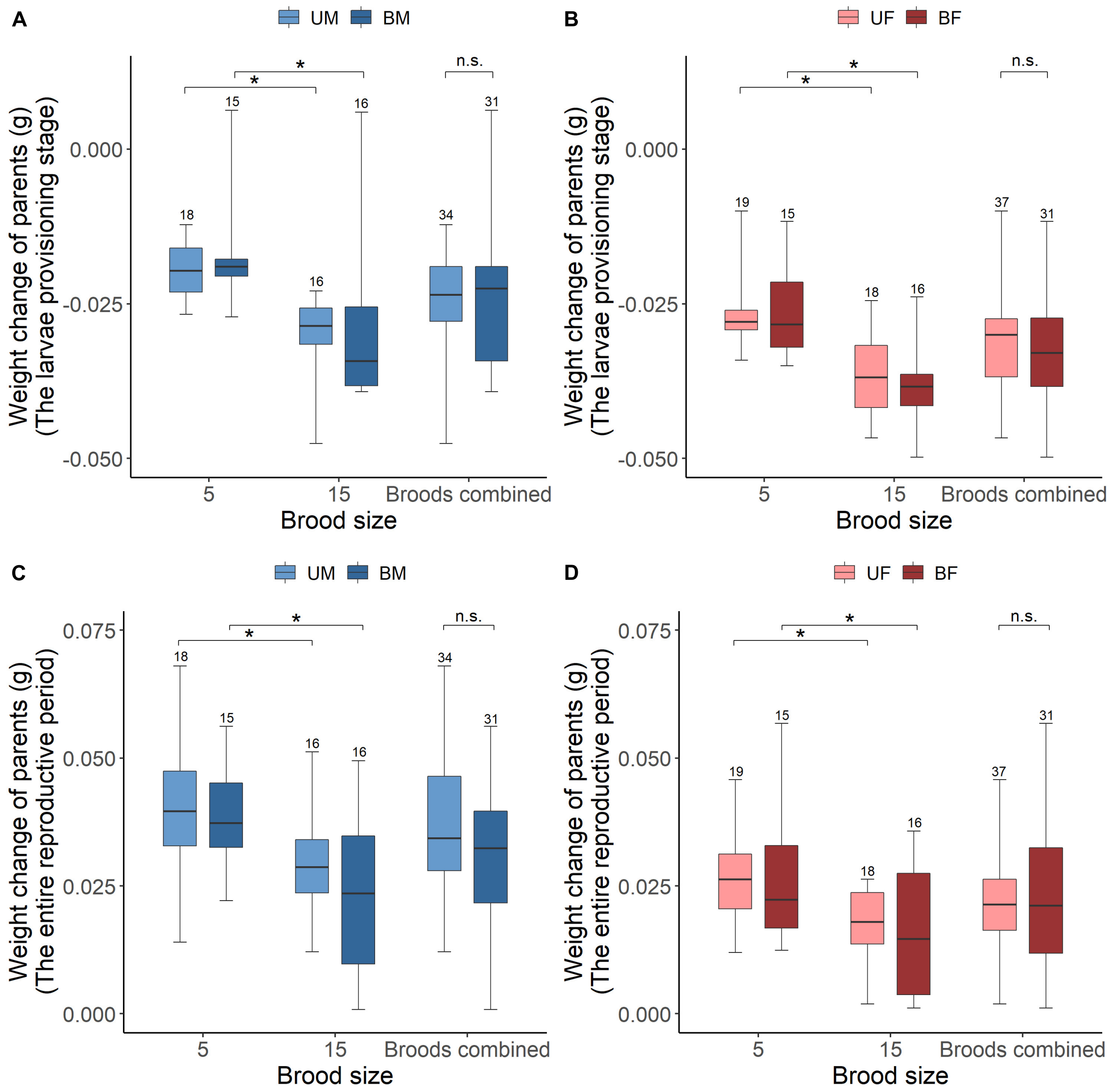

FIGURE 2 | The effect of parental care system (uniparental male, uniparental female, biparental male, and biparental female) and brood size (5 vs. 15 larvae) on weight change of burying beetle during the larvae provisioning stage [values below 0 indicate weight loss; **(A)** male, **(B)** female] and the entire reproductive period [values above 0 indicate weight gain; **(C)** male **(D)** female] in the first breeding. Boxplots show median, interquartile range, and minimum/maximum range. Numbers above error bars are sample sizes. For statistical analyses see **Tables 2**, **3**. Asterisks, significant ($P < 0.05$); n.s., not significant; UM, uniparental male; UF, uniparental female; BM, biparental male; BF, biparental female.

larvae number and average larval mass at dispersal between different parental care system treatments or brood size categories (**Figures 5A,B**). Our power analysis indicated that both the first ($N = 133$, $w = 0.286$) and second set of analysis ($N = 113$, $w = 0.311$) was adequate to detect a medium effect size.

DISCUSSION

Here, we report empirical and experimental evidence from a burying beetle that can have uni- or biparental care showing that parents adjust their contribution based on both partner's presence and brood size, and that this adjustment differs between the sexes. Specifically, males exhibited partial compensation behavior for mate loss regardless of brood size, whereas females fully compensated for mate loss in small broods but showed no response when caring for large broods. We found that sexes differ in their reproductive trade-off between current and future reproduction. Increased allocation to current reproduction due to brood size incurred a cost of reproduction in terms of reduced future parental care for males, but not for females. However, there was no evidence that increased allocation to current reproduction resulted in observed fitness costs during the subsequent breeding event in terms of larvae number and average larval mass at dispersal.

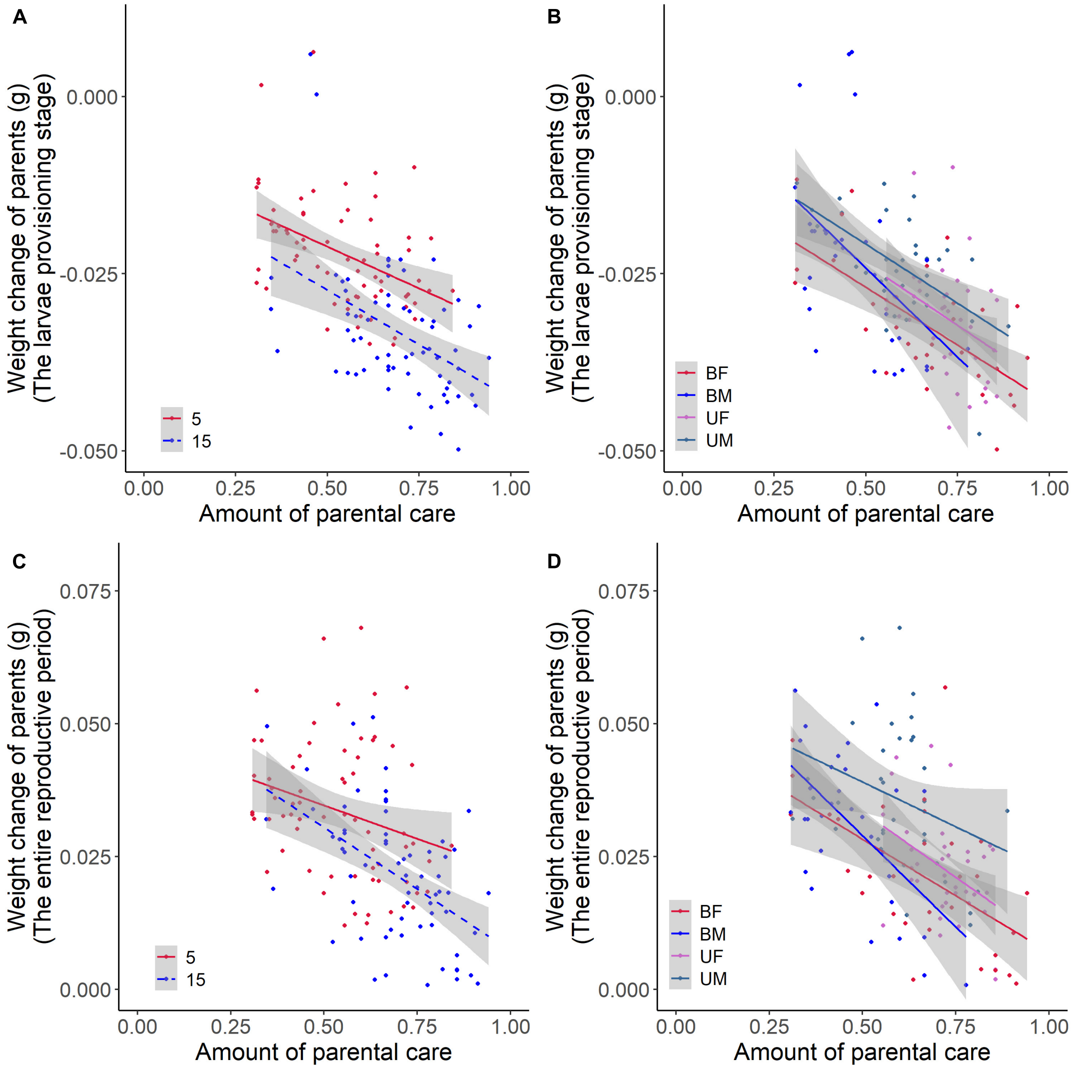

FIGURE 3 | Effects of the amount of parental care on weight change of burying beetle parents during the larvae provisioning stage and the entire reproductive period in the first breeding. The colored regression lines represent the relationship for each treatment (±95% confidence intervals). The larvae provisioning stage (values below 0 indicate weight loss) for **(A)** brood size manipulations (Brood 5, $y = -0.024x - 0.010$, $R^2 = 0.173$, $N = 67$, $P = 0.001$; Brood 15, $y = -0.031x - 0.012$, $R^2 = 0.200$, $N = 66$, $P < 0.001$) and **(B)** parental care system treatments (UM, uniparental male, $y = -0.033x - 0.004$, $R^2 = 0.329$, $N = 34$, $P < 0.001$; UF, uniparental female, $y = -0.035x - 0.006$, $R^2 = 0.112$, $N = 37$, $P = 0.0243$; BM, biparental male, $y = -0.050x - 0.001$, $R^2 = 0.201$, $N = 31$, $P = 0.007$; BF, biparental female, $y = -0.033x - 0.011$, $R^2 = 0.425$, $N = 31$, $P < 0.001$). The entire reproductive period (values above 0 indicate weight gain) for **(C)** brood size manipulations (Brood 5, $y = -0.025x + 0.047$, $R^2 = 0.061$, $N = 67$, $P = 0.025$; Brood 15, $y = -0.046x + 0.054$, $R^2 = 0.254$, $N = 66$, $P < 0.001$) and **(D)** parental care system treatments (UM, uniparental male, $y = -0.034x + 0.056$, $R^2 = 0.070$, $N = 34$, $P = 0.071$; UF, uniparental female, $y = -0.049x + 0.058$, $R^2 = 0.159$, $N = 37$, $P = 0.008$; BM, biparental male, $y = -0.069x + 0.063$, $R^2 = 0.319$, $N = 31$, $P = 0.001$; BF, biparental female, $y = -0.043x + 0.049$, $R^2 = 0.300$, $N = 31$; $P = 0.001$) in the first breeding.

Effects of Parental Care System and Brood Size and Their Interaction on the Amount of Care and Weight Change

We found that parental care system had a significant effect on the amount of parental care provided. Both uniparental males and uniparental females provided more care than biparental males and females, yet they spent less time on care than two parents together, indicating incomplete compensation. Parents respond to mate removal by compensating the amount of care, but also the kind of care if parental roles are sex-dependent. Although both male and female burying beetles are able to perform all parental duties, they focus on different tasks (Smiseth and Moore, 2004; Ratz and Smiseth, 2018). Therefore, one likely explanation may be that the incomplete compensation behavior for provisioning was caused by the sex-dependent roles in parental care and the ability of each sex to carry out the role normally performed by its partner. This is supported by the fact that males focus more on carcass maintenance and defense, whereas females spend more time provisioning larvae (Smiseth and Moore, 2004; Ratz and Smiseth, 2018). We also showed that females spent significantly more time on caring than males and terminated caring later

TABLE 5 | Effects of parental care system (parent traits: uniparental male, uniparental female, biparental male, biparental female; larvae traits: uniparental male, uniparental female, and biparental care) and brood size (5 vs. 15), and their interaction on the amount of parental care during the carcass maintenance stage and the larvae provisioning stage, and larvae number, average larval mass at dispersal in the second breeding.

Factor	*N*	Amount of parental care (the carcass maintenance stage)			Amount of parental care (the larvae provisioning stage)			Larvae number			Average larval mass		
		χ^2	*df*	*P*	χ^2	*df*	*P*	χ^2	*df*	*P*	*F*	*df*	*P*
Brood size	113	12.526	1	**<0.001**	0.001	1	0.97	0.276	1	0.59	0.274	1	0.60
Parental care system	113	47.130	3	**<0.001**	9.018	3	**0.029**	1.426	2	0.49	0.688	2	0.51
Interaction	113	7.473	3	0.058	0.637	3	0.89	0.711	2	0.70	1.556	2	0.22

df, degree of freedom; N, sample sizes; Significant P-values are indicated in bold.

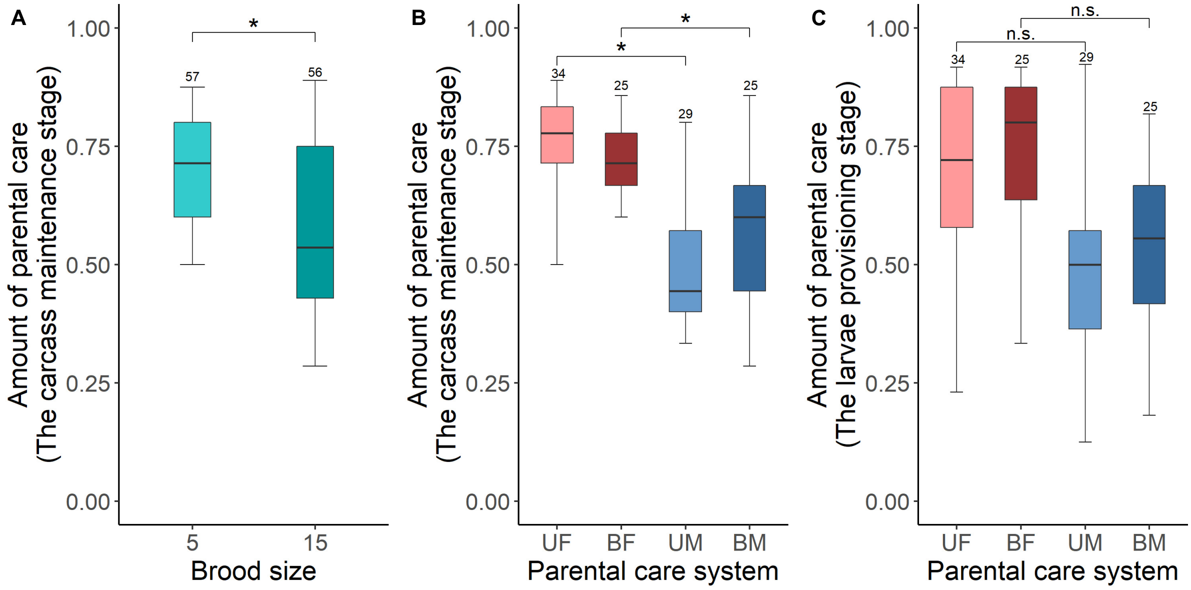

FIGURE 4 | The carry over effects of parental care system (uniparental male, uniparental female, biparental male, biparental female) and brood size (5 vs. 15 larvae) in the first breeding on the amount of parental care during the carcass maintenance stage [**(A)** brood size **(B)** parental care system] and the larvae provisioning stage [**(C)** parental care system] in the second breeding. Boxplots show median, interquartile range, and minimum/maximum range. Numbers above error bars are sample sizes. For statistical analyses see **Tables 5**, **6**. Asterisks, significant ($P < 0.05$); n.s., not significant; UM, uniparental male; UF, uniparental female; BM, biparental male; BF, biparental female.

TABLE 6 | Pairwise comparison for the effect of parental care system (uniparental male, uniparental female, biparental male, and biparental female) in the first breeding on the amount of parental care during the carcass maintenance stage and the larvae provisioning stage in the second breeding.

Comparison	Amount of parental care (the carcass maintenance stage)				Amount of parental care (the larvae provisioning stage)			
	Est	SE	*z*	*P*	Est	SE	*z*	*P*
UM- BM	−0.217	0.209	−1.039	0.67	−0.392	0.234	−1.677	0.29
UF-BF	0.214	0.234	0.915	0.75	−0.453	0.249	−1.822	0.22
UM-UF	−1.195	0.205	−5.825	**<0.001**	−0.314	0.264	−1.190	0.59
BM- BF	−0.764	0.237	−3.224	**0.005**	−0.374	0.218	−1.718	0.27

Est, estimate; SE, standard error; UM, uniparental male, N = 29; UF, uniparental female, N = 34; BM, biparental male, N = 25; BF, biparental female, N = 25; Significant P-values are indicated in bold.

than males. Other studies on this and other species of burying beetles showed that males normally desert the broods earlier and provide less care than females (Bartlett, 1988; Scott and Traniello, 1990; Trumbo, 1991). In our study, males had higher residual reproductive value than females after their first breeding because they provided less care and gained more weight at the entire reproductive period than females. It has been suggested that residual reproductive value predicts brood desertion in burying beetles (Ward et al., 2009). We therefore suggest that the total amount of care provided by males is expected to be lower than

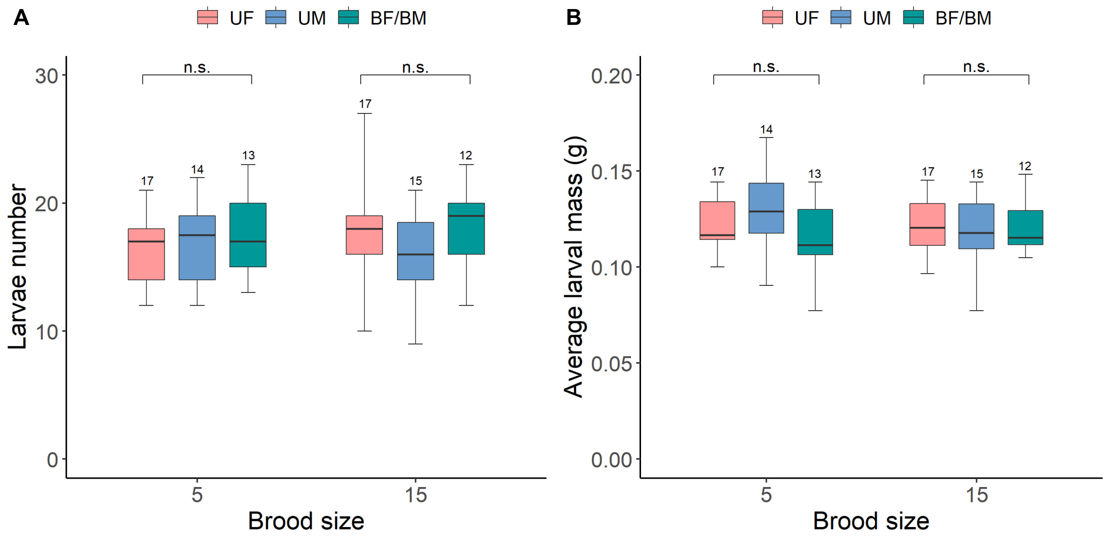

FIGURE 5 | The carry over effects of parental care system (uniparental male, uniparental female, biparental care) and brood size (5 vs. 15 larvae) in the first breeding on **(A)** larvae number and **(B)** average larval mass at dispersal in the second breeding. Boxplots show median, interquartile range, and minimum/maximum range. Numbers above error bars are sample sizes. For statistical analyses see **Table 5**. n.s., not significant; UM, uniparental male; UF, uniparental female; BM, biparental male; BF, biparental female.

females, and thus males provide less care and terminate caring earlier than females. In addition, we found that both males and females respond to brood size manipulations by increasing their provisioning toward larger broods, whereas the amount of parental care per larva was less in large broods than in small broods, indicating incomplete compensation for increased brood size. This is consistent with previous studies on this and other species (Sargent, 1988; Rauter and Moore, 2004; Karino and Arai, 2006; Ratz and Smiseth, 2018; Richardson et al., 2020), suggesting that parents usually provide more care toward larger broods because of higher benefits from providing care. However, our manipulation included both small and large broods which allowed us to test the effect of brood size on the compensation behavior of parents. We found that the amount of parental care was also significantly affected by the interaction between parental care system and brood size manipulation. Uniparental males exhibited partial compensation behavior for mate removal by increasing their food provisioning in both small and large broods compared to biparental males, whereas uniparental females fully compensated for mate removal in small broods but did not show compensation behavior when caring for large broods as they provided the same amount of care as biparental females. A previous mate removal study on the same species has also demonstrated that only males compensate for mate loss (Smiseth et al., 2005). However, this study did not investigate the combined effects of brood size and parental care system, nor did they manipulate brood size experimentally. Additionally, we found that the sex difference in response to mate removal corresponded with the previously found sex difference in parental care in response to larval begging. Males provided more care in response to higher larval begging, whereas females, in contrast, did not change their care (Smiseth and Moore, 2004). Considering that females normally stay longer and provide more care than males (Bartlett, 1988; Fetherston et al., 1990, 1994; Scott, 1998), we suggest that the sex difference in response to mate loss and larval begging may be due to the difference in reaching their maximum capacity of providing care. Females may not show compensation behavior when caring for large broods because they have already worked near their maximum capacity and thus their ability to increase their contribution after male removal is limited. This suggestion is supported by the results showing that females caring for small broods fully compensated for mate removal, whereas females caring for large broods did not. Meanwhile, parents lost more weight when providing higher amount of care during the larvae provisioning stage. This is in agreement with biparental birds in which incomplete compensation is often the result of physical limitations (Drent and Daan, 1980; Jones et al., 2002; Matysioková and Remeš, 2014; Cones and Crowley, 2020; Williams and DeLeon, 2020). In contrast, males exhibited compensation behavior regardless of brood size, and this may be because they provided less care and worked at a lower level than females and gained more weight than females.

We also found that in the first breeding females lost more weight during the larvae provisioning stage and gained less weight during the entire reproductive period than males, and this is consistent with the fact that females provided more care than males, suggesting that males expend less energy than females during breeding or they feed more from the carcass than females. Meanwhile, we found that parents lost more weight during the larvae provisioning stage and thus gained less weight during the entire reproductive period when caring for larger broods, reflecting that larger broods require more care and resource from parents.

Sex-Dependent Reproductive Trade-Offs Between Current and Future Reproduction: Effects of Parental Care System and Brood Size

We found a trade-off between current and future reproductive investment for males but not for females. In males, higher amount of care due to larger brood size resulted in less weight gain and in turn lower future reproductive investment. In females, increased allocation due to larger brood size resulted in less weight gain but did not incur future costs in terms of reduced parental care in the second breeding. The sex-dependent reproductive trade-offs may be explained by the sex difference in the functions of parental care. In burying beetles, males are more involved in the carcass maintenance stage, while females often spend more time on the larvae provisioning stage than males (Müller et al., 1998; Scott, 1998; Smiseth and Moore, 2004; Ratz and Smiseth, 2018). Therefore, the carcass maintenance stage should be the most stressful for males. The benefits gained by feeding from the carcass during the subsequent breeding might be not enough to cancel out the costs of prior reproduction for males, and thus they exhibited reproductive trade-offs. However, females focused on the larvae provisioning stage thus they might have a higher probability to recover energy and have more chance to offset the costs of prior reproduction by feeding from the carcass during the carcass maintenance stage. Additionally, we demonstrated the predicted reproductive trade-off during the carcass maintenance stage, but not during the larvae provisioning stage. It has been suggested that the weight gained during the initial breeding attempt can serve as a proxy for investment in future reproduction (Creighton et al., 2009; Billman et al., 2014; Pilakouta et al., 2016; Richardson et al., 2020). In our experiment, all parents gained weight during breeding, which improved their own body condition. Therefore, a likely explanation is that the costs of reproduction were offset to some degree by the benefits of feeding from the carcass during the carcass maintenance stage, then resulting in undetectable costs in subsequent stages. That is, the carry-over effects of reproductive costs may diminish with time as parents gained benefits from the carcass. Another explanation is that the costs of reproduction were reduced under laboratory conditions compared with natural conditions, because burying beetles did not have to search and compete for food, carcasses and mates between two breeding attempts. The breeding opportunities for *N. vespilloides* are typically limited by the availability of resources in the field and it is costly to compete for and protect the carcasses (Scott, 1998). By providing them with food, carcasses and mates before the subsequent breeding attempt, the potential cost for not storing enough energy during their initial breeding was canceled out to some degree and thus leading to the non-significant results during the larvae provisioning stage. We found no evidence that increased allocation to current reproduction resulted in future costs for reproductive performances in subsequent reproduction as there were no differences in larvae number and average larval mass at dispersal among different treatments. The weight change during the entire reproductive period in the first breeding had no effect on larvae number and average larval mass at dispersal in the second breeding. It has been reported that pre-hatching care (i.e., the carcass maintenance stage) did not affect offspring number and brood mass in burying beetles (Capodeanu-Nägler et al., 2016). In the subsequent breeding, although males caring for small and large broods provided different amount of care during the carcass maintenance stage, there were no differences in post-hatching care (i.e., the larvae provisioning stage) among the different treatments. Considering the fact that males are more involved in carcass preparation and brood guarding, and females spend more time provisioning food for larvae (Smiseth and Moore, 2004), we suggest that offspring performances were more likely to be influenced by post-hatching care and female care in burying beetles. Finally, we showed that weight gain during the entire reproductive period, but not the amount of prior care of parents, had a significant effect on parental allocation in subsequent breeding. This may be the reason for our findings that the observed increased allocation due to the variation in parental care system did not incur future costs, as parental care system (uniparental vs. biparental) was of no effect on weight change for both male and female parents. However, brood size may affect the reproductive trade-off through the weight change of parents during the entire reproductive period, because parents that had cared for large broods provided more care and gained less weight in the initial breeding, and provided less parental care in the subsequent breeding. Prior work on the same species demonstrated that increased allocation to current reproduction resulted in reduced weight gain during the initial breeding and incurred a future cost of reproduction in terms of reduced competitive ability (Richardson et al., 2020). Our results are in line with these findings and provide further evidence for reproductive trade-offs by demonstrating that an increase in current parental effort can incur costs in future reproduction. Furthermore, our experimental design included a novel manipulation that allowed us to tease apart effects of parental care system and brood size.

CONCLUSION

In this study, we experimentally demonstrated the effects of parental care system and brood size and the interaction between these two factors on parental investment and reproductive trade-off between current and future reproduction. Males and females differ in compensation behavior: males compensated for mate loss by significantly increasing the level of care regardless of brood size, while females exhibited such compensation only for small brood size. The effects of brood size manipulation but not of parental care system carried-over into sex-dependent reproductive allocation. With an increase in allocation to current reproduction due to larger brood size, males but not females showed decreased parental investment during subsequent breeding. However, increased investment due to parental care system did not incur future costs in terms of reduced parental care. This is, to our knowledge, the first study on the combined effects of parental care system and brood size on reproductive allocation and future fitness, and the results enhanced our understanding of sex roles in parental investment and furthered our knowledge of sex-dependent reproductive trade-offs in burying beetles. However, we tested the trade-off under laboratory conditions where the potential future costs for searching and competing for food, carcasses, and mates was not paid. It is unclear what the consequence will be when breeding in natural situations where the inter- and intra-specific competition for limited food and mates is higher. In addition, considering that the benefits gained by feeding from the resource may mask the costs of reproduction and thus have important consequence for subsequent reproduction, we suggest that future work on reproductive trade-offs in such species should consider the potential impact of resource variability and how much benefits they could gain when reproductive resource changes.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

WW, LM, JK, and HW conceived and designed the study. WW performed the experiments and wrote the initial manuscript. MV and WW analyzed the data with significant help of LM. JK led the manuscript writing and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a Ph.D. grant from the China Scholarship Council (CSC, 201906770043) and a Dobberke foundation from the Royal Netherlands Academy of Arts and Sciences (KNAWWF/Dobberke/2785) to WW, an Ecology Fund of the Royal Netherlands Academy of Arts and Sciences (KNAWWF/807/19021) to LM, and NWO grants (854.11.003 and 823.01.014) to JK.

ACKNOWLEDGMENTS

We thank VB and two reviewers for their constructive comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.739396/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 21 October 2021
doi: 10.3389/fevo.2021.756476

Sex-Biased Mortality and Sex Reversal Shape Wild Frog Sex Ratios

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Population sex ratio is a key demographic factor that influences population dynamics and persistence. Sex ratios can vary across ontogeny from embryogenesis to death and yet the conditions that shape changes in sex ratio across ontogeny are poorly understood. Here, we address this issue in amphibians, a clade for which sex ratios are generally understudied in wild populations. Ontogenetic sex ratio variation in amphibians is additionally complicated by the ability of individual tadpoles to develop a phenotypic (gonadal) sex opposite their genotypic sex. Because of sex reversal, the genotypic and phenotypic sex ratios of entire cohorts and populations may also contrast. Understanding proximate mechanisms underlying phenotypic sex ratio variation in amphibians is important given the role they play in population biology research and as model species in eco-toxicological research addressing toxicant impacts on sex ratios. While researchers have presumed that departures from a 50:50 sex ratio are due to sex reversal, sex-biased mortality is an alternative explanation that deserves consideration. Here, we use a molecular sexing approach to track genotypic sex ratio changes from egg mass to metamorphosis in two independent green frog (*Rana clamitans*) populations by assessing the genotypic sex ratios of multiple developmental stages at each breeding pond. Our findings imply that genotypic sex-biased mortality during tadpole development affects phenotypic sex ratio variation at metamorphosis. We also identified sex reversal in metamorphosing cohorts. However, sex reversal plays a relatively minor and inconsistent role in shaping phenotypic sex ratios across the populations we studied. Although we found that sex-biased mortality influences sex ratios within a population, our study cannot say at this time whether sex-biased mortality is responsible for sex ratio variation across populations. Our results illustrate how multiple processes shape sex ratio variation in wild populations and the value of testing assumptions underlying how we understand sex in wild animal populations.

Keywords: amphibian, endocrine disruption, population dynamics, sex determination, suburban, urban ecology

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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *10 August 2021*
Accepted: *29 September 2021*
Published: *21 October 2021*

Citation:
Lambert MR, Ezaz T and
Skelly DK (2021) Sex-Biased Mortality
and Sex Reversal Shape Wild Frog
Sex Ratios.
Front. Ecol. Evol. 9:756476.
doi: 10.3389/fevo.2021.756476

INTRODUCTION

Sex ratio is a fundamental characteristic of a population capable of affecting its demography and fate (Cotton and Wedekind, 2009; Earl, 2019). In species where gonadal sex is determined by sex chromosomes, Fisher (1930) predicted that sex ratios should be equal at birth, hatching, or metamorphosis. However, observations of sex ratios at early life stages are frequently biased from parity in a variety of species (Hardy, 2002). In mammals and birds, most research on the drivers of early-life sex ratio variation has stressed parental sex allocation – where parents manipulate the sex of their offspring prior to or soon after fertilization by disproportionately allocating resources to a given sex – as a leading mechanism modulating sex ratio in early life stages (Charnov, 1982; West, 2009). However, research in other vertebrates, like many fishes and non-avian reptiles, emphasizes a role for sex determination in shaping offspring sex ratios (Sarre et al., 2004; Ospina-Alvarez and Piferrer, 2008). Sex determination in vertebrates occurs along a spectrum (Sarre et al., 2004). At one end, an organism's phenotypic sex is controlled entirely by genes (genotypic sex determination) and at the opposite end by environmental conditions like temperature (environmental sex determination). In between these extremes there can be roles for both genetic and environmental factors to contribute to sex determination. Such species can sex reverse, where environmental conditions cause individuals to develop their phenotypic gonadal sex opposite from their genotypic sex (Sarre et al., 2004; Quinn et al., 2007; Ospina-Alvarez and Piferrer, 2008; Alho et al., 2010; Holleley et al., 2015, 2016; Lambert et al., 2019; Miko et al., 2021). In mammals and birds, phenotypic sex is genetically determined whereas fishes and reptiles show a diversity of sex-determining modes, including environmental sex reversal (Bachtrog et al., 2014).

Amphibians have been used as model organisms for over a century to study how environmental conditions influence sex ratios in developing animals (King, 1909, 1910; Witschi, 1929; Wallace and Wallace, 2000; Pettersson and Berg, 2007; Lambert, 2015; Lambert et al., 2016a, 2018; Miko et al., 2021). Sex ratio variation in metamorphosing amphibians is largely assumed to be the result of sex reversal and phenotypic sex ratios at metamorphosis are a proxy of sex reversal in laboratory and field studies (Wallace and Wallace, 2000; Pettersson and Berg, 2007; Papoulias et al., 2013; Lambert, 2015; Lambert et al., 2015, 2018). In such studies, female-biased phenotypic sex ratios are assumed to result from male-to-female sex reversal and male-biased phenotypic sex ratios from female-to-male sex reversal. However, this assumption is rarely tested. Evaluating whether sex ratio variation is due to sex reversal has remained elusive, in large part because genotypically sexing amphibians is notoriously challenging (Alho et al., 2010; Lambert et al., 2016b, 2019; Nemeshazi et al., 2020). Recent genomic advances permit the development of sex-linked molecular markers that allow researchers to genotypically sex large numbers of individual amphibians (Lambert et al., 2016b, 2019; Nemeshazi et al., 2020). Sex-linked markers have recently been used to demonstrate that sex reversal is occurring in wild amphibian populations (Lambert et al., 2016b, 2019; Nemeshazi et al., 2020). Even so, no study has yet to directly evaluate sex reversal as a mechanism underlying phenotypic sex ratio variation.

An alternate, and non-exclusive, hypothesis to sex reversal is that amphibian sex ratio variation is driven by sex-biased mortality (**Figure 1**). Sex-biased mortality prior to birth or hatching has been increasingly observed in wild animal populations, particularly in mammals and birds, but also occasionally in fish (Kruuk et al., 1999; Cichon et al., 2005; Svensson et al., 2007; Orzack et al., 2015; Moran et al., 2016; Kato et al., 2017; Firman, 2019). Multiple factors, including temperature stress and parental condition, have been identified as factors underlying sex-biased mortality, suggesting that sex-biased mortality may not be an adaptive process, as has been suggested for sex allocation, but a maladaptive response to stressful rearing conditions (Kruuk et al., 1999; Goth and Booth, 2005; Eiby et al., 2008; DuRant et al., 2016; Firman, 2019). Thus, sex-biased mortality is important to consider as it can directly shape sex ratios (Szekely et al., 2014).

To our knowledge, sex-biased mortality in pre-metamorphic amphibians remains largely unexplored. As with sex reversal, sex-biased mortality has likely remained understudied due to the technical limitations in genotypically sexing amphibians. Only two studies that we are aware of have considered sex-biased mortality in developing amphibians. One study attempted to infer sex-biased mortality by regressing phenotypic sex ratio variation against mortality rates at metamorphosis (Lambert et al., 2016a). This study concluded that sex ratio variation was due to sex reversal and not sex-biased mortality, however, molecular sex data are necessary to better clarify the processes underlying sex ratio variation. Another laboratory experiment found evidence for sex-biased mortality in tadpoles in response to high temperature even though that was not the intention of the study (Miko et al., 2021). Because sex reversal and sex-biased mortality could co-occur, distinguishing the relative contribution of these processes is crucial as both may produce similar phenotypic sex ratios but different genotypic sex ratios which can have implications for the evolutionary ecology and persistence of populations (Cotton and Wedekind, 2009; Earl, 2019).

In this study, we explore sex-biased mortality from embryogenesis to metamorphosis in two natural populations of the Green Frog (*Rana clamitans*) by sampling the genotypic sex of multiple developmental stages directly from a forested pond and a suburban pond. One of the few studies to assess phenotypic sex ratios in wild metamorphosing amphibians found that metamorphosing *R. clamitans* tadpole phenotypic sex ratios were male-biased in uncontaminated forest ponds and equal or female-based in suburban ponds that were contaminated by chemicals known to impact sexual differentiation (Lambert et al., 2015). Thus, *R. clamitans* sex ratio variation could suggest female-to-male sex reversal in natural populations and perhaps male-to-female reversal in environments impacted by suburban pollutants. Subsequent work used a novel molecular sexing approach and found that sex reversal was prevalent across 16 populations of free-living *R. clamitans* in both forest and

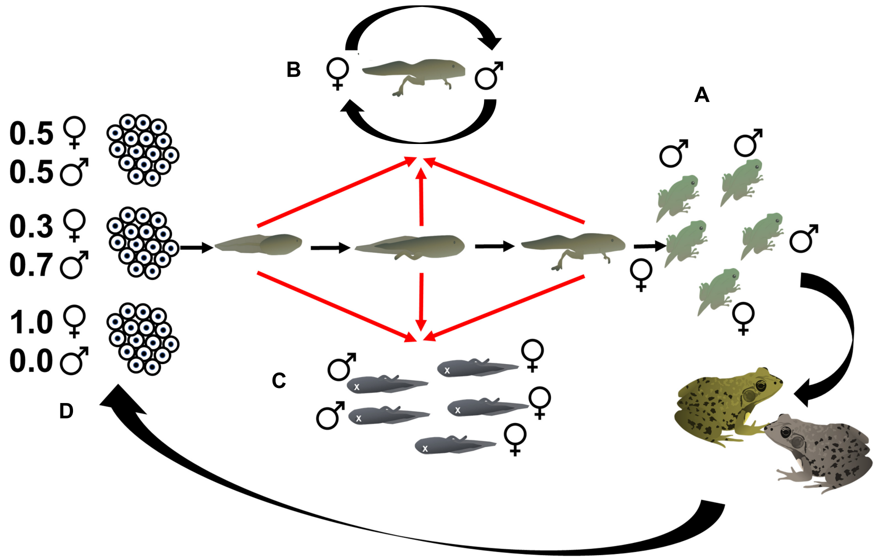

FIGURE 1 | Phenotypic sex ratios **(A)** in metamorphosing tadpoles may have contributions from the direct effect of sex reversal as tadpoles develop **(B)**, the effects of genotypic sex-biased mortality **(C)**, and indirect effects of sex reversal whereby sex-reversed adults breed and produce clutches with skewed genotypic sex ratios that bias entire cohort sex ratios **(D)**.

suburban ponds and that both female-to-male and male-to-female reversal occur (Lambert et al., 2019). Although this study demonstrated that sex reversal is common in wild *R. clamitans*, it assessed sex reversal in adult frogs. Because adult *R. clamitans* populations represent multiple overlapping offspring cohorts, this study could not determine whether sex reversal contributes to sex ratio variation in metamorphosing *R. clamitans* larvae. Here, our two goals were to (1) document whether sex-biased mortality occurs and alters phenotypic sex ratios and (2) test whether sex reversal is correlated with phenotypic sex ratio variation. We genotype the sex of over 900 *R. clamitans* across ontogeny from embryo to free-swimming tadpole and metamorphosis, illustrating the important contribution of sex-biased mortality, but secondary and inconsistent effects of sex reversal, on phenotypic sex ratios.

MATERIALS AND METHODS

All methods were approved by Yale IACUC protocols 2013-10361 and 2015-10681 and CT DEEP Permit 0116019b.

Ontogenetic Genotypic Sex Ratio Variation

Our goal was to evaluate the existence of sex-biased mortality in wild *R. clamitans* cohorts, inferring bias based on changes in genotypic sex ratios across ontogeny from embryogenesis to metamorphosis (Orzack et al., 2015). We sampled genotypic sex ratios at three developmental points: (1) embryogenesis, when clutches are freshly laid in ponds, (2) the free-swimming tadpole stage, multiple months after hatching and prior to overwintering, and (3) at metamorphosis, a developmental stage analogous to birth in mammals or hatching in birds when aquatic tadpoles metabolize their tails and develop all four limbs to become more terrestrial. We sampled two ponds – Septic7 and Forest5 – which have been central to our study of sex research in *R. clamitans* and which represent ends of a land use spectrum from undeveloped, forested environments (Forest5) and dense, residential suburbs (Septic7; Lambert et al., 2015).

Clutch Sampling

For clutch sampling, we visited the two ponds between 01 June and 12 July, 2017 every 1–3 days to ensure we sampled all new clutches. We note that this period of time does not typically encompass the entire breeding season of the species but, because of drought conditions, we detected few clutches past 26 June, 2017. We saw reduced breeding after this time because water within the two pond basins dried sufficiently below the emergent vegetation at the ponds' edges that is necessary for *R. clamitans* to deposit clutches. While this survey period is not as long as it would be in most years, it is comprehensive of the breeding season for this year and allows us to begin addressing the degree of sex ratio variation in *R. clamitans* clutches and the genotypic sex ratio for the year's cohorts. From each fresh clutch observed, we collected a subsample of ca. 150 embryos prior to neurulation (Gosner 14 and below), attempting to minimize any environmental effects on embryo survival. This species produces clutches with well over 1,000 embryos and so our samples represent at most 10% of a clutch. Because *R. clamitans* lays a single-layer film of embryos on the water's surface, sampling randomly throughout the egg mass is impossible without destroying the entire mass. As such, we haphazardly sampled around each clutch's periphery in a way that minimized damage to collected embryos or remaining embryos and in a way that did not detach the egg mass from emergent vegetation.

We maintained each clutch in a separate aquarium with reconstituted distilled water at 18°C until embryos freed themselves from jelly which is particularly important for *R. clamitans* because the jelly coat is too sticky to extract individual embryos without destroying them and collecting embryos singly (Sakisaka et al., 2000; Alho et al., 2010). The assumption in our approach is that the lab hatching accurately reflects the genotypic sex ratios of the clutches when collected in the field. We haphazardly collected 28–30 recently hatched embryos from each clutch, shipped tissue samples to Diversity Arrays Technology (Bruce, ACT, Australia), and genotypically sexed all individuals using DArTmp methods including two rounds of polymerase chain reaction and sequencing on an Illumina HiSeq 2500 using a sex-specific molecular marker for *R. clamitans* as described previously (Lambert et al., 2016b, 2019). For the sex-specific marker, we used the locus RaclCT001 which has previously been shown to be perfectly linked to genotypic sex, can accurately diagnose sex reversal on its own in the absence of other sex markers, and for which DArTmp methods consistently provide substantial reads to confidently call sex genotypes (Lambert et al., 2016b, 2019). Importantly, these markers have all been shown to be effective at genotypically sexing *R. clamitans* in all the focal ponds studied here (Lambert et al., 2019). Clutch sex ratio data are provided as **Supplementary Data Sheet 1**.

Larval and Metamorph Sampling

Larval *R. clamitans* have a relatively long larval development for amphibians where larvae typically hatch in late summer, overwinter as larvae in their natal pond, and metamorphose the following summer roughly 1 year after fertilization. From the two cohorts for which we had genotyped sex ratios in wild clutches in June and July 2017, we followed genotypic sex ratios during larval development. Specifically, we collected larvae in October 2017 prior to overwintering and we collected metamorphs in June 2018. Thus, we sampled tadpoles and metamorphs ∼95 and 337 days post-hatching, respectively, after we collected the final clutch in July 2017. We sampled larvae and metamorphs by comprehensively dipnetting the entirety of each waterbody, which should provide a relatively unbiased sample of each population. The phenotypic (i.e., gonadal) sex of tadpoles collected in October 2017 were not developmentally differentiated enough to be identifiable as ovaries or testes via dissection (Foote and Witschi, 1939; Lambert et al., 2016b) and so were only genotypically sexed. However, metamorphosing *R. clamitans* have well-differentiated gonads and so we assessed metamorph phenotypic sex by dissection as described previously (Lambert et al., 2015, 2016b). We also genotypically sexed all dissected metamorphs as described above. Larval and metamorph data are provided as **Supplementary Data Sheets 2, 3**.

Analysis

To evaluate whether genotypic sex ratios varied across ontogeny, we took an information theoretic approach to model our data. Specifically, we used binomial generalized linear models (GLMs) to model the frequency of each genotypic sex as a function of Pond (Forest5 or Septic7), developmental Stage (embryo, tadpole, and metamorph), and the effects of Pond and Stage together. We used the *aictab* function in the R (v 3.4.0) package "AICcmodavg" to compare AICc values and model weights for a null (intercept-only) model, each univariate model, and the additive model. We considered models to provide important explanatory power if they were within ΔAICc $\leq$ 2.0 of the top-ranked model (i.e., model with lowest AICc) and carried $\geq$ 0.10 of model weight. If the null model was the top-ranked model, a candidate model was not considered strong evidence but its trend was considered of interest if ΔAICc $\geq$ 2.0 of the null model and carried $\geq$ 0.10 of model weight. We constructed models using individuals as the unit of measurement and, because the number of embryo samples were much larger than the tadpole or metamorph samples, we excluded the tadpole samples to minimize the number of contrasts our models had to make between developmental stages. As such, this approach analyzes changes in genotypic sex frequencies from embryo to metamorphosis but does not incorporate an intermediate developmental stage, although these data are presented (**Figure 2**). We checked for the top models' fits by plotting residuals against predicted model estimates because small sample sizes in terms of the number of populations studied did not allow for more integrative model diagnostics. The associated R script and data csv are in **Supplementary Data Sheets 4, 5**.

We also explored whether individual clutch sex ratios in *R. clamitans* had the potential to differ from parity. Because sex ratios can require large sample sizes to detect deviations from parity using traditional frequentist statistical binomial approaches (e.g., Chi-squared analysis), we analyzed individual clutch sex ratio data within a probabilistic Bayesian framework using the maximum *a posteriori* (*map*) function in the R package "rethinking" following McElreath (2016). We only performed this analysis on clutch sex ratios as we did not analyze deviations from parity in sex ratios from other life stages. To test whether clutch genotypic sex ratios were skewed from parity, we used the *map* function and a binomial likelihood to assess whether the proportion of females in a sample differed from a flat prior (i.e., differed from an equal sex ratio). For each clutch we assessed deviation from parity using, for example, a script like: *precis(map(alist(f∼ dbinom(30, p), p∼ dunif(0,1)), data = list(f = 11)))*. In this example, the script tests for a sex ratio bias in a sample of 30 individuals where 11 are females.

Phenotypic Sex Ratio Relationships With Sex Reversal

Sex Ratios and Sex Reversal

Next, our goal was to understand the relationship – if any – between phenotypic sex ratio variation at metamorphosis and the frequency of sex reversal in either direction (female-to-male or male-to-female reversal). If sex reversal is the primary mechanism underlying phenotypic sex ratio variation, then male-biased phenotypic sex ratios would be correlated with higher frequencies of female-to-male sex reversal, and *vice versa* for female-biased phenotypic sex ratios. To do so, we leveraged specimens collected the prior year across six populations. In 2016, we collected metamorphosing *R. clamitans* tadpoles from six

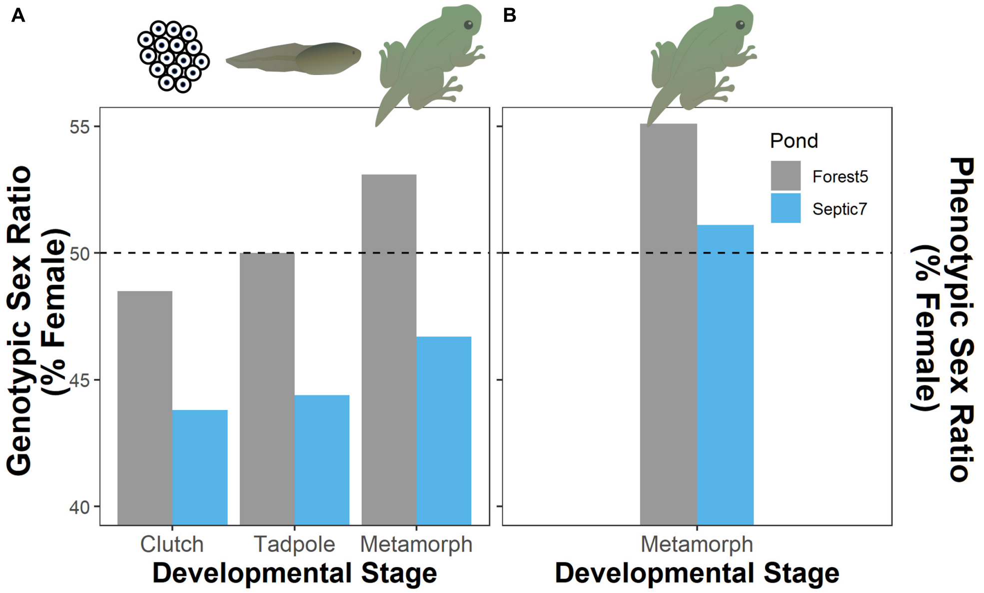

FIGURE 2 | Evidence for genotypic male-biased mortality as genotypic sex ratios **(A)** in both ponds (Forest5 and Septic7) become increasingly comprised of females as cohorts develop from clutches of embryos through the tadpole stage and into metamorphosis. Final phenotypic sex ratios in these cohorts **(B)** are comprised of more females than would be expected from genotypic sex ratios due to sex reversal in both cohorts (4 and 12.5% of genotypic males reversing in Forest5 and Septic7, respectively). The horizontal dashed line represents a hypothetical 50:50 sex ratio (phenotypic or genotypic).

ponds along a forest-suburban environmental gradient, including the two ponds (Forest5 and Septic7) sampled for the sex-biased mortality study. Because larval *R. clamitans* have a relatively long larval development for amphibians (∼1 year), *R. clamitans* larvae are sensitive to drought as ponds can dry in late summer or fall, resulting in complete mortality of entire larval cohorts prior to metamorphosis the following year. Because of the drought conditions, metamorphs were only present in a subset of ponds (*n* = 6; AV08, Septic3, Septic7, Septic10 Sewer3, Forest5) used in prior studies and which maintained water over winter (Lambert et al., 2015, 2019). Even so, the ponds sampled here cover the extent of a forest-suburban environmental gradient used in prior studies of sex ratios and sex reversal (Lambert et al., 2015, 2019). We surveyed ponds at least twice per week, collecting metamorphosing *R. clamitans* larvae from these six ponds as they emerged in summer 2016 and assessed gonadal sex via dissection to estimate phenotypic sex ratios. To infer sex reversal, we genotyped a random subset of metamorphs' sex (minimum of 55% of individuals in a cohort, but typically 70% or more) and identified individuals where genotypic sex differed from phenotypic sex.

Environmental Correlates

We also used these data to explore whether sex ratios and sex reversal were associated with differences in environmental conditions across populations. Although in our prior work we found that sex reversal occurred in both the female-to-male and male-to-female directions and was prevalent across 16 *R. clamitans* populations, including four forested and 12 suburban populations, we were unable to confidently assess environmental relationships with sex reversal frequencies (Lambert et al., 2019). This is because our prior study assessed sex reversal in adult *R. clamitans* and relating a single year of environmental data to sex reversal frequencies in adults is not possible because this species survives up to 7 years and adult populations comprise multiple overlapping cohorts (Shirose and Brooks, 1995). By assessing sex ratios and sex reversal at metamorphosis here, we can more confidently explore environmental relationships with sex ratios and sex reversal. We collected water condition data [specific conductance (i.e., conductivity), pH, dissolved oxygen, and temperature] at each survey using an Oakton PCSTestr 35 Multiparameter probe (conductivity, pH, and temperature) and a YSI ProODO Optical Meter for dissolved oxygen as described previously (Lambert et al., 2019). We used the mean measurement across surveys for each environmental condition in our statistical analyses. Using GIS and custom high-resolution suburban land use data, we also calculated the percent of land cover surrounding each pond that was either forest or suburban development (Lambert et al., 2019). Our prior work has found that, although environmental conditions certainly fluctuate throughout the year within a pond, relative differences among ponds are generally constant (Lambert et al., 2019). This is important here because the developmental period where *R. clamitans* larval sex determination is sensitive to environmental conditions is unclear. As such, our environmental correlates can help identify patterns of relative environmental conditions associated with sex.

Analysis

We used a similar modeling approach as described in the sex-biased mortality portion of our study. To analyze whether sex ratios varied as a function of sex reversal frequency, we again used binomial GLMs with phenotypic sex frequency as a binary response variable and assessed the independent and additive influence of female-to-male and male-to-female sex reversal frequencies on phenotypic sex ratios. We similarly modeled the relationship between environmental conditions and phenotypic sex ratios using the same approach except that we used each pond as the unit of measurement and express sex ratios as the proportion of females (individuals with phenotypic ovaries) and sex reversal rates as proportion of genotypic females with testes (female-to-male reversal) or genotypic males with ovaries (male-to-female reversal). Finally, we modeled environmental relationships and either direction of sex reversal separately. For female-to-male sex reversal, our response variable was a binary probability of a genotypic female having either testes or ovaries (i.e., a phenotypic male or female, respectively). We used the same modeling approach for male-to-female sex reversal but using genotypic males. Because the number of sampled populations ($n = 6$) was relatively low due to drought conditions, we only modeled the additive effects of at most two explanatory variables in a single model to avoid overfitting. The associated R script and data csv are in **Supplementary Data Sheet 6**.

RESULTS

Ontogenetic Genotypic Sex Ratio Variation

In total, we genotyped 598 embryos from seven and 13 clutches, respectively, from the two ponds Septic7 (n = 208 embryos total) and Forest5 (n = 390 embryos total). We sampled 36 tadpoles from each pond and genotyped the sex of all tadpoles. For metamorphs, we sampled 49 frogs from Forest5 and 45 from Septic7, genotyping all metamorphs. By tracking genotypic sex ratios from embryogenesis through metamorphosis in these cohorts, we found that genotypic sex-biased mortality – specifically male-biased mortality – contributed to phenotypic sex ratios in metamorphosing frog cohorts. In both ponds, genotypic sex ratios become more female-biased as larvae developed from embryos to tadpoles through metamorphosis (**Figure 2**). Our analysis found evidence for sex-biased mortality (**Table 1**). In this analysis, an intercept-only model was ranked highest but a model including Stage received sufficient support to infer consistent male-biased mortality between both ponds. Additionally, male-to-female sex reversal caused the final phenotypic sex ratios to become further female-biased relative to the initial genotypic sex ratios of these cohorts' clutches (**Figure 2**).

Interestingly, individual clutch genotypic sex ratios varied from 36.7 to 63.3% female. Our analyses support male-biased genotypic sex ratios for four clutches, two from Septic7 (S7_1 and S7_6) and two from Forest5 (F5_1 and F5_2; **Figure 3**). The cohort genotypic sex ratio estimated by summing across clutches for Septic7 also had a significant male bias (**Figure 3**). There was also support for two clutches from Forest5 (F5_4 and F5_13) exhibiting a genotypic female-bias (**Figure 3**).

Phenotypic Sex Ratio Relationships With the Environment and Sex Reversal

From across the six ponds, we phenotypically sexed 221 (n = 29–56) metamorphosing *R. clamitans*. Phenotypic sex ratios in metamorphs from the six focal ponds in 2016 ranged from 39–52% female. We did not identify any environmental correlates of phenotypic sex ratios. Of our models evaluating possible environmental correlations with phenotypic sex ratios, no model including environmental variables improved model fit over a null, intercept-only model all models (ΔAICc > 4.3 over null model, AICc Weight $\leq$ 0.08; **Table 2**).

We genotyped the sex of a random subset of these metamorphosing *R. clamitans* larvae from each pond (n = 164 total; n = 18–31 per pond) and inferred sex reversal by identifying differences between each metamorph's genotypic and gonadal sex (i.e., ovaries vs. testes). Consistent with prior work in adult *R. clamitans* (Lambert et al., 2019), we found both female-to-male and male-to-female sex reversal directions, each occurring across roughly half of the study populations. Neither direction of sex reversal provided a better fit to the phenotypic sex ratio data than an intercept-only model (**Table 2**), suggesting sex reversal is not tightly or consistently associated with phenotypic sex ratios.

Environmental Predictors of Sex Reversal

Female-to-male sex reversal was best predicted by dissolved oxygen concentrations in ponds with a univariate model carrying > 50% of model weight (**Figure 4** and **Table 3**). Models containing pH, dissolved oxygen + temperature, and temperature alone were also relevant candidate models for female-to-male sex reversal in metamorphosing *R. clamitans* (**Figure 4** and **Table 3**). In sum, rates of female-to-male sex reversal were highest in metamorphosing tadpoles developing in ponds with low dissolved oxygen, more acidic pH, and cooler pond temperatures.

No environmental variables explained male-to-female sex reversal (i.e., an intercept-only model was the best supported model; **Table 3**). However, univariate models containing suburban land use and mean pond water temperatures carried > 24% of AICc model weight each and were within 2.0 ΔAICc from the null model (**Table 3**). These results provide limited evidence that rates of male-to-female sex reversal were highest in ponds impacted by surrounding suburban land use and ponds with warmer average water temperatures.

We note that, for the two focal populations we studied for the sex-biased mortality part of the study in 2018 and in the sex reversal part of our study, the extent of sex reversal in metamorphosing cohorts varies between populations and among years within a population (**Figure 5**). Additionally, our focal environmental variables (dissolved oxygen, temperature, and pH) and suburban land use were largely uncorrelated in this study (linear regression, all $p \geq 0.14$), except for a correlation between pond pH and dissolved oxygen (linear regression, $p = 0.009$, $R^2 = 0.81$).

TABLE 1 | Analysis of models of genotypic sex ratio across developmental stages (a proxy of sex-biased mortality) and between ponds using individual sample as the unit of measurement.

	Variables	AICc	Delta_AICc	AICcWt	Cum.Wt
Genotypic sex ratio: Both ponds	Intercept only	959.23	0.00	0.38	0.38
	Pond	959.82	0.58	0.28	0.66
	Stage	960.92	1.68	0.16	0.82
	Stage + Pond	961.36	2.13	0.13	0.95
	Stage × Pond	963.36	4.13	0.05	1.00
Genotypic sex ratios: Forest5	Intercept only	610.41	0.00	0.70	0.70
	Stage	612.06	1.65	0.30	1.00
Genotypic sex ratios: Septic7	Intercept only	349.42	0.00	0.72	0.72
	Stage	351.32	1.90	0.28	1.00

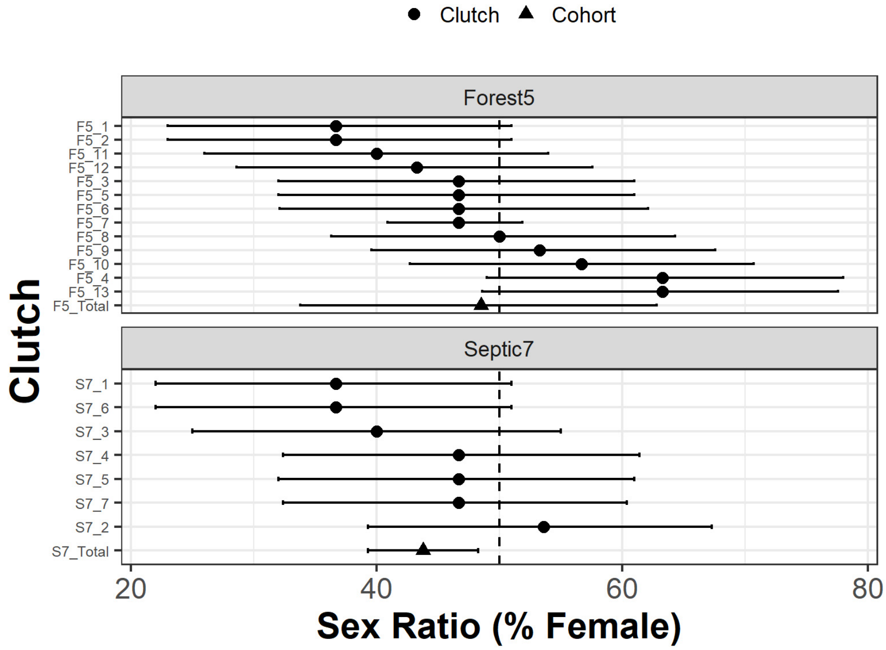

FIGURE 3 | Genotypic sex ratios for individual clutches (circles) in two *R. clamitans* populations. Clutch identifiers are on the *y*-axis. Cohort genotypic sex ratios (triangles) are pooled across clutches. The vertical dashed line indicates sex ratios at parity (50% female and 50% male). Horizontal bars are Bayesian credible intervals and higher support for biased sex ratios is observed in clutches with credible intervals that minimally overlap or do not overlap the vertical line at parity.

DISCUSSION

The pattern of genotypic sex ratio in multiple populations from the embryo stage to metamorphosis is consistent with an important role for sex-biased mortality in amphibians. Although there is evidence of sex-biased mortality in juvenile mammals, birds, and even some fish (Kruuk et al., 1999; Cichon et al., 2005; Svensson et al., 2007; Orzack et al., 2015; Moran et al., 2016; Kato et al., 2017; Firman, 2019), to our knowledge, this is the first record of sex-biased mortality in larval amphibians in the wild. Importantly, we find the same pattern of male-biased mortality across two independent frog populations which inhabit contrasting environments, suggesting sex-biased mortality may be a relatively common phenomenon. Although we identified both directions of sex reversal, we found little evidence that sex reversal frequencies are consistently associated with phenotypic sex ratios. Even though sex reversal may not be the dominant mechanism undergirding phenotypic sex ratio variation, sex reversal still contributes to phenotypic sex ratios. In the populations we studied, male-biased mortality produced proportionally more genotypic females over larval development and additional sex reversal led to proportionally more phenotypic females than expected from genotypic sex ratios alone. The degree of sex-biased mortality we detected was modest (3–5% percentage change) but the degree of sex ratio change due to sex-biased mortality is sufficient to have important demographic consequences (Wedekind, 2017). To be clear, we identified sex-biased mortality as a mechanism that shapes sex ratios of individual wild amphibian populations, but our study was not designed to document whether sex-biased mortality shapes sex ratio variation across populations. Additionally, we note that our limited sample size of six populations may have had

TABLE 2 | Models of phenotypic sex ratios as a function of environment and sex reversal.

	Variables	AICc	Delta_AICc	AICcWt	Cum.Wt
Environmental predictors	Intercept only	26.92	0.00	0.71	0.71
	pH	31.31	4.39	0.08	0.79
	Temp	31.38	4.47	0.08	0.86
	DO	31.48	4.56	0.07	0.94
	Suburban	31.88	4.97	0.06	1.00
	Suburban + Temp	40.54	13.63	0.00	1.00
	pH + Temp	41.03	14.11	0.00	1.00
	Temp + DO	41.09	14.17	0.00	1.00
	pH + DO	41.29	14.37	0.00	1.00
	Suburban + pH	41.31	14.39	0.00	1.00
	Suburban + DO	41.48	14.56	0.00	1.00
Sex reversal predictors	Intercept only	26.92	0.00	0.83	0.83
	Female-to-Male sex reversal	31.27	4.35	0.09	0.93
	Male-to-Female sex reversal	31.81	4.90	0.07	1.00
	Female-to-Male + Male-to-Female sex reversal	41.22	14.31	0.00	1.00

limited power to detect a relationship between sex reversal and phenotypic sex ratios, although the lack of a detectable pattern with these six population is notable. Sex-biased mortality, to our knowledge, is a previously undocumented process in larval amphibians and acts in concert with other processes like sex reversal to shape the phenotypic sex ratios of entire cohorts.

The causes for sex-biased mortality in this system remain to be studied, but both chromosomal and ecological processes may contribute. Heterogametic sex chromosomes (Y-chromosomes in species with XX-XY sex chromosome and W-chromosome in species with ZZ-ZW sex chromosomes) are often degenerate and accumulate deleterious alleles (Charlesworth and Charlesworth, 1997; Gordo and Charlesworth, 2001; Bachtrog, 2008; Miura et al., 2012; but see Kratochvil et al., 2021). As such, these degraded sex chromosomes can reduce survival and longevity in a diversity of animals (Xirocostas et al., 2020). Given *R. clamitans* has an XX-XY sex chromosome system (Lambert et al., 2016b), *R. clamitans* Y-chromosomes may be degraded (although there is no evidence for sex chromosome heteromorphy) which could explain higher rates of genotypic male mortality early in life. The absence of YY-individuals in our study may provide additional support for this hypothesis if YY-embryos are frequently unfit, as is often expected (Bull, 1983; Charlesworth, 1996). However, embryos with two Y-chromosomes may also never exist in wild populations if ecological factors limit reproduction by male-to-female reversed frogs.

Ecological differences between genotypic female and male larvae may also contribute to sex-biased mortality. For instance, pilot laboratory data suggest that male wood frog (*Rana sylvatica*) tadpoles exhibit higher activity patterns than female tadpoles (Skelly et al., unpubl.). Higher activity in tadpoles is associated with higher mortality due to predation (Skelly, 1994). If *R. clamitans* larvae similarly display sex-specific behaviors, then there is the possibility for sex-biased predation that could contribute to the sex-biased mortality we observed. Further, sex-specific behaviors may also influence sex-specific sampling efficacy. For instance, if sexes differ in microhabitat preferences, different types of sampling approaches may be biased toward one sex or another. Although identifying this issue is beyond the scope of our study, we sampled through the entire water body of each pond, including the benthos and surface and so presumably this bias should not influence our results. In mammals and birds, evidence also suggests that stress or poor health in parents can lead to sex-biased mortality in embryos and offspring (Kruuk et al., 1999; Muller et al., 2005; Perez et al., 2006; Firman, 2019). It is possible that reduced body condition in breeding *R. clamitans* similarly contributes to sex-biased mortality of embryos and tadpoles. Additionally, sex-biased mortality may be a result of female and male differences in tolerance of various environmental conditions like temperature, as has been seen in birds (Goth and Booth, 2005; DuRant et al., 2016; Eiby et al., 2008). Support for these and other hypotheses require further research.

Our analyses provide some evidence for environmental conditions associated with sex reversal. Specifically, our data suggest that natural water chemistry (dissolved oxygen, pH, and temperature) are related to genotypic female *R. clamitans* developing as phenotypic males. Experimental evidence in fishes shows that sex reversal can result from variation in dissolved oxygen and pH, and so these conditions may be relevant to amphibian sexual development (Romer and Beisenherz, 1996; Oldfield, 2005; Shang et al., 2006). We also have correlative evidence of temperature-mediated sex reversal where cooler temperatures contribute to higher rates of female-to-male sex reversal. Intriguingly, the opposite trend occurred for male-to-female sex reversal where warmer temperatures contribute to more male-to-female reversal, although the strength of evidence was weak. Temperature-mediated sex reversal in amphibians has been explored in laboratory experiments (Witschi, 1929; Wallace and Wallace, 2000; Lambert et al., 2018) but, to our knowledge, this is the first evidence from wild populations. Sex reversal in amphibians has, in recent decades, been assumed to be strictly caused by environmental pollutants or to unnatural conditions imposed by laboratory experiments (Hayes, 1998;

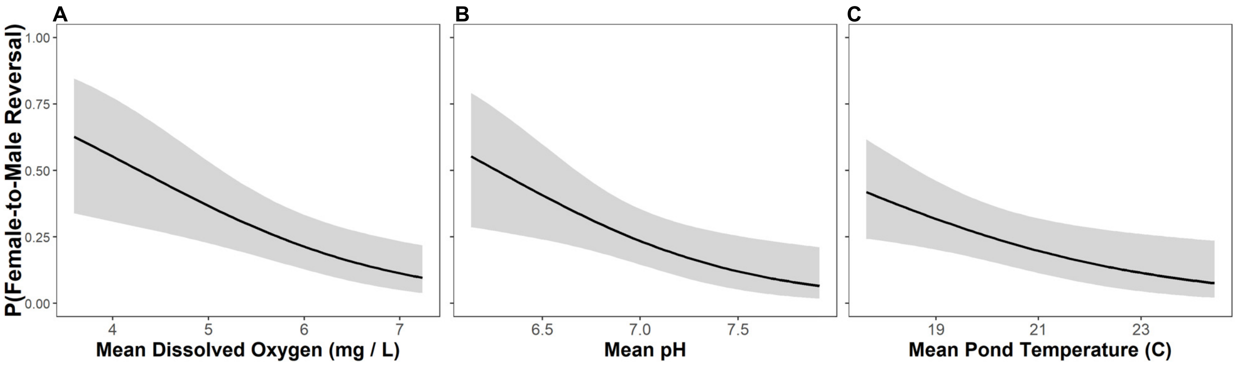

FIGURE 4 | Female-to-male sex reversal rates are highest in metamorphosing green frog (*Rana clamitans*) tadpoles developing in ponds with lower dissolved oxygen **(A)**, more acidic pH **(B)**, and cooler water temperatures **(C)**.

TABLE 3 | Model ranking for environmental predictors of sex reversal.

	Variables	AICc	Delta_AICc	AICcWt	Cum.Wt
Female-to-Male sex reversal	DO	30.46	0.00	0.51	0.51
	pH	32.95	2.49	0.15	0.66
	DO + Temp	33.36	2.89	0.12	0.78
	Temp	33.81	3.35	0.10	0.88
	Intercept only	35.56	5.10	0.04	0.92
	pH + Temp	35.71	5.24	0.04	0.95
	Suburban + DO	36.60	6.14	0.02	0.98
	Suburban	38.59	8.13	0.01	0.99
	Suburban + pH	38.94	8.48	0.01	0.99
	DO + pH	39.70	9.23	0.01	1.00
	Suburban + Temp	41.56	11.09	0.00	1.00
Male-to-Female sex reversal	Intercept only	24.87	0.00	0.32	0.32
	Suburban	25.13	0.26	0.28	0.61
	Temp	25.41	0.54	0.25	0.86
	DO	28.36	3.49	0.06	0.91
	pH	29.09	4.22	0.04	0.95
	Suburban + pH	30.97	6.10	0.02	0.97
	Suburban + Temp	31.49	6.62	0.01	0.98
	pH + Temp	32.02	7.15	0.01	0.99
	DO + Temp	32.26	7.39	0.01	1.00
	Suburban + DO	35.06	10.19	0.00	1.00
	DO + pH	37.87	13.00	0.00	1.00

Eggert, 2004; Nakamura, 2013; Orton and Tyler, 2015). This contrasts with work over a century ago that assumed sex reversal was largely a natural process (King, 1909, 1910; Witschi, 1929). Our findings here and previously in both metamorphosing and adult *R. clamitans* – in addition to recent work on European Agile Frogs (*Rana dalmatina*) – suggest that sex reversal can be natural and not necessarily a response to abnormal conditions because sex reversal has been observed under natural conditions in the wild for both species (Lambert et al., 2019; Nemeshazi et al., 2020). The conditions (dissolved oxygen, pH, and temperature) correlated with sex reversal vary naturally but can also be influenced by human land use (Holgerson et al., 2018; Lambert et al., 2019). However, in our study, these variables were uncorrelated with suburban land use (all $p \geq 0.14$), suggesting that female-to-male reversal is largely natural. Although, our analysis did provide limited support that suburban land used increased the rate of male-to-female sex reversal. These patterns should encourage further work on sex reversal and the relative effect of natural and anthropogenic causes in wild amphibians.

Temporal variation may complicate identifying relationships between the environment, sex ratios, and sex reversal. For instance, in our two focal populations (Forest5 and Septic7), the presence, direction, and frequency of sex reversal varied not only between the two populations but also between years (**Figure 5**). This resulted in among-year differences in phenotypic and genotypic sex ratios (**Figure 6**). Among-year variation may explain why our results seemingly contrast with previous research. In prior work in this system, metamorphs

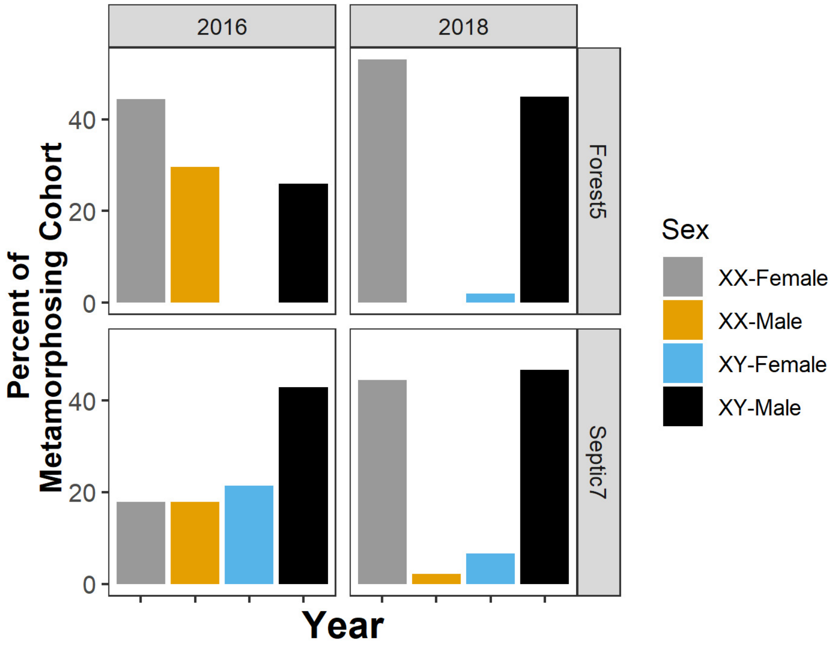

FIGURE 5 | The presence, direction, and frequency of sex reversal in metamorphosing cohorts varies between populations and among years within a population. Sexual genotype-phenotype data from two focal ponds – Forest5 and Septic7 – in the years 2016 and 2018. Orange and blue bars represent frequencies of sex-reversed metamorphs and gray and black bars represent non-reversed frogs.

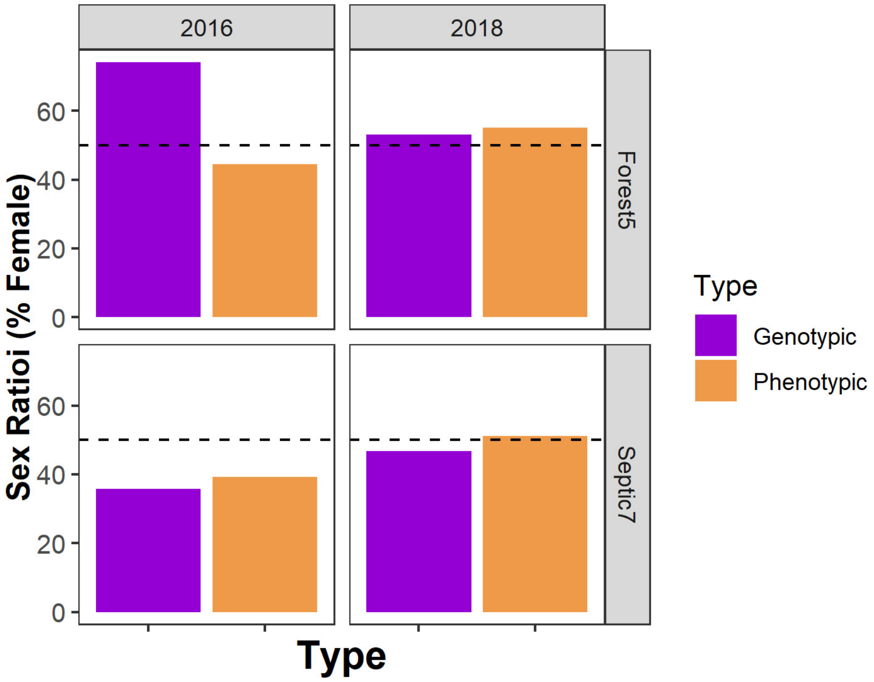

FIGURE 6 | Genotypic and phenotypic sex ratios vary not only among populations but also among years within a population. Horizontal dashed lines indicate a hypothetical 50:50 sex ratio. Here are genotypic and phenotypic sex ratio data from two focal ponds – Forest5 and Septic7 – in the years 2016 and 2018.

sampled across 13 populations in 2012 consistently had male-biased phenotypic sex ratios across multiple forest ponds but equal or female-biased sex ratios in suburban ponds (Lambert et al., 2015). We found no such association between the environment and sex ratios in metamorphs sampled in 2016. Additionally, due to drought, we had a smaller number of populations ($n = 6$) available for study. Although this sample size is like what is often used in similar studies (Hayes et al., 2003; Orton and Routledge, 2011; Papoulias et al., 2013), it is half of the sample size of our prior work (Lambert et al., 2015). How yearly differences in environmental conditions influence sex ratios and sex reversal is unclear but documenting interannual variation is likely important for understanding the natural and anthropogenic contributions to wild amphibian sexual demography. Importantly, work in fishes and reptiles suggests that climate change is having and will continue to shape the sexual demography of wild animal populations (Ospina-Alvarez and Piferrer, 2008; Montalvo et al., 2012; Holleley et al., 2015). Additionally, experimental research in amphibians provides unambiguous evidence that temperature can modulate amphibian sex reversal (Lambert et al., 2018; Miko et al., 2021). Given the associations between drought, temperature, sex reversal, and sex ratios observed in *R. clamitans* here, there is a need to understand how climate change will shape sex reversal and population demography in wild amphibians.

We also found that genotypic sex ratios of individual clutches are variable and can deviate from parity. Breeding by sex-reversed individuals could produce a diversity of potential genotypic sex ratios (**Figure 7**) which, in aggregate, could hypothetically shape cohort sex ratios. Four clutches (two from each pond) had male-biased genotypic sex ratios. The most likely explanation for this pattern is that male-to-female reversed adults successfully breed, although further work is needed to document breeding by sex-reversed frogs. Interestingly, we also found two clutches in our focal forest pond which had female-biased genotypic sex ratios. Mating between only two frogs – a sex-reversed genotypic female and a non-sex reversed female – would produce a clutch with only genotypically female embryos (i.e., all XX sex chromosome genotypes). However, the female-biased clutch genotypic sex ratios we observed were ∼ 2/3 female, not entirely genotypically female. The only other study, to our knowledge, to assess clutch sex ratios was on European Common Frogs (*Rana temporaria*) and also found several clutches that were ∼ 2/3 genotypic female (Alho et al., 2010). Alho et al. (2010) interpreted this as a

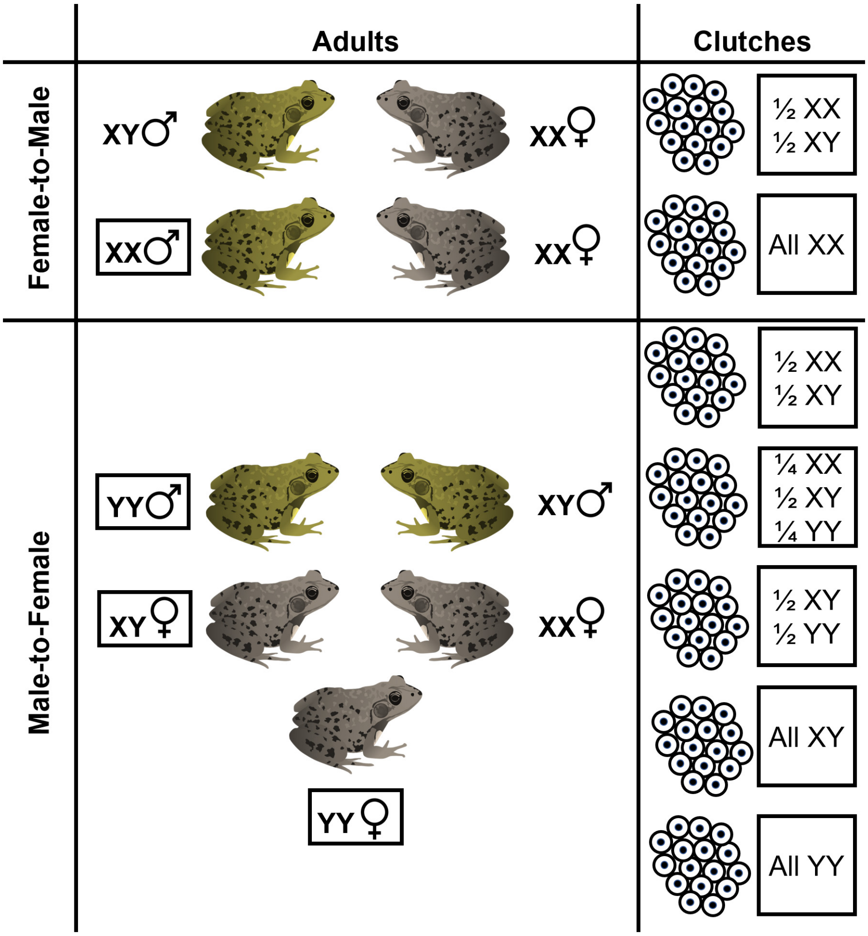

FIGURE 7 | Breeding by sex-reversed adults, either due to female-to-male or male-to-female sex reversal, can result in a diversity of hypothetical clutch genotypic sex ratios. In species such as *R. clamitans* which have an XX-XY male heterogametic sex chromosome systems, if YY individuals are viable, sex reversal may produce phenotypic females that have two Y-chromosomes. Shown are the various hypothetical genotypic-phenotypic sex combinations for adult frogs and associated clutch genotypic sex ratios that can result from various crossings. Other genotypic sex ratios may occur for clutches if mutliple paternity or clutch piracy occurs.

result of multiple paternity where one father was a sex-reversed XX-male. While the drivers of female-biased (but not entirely female) clutches are unclear, multiple paternity and "clutch piracy" is known to occur in frogs (Laurila and Seppa, 1998; Vietes et al., 2004) and so these sex ratios could potentially be the result of mating between a sex-reversed genotypic female, a sex-concordant male, and a sex-concordant female. Our individual clutch genotypic sex ratios provide suggestive evidence that sex-reversed frogs breed in the wild which may skew individual clutch and entire cohort genotypic sex ratios at fertilization (**Figure 1**). Direct observations are still needed to confirm whether sex-reversed adults successfully reproduce and whether this breeding can sufficiently skew entire cohort genotypic sex ratios. We note that because our clutch samples represent field-collected embryos that were hatched in the lab, it is possible that sex-biased mortality occurred during lab hatching, skewing our inferences of clutch sex ratios. Even so, mortality in our lab-hatched embryos was negligible and likely did not impact our inferences here.

Our work has implications for how we study the role sex variation plays in population, community, and ecosystem biology. The growing recognition that sex reversal in amphibians is common including in environments assumed to be natural and minimally impacted by people – including our observations here – highlights how a binary model of sex (i.e., female vs. male) limits how we study the biology of sex (Alho et al., 2010; Lambert et al., 2019; Nemeshazi et al., 2020). In the case of amphibian sex reversal, we can conceive of at least four sex categories depending on the combination of an individual's genotypic and phenotypic sex. However, these sex categories may still be incomplete as sexual phenotypes and functions may occur along a gradient rather than discrete categories and sex-reversal may create new emergent behavioral and morphological phenotypes that differ from expectations based on constituent phenotypic or genotypic sexes (Li et al., 2016). Sex ratios can influence intraspecific interactions in a population and sex differences in behavior or morphology can shape predation rates and impacts on community and ecosystem processes (Lode et al., 2004, 2005; Weir et al., 2011; Liker et al., 2014; Fryxell et al., 2015). Given this, sex reversal combined with sex-biased mortality has the potential to influence the role of individuals within populations as well as how populations of different genotypic and phenotypic sex compositions influence the broader biological community and ecosystem properties. There has been an increasing call for biologists to rethink our understanding of sex roles and sexual variation in amphibians may open opportunities to expand this area of inquiry (Ah-King and Ahnesjo, 2013; Monk et al., 2019; Warkentin, 2021).

We document multiple processes that work together to shape phenotypic sex ratios of metamorphosing tadpoles. Particularly noteworthy is the consistent pattern of male-biased mortality between two disparate populations. Sex-biased mortality among pre-metamorphic life stages has, to our knowledge, never been documented before in amphibians and represents a previously-unknown contribution to amphibian population demography. Sex-biased mortality, particularly in the context of sex reversal, has the potential to shape amphibian ecological and evolutionary dynamics due to the underappreciated influence on both the genotypic and phenotypic sexual makeup of populations. We further highlight how phenotypic sex ratios may not be a reliable proxy for important biological processes. For example, sex-biased mortality could skew *genotypic* sex ratios in one direction, but sex reversal could bring *phenotypic* sex ratios back to parity; superficially this equal sex ratio would appear unremarkable whereas it is in fact shaped by the joint effects of sex-biased mortality and sex reversal. Continued exploration of the processes underlying the sexual demography of wild populations will be essential not only to our general understanding of population biology but to conserving populations and species in the face of global environmental change.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Yale IACUC Prototocols 2013-10361 and 2015-10681.

AUTHOR CONTRIBUTIONS

ML, TE, and DS conceived the project idea, contributed to the writing and editing of the manuscript. ML performed field and laboratory procedures and conducted statistical analyses. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by NSF DDIG-1701311 to ML and DS.

ACKNOWLEDGMENTS

We thank R. Denton, A. Georges, A. Kamath, J. Monk, M. Packer, O. Schmitz, G. Wagner, and A. Wesner for conversations about the biology of sex.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.756476/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 21 October 2021
doi: 10.3389/fevo.2021.750483

Sex-Specific Routes to Independent Breeding in a Polygynous Cooperative Breeder

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: 30 July 2021
Accepted: 20 September 2021
Published: 21 October 2021

Citation:
Josi D, Flury JM, Reyes-Contreras M, Tanaka H, Taborsky M and Frommen JG (2021) Sex-Specific Routes to Independent Breeding in a Polygynous Cooperative Breeder. Front. Ecol. Evol. 9:750483. doi: 10.3389/fevo.2021.750483

How can individuals obtain a breeding position and what are the benefits associated with philopatry compared to dispersal? These questions are particularly intriguing in polygamous cooperative breeders, where dispersal strategies reflect major life history decisions, and routes to independent breeding may utterly differ between the sexes. We scrutinized sex-dependent life-history routes by investigating dispersal patterns, growth rates and mortality in a wild colony of the cooperatively breeding cichlid *Neolamprologus savoryi*. Our data reveal that female helpers typically obtain dominant breeding positions immediately after reaching sexual maturity, which is associated with strongly reduced growth. In contrast, males obtain breeder status only at twice the age of females. After reaching sexual maturity, males follow one of two strategies: (i) they may retain their subordinate status within the harem of a dominant male, which may provide protection against predators but involves costs by helping in territory maintenance, defence and brood care; or (ii) they may disperse and adopt a solitary status, which diminishes survival chances and apparently reflects a best-of-a-bad-job strategy, as there are no obvious compensating future fitness benefits associated with this pathway. Our study illustrates that sex-dependent life history strategies strongly relate to specific social structures and mating patterns, with important implications for growth rates, the age at which breeding status is obtained, and survival.

Keywords: benefits-of-philopatry, polygamy, delayed dispersal, territory inheritance, cooperation, cichlid fish, *Neolamprologus savoryi*, ecological constraints

INTRODUCTION

Understanding the evolutionary mechanisms underlying variation in dispersal and life-history decisions is a major challenge in evolutionary biology and behavioural ecology (Clobert et al., 2012; Li and Kokko, 2019). Resource competition between relatives, risk of inbreeding, and environmental stochasticity are major factors affecting natal dispersal (Hamilton and May, 1977; Bonte et al., 2012; Clobert et al., 2012). Delayed dispersal is common in animals, and it is often caused by ecological constraints, such as a lack of suitable breeding vacancies or enhanced predation risk. These constraints prevent individuals from leaving home to acquire an independent breeding position elsewhere ("ecological constraints hypothesis"; Emlen, 1982; Pruett-Jones and Lewis, 1990; Komdeur, 1992; Heg et al., 2004; Tanaka et al., 2016). Individuals may furthermore gain higher fitness returns from remaining in the natal territory and reaping benefits from group membership and the potential territory inheritance ("benefits of philopatry hypothesis"; Stacey and Ligon, 1991; Kokko and Ekman, 2002; Kingma et al., 2014). Nevertheless, dispersal is the only possibility to expand one's range beyond the natal territory and to potentially access new resources such as breeding positions. Elucidating why, when and where individuals disperse is hence crucial if we aim to comprehend the evolution of complex sociality and cooperative breeding (Koenig and Dickinson, 2016; Rubenstein and Abbot, 2017; Kingma et al., 2021).

Dispersal decisions are selected to take account of various life-history traits such as growth, maturation and survival probability, which feedback on the breeding system by influencing resource access, mate choice, and parental care (McNamara et al., 2000; Kokko and Jennions, 2008; Mabry et al., 2013; Székely et al., 2014). A close relationship between the predominant mating pattern in a population and sex-specific dispersal has been hypothesised, with male-biased dispersal being associated with polygynous mating, and female-bias or no bias going along with monogamous mating (Greenwood, 1980; Perrin and Mazalov, 2000; Mabry et al., 2013; Li and Kokko, 2019). In other words, a particular mating system is likely to coincide with sex-specific life-history strategies that are characterised, for instance, by differences in growth and size, age at maturity, adult life-span, and dispersal (Badyaev, 2002; Hamilton and Heg, 2008; Székely et al., 2014; Trochet et al., 2016). For example, competition for territories and reproductive opportunities is typically stronger in males than in females due to sex-specific differences in the possibility to economically monopolise resources and mates (Emlen and Oring, 1977). This may select for a delayed start of reproduction in males in order to attain a large body size providing superiority in contests over resources and mating partners (Warner, 1984; Taborsky, 2016). The reproductive success of females, on the other hand, depends on brood size and hence the volume of the body cavity, but also on the number of broods she can produce and successfully raise (sensu fecundity selection; Hernaman and Munday, 2005). These fitness correlates in females can be strongly affected by the quality of the mate and his contribution to care (Webster, 1991; Huk and Winkel, 2006), which again may be size-dependent. Optimal dispersal decisions of females may consequently diverge from those of males (Promislow et al., 1992; Pakanen et al., 2016; Suh et al., 2020).

In cooperatively breeding species, groups are usually composed of individuals belonging to different generations, which implies divergence in important life history parameters such as growth, maturation, survival, and offspring care (Devillard et al., 2004; Koenig and Dickinson, 2016; Rubenstein and Abbot, 2017). The routes to independent breeding in such highly social animals are influenced by the likelihood to inherit resources from the breeders (Stiver et al., 2006; Leadbeater et al., 2011), reproductive skew within social groups (Johnstone and Cant, 1999; Taborsky, 2009), and costs of alloparental care (Taborsky and Grantner, 1998; Heinsohn and Legge, 1999), which all may affect dispersal decisions of group members (Clutton-Brock et al., 2002; Kingma et al., 2016). Furthermore, ecological factors such as the availability of suitable territories (Hatchwell and Komdeur, 2000), predation risk (Tanaka et al., 2016), and climatic conditions (Jetz and Rubenstein, 2011) may select for delayed dispersal and alternative routes to independent breeding (Eikenaar et al., 2009; Rubenstein, 2011). In many cooperative breeders individuals leaving their natal group go through a solitary phase before starting to breed independently (Kokko and Ekman, 2002; Koenig and Dickinson, 2016). The occurrence and duration of such "floating" phases often differs between the sexes (Eikenaar et al., 2009) and may be associated with survival costs due to increased predation risk (Taborsky, 1984; Ridley et al., 2008). Sex-specific costs and benefits of philopatry and dispersal (e.g., increased risk of mortality or reduced lifetime reproductive success) may yield alternative ways to become a breeder in highly social animals (Stiver et al., 2007; Kingma et al., 2016). To understand the evolutionary mechanisms underlying dispersal decisions in cooperative breeders it is hence important to trace individual life-histories and relate them to the way by which group members obtain breeder status (Kingma et al., 2016).

Here we studied the costs and benefits associated with different life-history routes to obtain breeder status in the cooperatively breeding cichlid, *Neolamprologus savoryi* (Heg et al., 2005). In this species, polygynous males monopolize several females that defend their own sub-territories, often together with brood care helpers (Josi et al., 2020a,b). Male territories usually cluster together and form colonies with complex relatedness structures (Josi et al., 2021). As in many other polygynous systems males exceed females in size (Heg et al., 2005). Offspring delay dispersal and support the breeder female in egg care, territory maintenance, and defence (Heg et al., 2005; Josi et al., 2019, 2020a,b). With increasing helper age, relatedness to the dominants decreases due to breeder turn-over, dispersal, and extra-pair parentage (Josi et al., 2021). Before reaching sexual maturity male and female helpers are of similar body shape and colour (**Figure 1**). Sexes take different life-history routes to reproduction: Females become breeders either by taking over their natal or a foreign territory, or by establishing a new territory (Josi et al., 2021). Males usually remain as helpers in a sub-territory until reaching a body size similar to the breeder female. Thereafter, they show two alternative trajectories before eventually becoming breeder: (i) They defend an own sub-territory, which has overlap with a dominant male's territory and with the territories of females in that male's harem. Here, they continue helping in territory defence and show submission to the dominant male (in the following these males are termed "subordinate males"; **Figure 2**). (ii) They disperse and defend an independent territory with little overlap to any other territories. These males (termed "solitary males"; **Figure 2**) do not show helping behaviour or submission to other males. Both trajectories may eventually result in either establishing a new harem or taking over a

FIGURE 1 | A breeding group of *Neolamprologus savoryi* consisting of a breeder male (largest fish on top), a breeder female (second largest fish below), as well as one large and one small helper of unknown sex. The group defends the female's sub-territory, with the main shelter being under the rock in the centre of the picture.

territory of a harem owner. Males and females that lose their breeder position were never observed to remain as a helper in the territory, but usually got evicted and most likely predated eventually.

This intersexual and intrasexual variation in the routes to independent breeding renders *N. savoryi* an intriguing model to elucidate sex-specific costs and benefits of alternative dispersal strategies. To that end we traced a wild colony of *N. savoryi* over two consecutive years and monitored dispersal patterns of males and females. Repeated capture of all colony members enabled us to estimate growth, age, dispersal trajectories and survival in and around the entire colony. With these data we aimed at answering the following questions: (1) How does a polygynous mating system in a cooperatively breeding species relate to sex-specific differences in life-histories (i.e., growth, age at maturity, and survival) and routes to independent breeding? The polygynous mating pattern suggests that the higher competition for a breeding position among males selects for delaying the onset of independent reproduction to attain large size for competitive superiority, as compared to females. This predicts intersexual differences in growth rates, age at maturity, dispersal decisions, survival, and the routes to independent breeding. (2) How does the sex-specific role of subordinate group members (i.e., brood care helpers) affect fundamental life history decisions, such as dispersal? The potential to inherit the territory should be greater in females than in males due to the polygynous group structure, causing more places to fill in the territory of residence for females than for males. Hence, the cooperative breeding pattern suggests that females should remain philopatric and reach a breeding position earlier than males. Furthermore, male helpers are a greater risk to male breeders than female helpers are to female breeders, due to the sex-specific costs of reproductive parasitism (Josi et al., 2021). This implies that males should be forced to leave their natal territory more readily than females, which may favour a solitary phase. Interestingly, regarding the timing of dispersal the mating pattern and the cooperative breeding system predict contrasting sex-specific decisions.

MATERIALS AND METHODS

Data were collected by SCUBA diving at the southern tip of Lake Tanganyika at Kasakalawe Point, Republic of Zambia, in September and October 2016 and 2017. Here, groups of *N. savoryi* defend territories containing shelters under rocks in 9–11 m depth (**Figure 1**; Heg et al., 2005). In both study years a topographic map was established to assess group structures and to mark territories (**Figure 2**; see Josi et al., 2020b for details). Home ranges of all individuals larger than 1.5 cm were assessed by observing individuals continuously for 20 min (cf. Tanaka et al., 2015; Josi et al., 2020b). Group compositions were determined based on home ranges, social interactions, and breeding chamber visits. Afterward, all fish were captured to determine their sex and standard length (SL). A small piece of fin tissue (approx. 1 mm^2) was collected for genetic analyses. In all cases the tissue re-grew after a short period of time. Within years, individuals could be reliably identified using a combination of their size, individual colour patterns and location (Josi et al., 2020b). We re-identified individuals between the 2 years using 14 polymorphic microsatellites (Josi et al., 2019). Additionally, we took genetic samples from individuals in all isolated territories scattered around the focal colony in 2017 in search for potential long-distance dispersers. In both years, group compositions were repeatedly checked to identify potential dispersers. Details on microsatellites and genetic analyses are given in Josi et al. (2019).

We sampled fish from 48 different harems. The social structure of the colony changed between years. In 2016 we captured 182 individuals from which 9 were captured twice within a 3-months period (**Table 1**). In 2017 we captured 177 individuals in the focal colony and in close-by isolated territories to identify recaptures (**Table 1**). Of this sample, 34 individuals were recaptures from 2016 (recapture rate between years = 18.57%). In 2017 we also recaptured most individuals within the 3-months observation period (**Table 1**). We only included individuals as recaptures if a minimum interval of 20 days was given between the catches. In both years combined we recaptured 82 females and 94 males. In total, nine individuals were too small to be reliably sexed (**Table 1**).

Statistical Analysis

The individual growth rate (mm/day) was calculated as the difference in SL between the two capture events, divided by the number of days in between. A linear mixed effect model (LMM) was fitted to compare the growth rates of males (N = 94) and females (N = 82) in relation to the initial size [ln(SL)] to account for exponentially diminishing growth over time. Growth rate was set as response variable and the initial size and sex as well as the respective interaction were included as predictors. Additionally, *Harem ID* and *Catcher ID* were included as random factors to account for non-independence of group members and for potential differences in size measurements by different persons

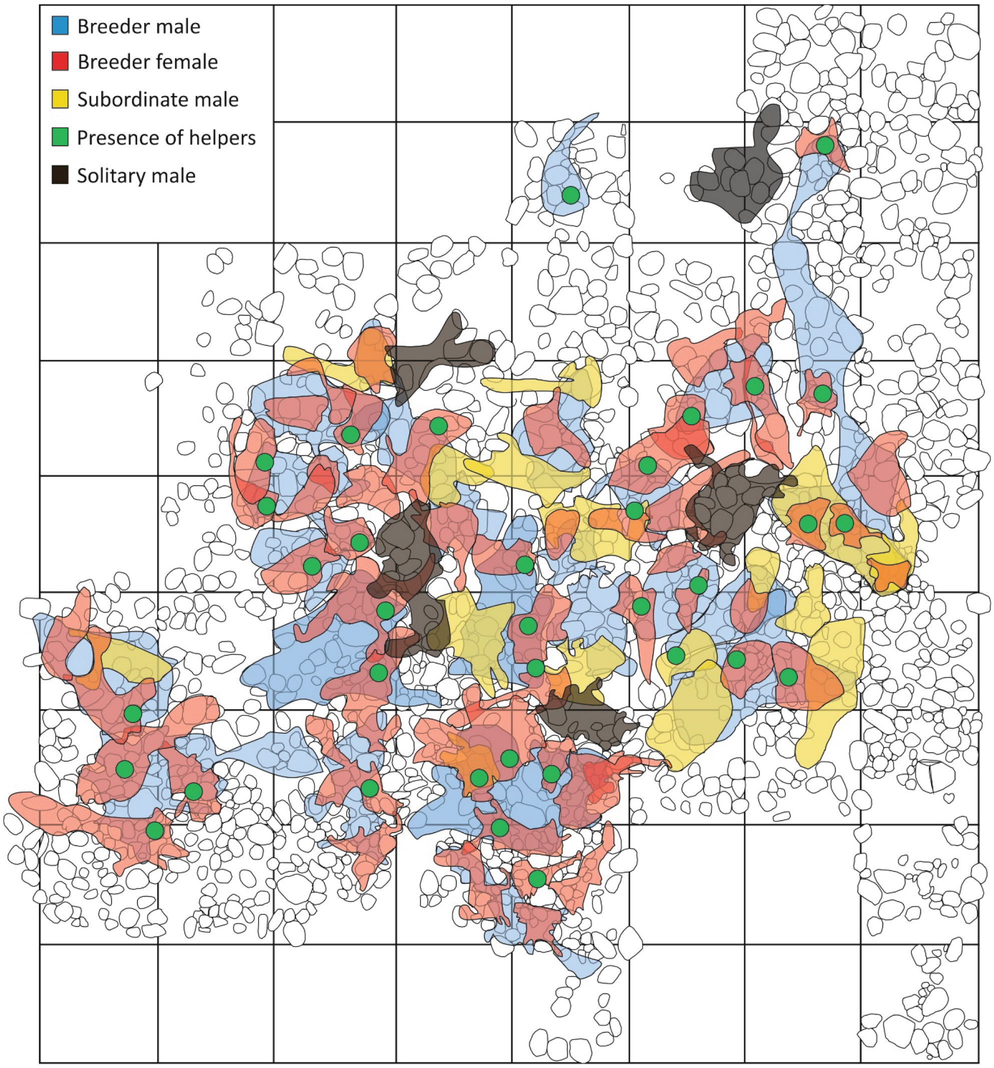

FIGURE 2 | Map of the home ranges of individuals belonging to different social classes of the focal colony in 2016. Different colours indicate different social classes. Green dots indicate helper home ranges, which were small and always within the respective dominant female's home range. Cobble and rock outlines are by depicted in black lines. Each square measures 1 × 1 m.

TABLE 1 | Total number of (re-)captured individuals.

Social status	Captures in colony 2016	Recaptures in 2016	Captures in colony 2017	Recaptures in 2017	Recaptures between years	Recaptures total
Breeder males	28	2	6	26	6	34
Breeder females	54	1	14	42	14	57
Subordinate males	18	1	7	15	5	21
Solitary males	4	0	8	5	8	13
Helper males	24	4	5	22	0	26
Helper females	22	0	12	25	1	26
Unsexed helpers	32	1	3	8	0	9
Total	182	9*	55	143**	34***	186

**In 2016 recaptures within the year were only conducted in cases where individuals were observed dispersing.*
***Individuals were captured in the focal colony as well as in all close-by territories in the area.*
****Sample sizes indicate social status at the time point of recapture.*

catching the fish. Using the intercept and the slope from the model, the age at which females and males became breeders was calculated (cf. Skubic et al., 2004). To analyse sex-dependent survival rates between the years, we fitted a generalized models (GLM) with binomial error distribution with recapture (yes/no) as response variable and sex as predictor. For comparing the growth rates among the different male types [subordinate males ($N = 20$), solitary males ($N = 6$) and breeder males ($N = 28$)] we fitted a LMM using the daily growth rate as response variable, with body size and male type as predictors. *Harem ID* and *Catcher ID* were included as random effects.

To compare the survival rates between male types living either solitary or associated with a group (subordinate and dominant males) over a period of 37 days in 2016 and 2017 (beginning and end of each field season), we sampled the entire focal colony and all surrounding territories in the area. Predation risk is high at the study side, and long-distance dispersal is scarce in cooperatively breeding cichlids (see Taborsky, 2016 for review). Therefore, we conclude that males that disappeared were most likely dead. A GLM with a binomial error distribution was fitted with survival as response variable and social status (solitary males and group males) as predictor.

Differences in transition probability between the sexes from helper stage to solitary living were tested with Fisher's exact test.

Data were analysed using R version 3.3.3 (R Core Team, 2017). We fitted LMMs, and binomial GLMs using the package lme4 (Bates et al., 2013). Models were checked for overdispersion and normality. Parameter significances were tested using likelihood ratio tests.

RESULTS

Growth and Survival

Growth rates differed between males and females, depending on initial body size (LMM: body size × sex: $\chi^2_1 = 4.62$, $p = 0.03$; **Figure 3A**). In both sexes, growth rate decreased with increasing body size/age (males: intercept: 0.481, size: $\beta \pm SE = -0.12 \pm 0.008$, $\chi^2_1 = 110.06$, $p < 0.001$; females: intercept: 0.553, size: $\beta \pm SE = -0.145 \pm 0.013$, $\chi^2_1 = 76.7$, $p < 0.001$), but at a size where females already decreased growth and started to breed males continued growing at a higher rate and for a longer period of time (see **Figure 3B**). The smallest breeder female measured 38 mm, which corresponds to an age of 412 ± 41 days (**Figure 3B**). The smallest breeder male measured 52 mm (age estimate 1,160 ± 200 days, **Figure 3B**).

The growth rates did not differ significantly between the three male types ($\chi^2_2 = 1.63$, $p = 0.44$). Initial body size differed slightly between the categories and seemed to explain more variation in growth than male status (body size: $\chi^2_1 = 1.44$, $p = 0.23$). Solitary males had a lower survival probability compared to males living in a group ($\chi^2_1 = 5.15$, $p = 0.02$, **Figure 4**).

Dispersal Events

Within Year

In total, we observed 15 dispersal events occurring within the two observation periods of 3 months each. Eight of these were not associated with a change in social status (four helpers and four breeder females; **Table 2**). The helpers were two males and two females, which dispersed and took up a helper position in a group of a different female within the same harem. All four dispersing breeder females moved to a breeder male that was larger than their original partner. The dispersal of the other seven individuals was associated with a change in social status. Four of these cases involved subordinate males that either became solitary ($n = 3$) or took over a harem ($n = 1$; **Table 2**). The other three individuals included one solitary male that became a breeder, one helper female changing to breeder status by establishing an own sub-territory within the original harem, and one helper female obtaining a breeder position in another harem defended by a larger male (**Table 2**).

Between Years

Out of the 34 recaptures between years, 19 individuals (56%) had changed their social status: Three out of 17 subordinate males caught in 2016 had become breeders in 2017 (18%), and eight male helpers had become solitary (47%; **Table 3**). One out of five solitary males captured in 2016 changed to breeder status in the subsequent year (20%). Seven out of 26 female helpers caught in 2016 had become breeders in 2017, either in the same or a different harem (27%; **Table 3**). Between years, we further recaptured six helpers (8%) that had not changed social status (five males and one female; **Table 3**), 5 of which had remained in the same location. The recapture rate of breeders between years was low (7/52 females, 2/32 males) and did not differ between the sexes (GLM; $\chi^2_1 = 0.36$, $p = 0.55$). Most recaptured female breeders (6/7) had changed the harem between years (86%; **Table 3**).

Territory Inheritance and Status Persistence of Helpers

Male and female helpers differed in their routes to independent breeding (**Tables 2**, **3**). In 2016 we caught 24 male and 22 female helpers. Three of 24 male helpers (12.5%) had become breeders in the following year (one inherited the territory), whereas 7 of 22 female helpers (32%) attained breeder status in the same time interval (three inherited the territory). Most of the male helpers became solitary (8/24; 33%) or remained as helpers/subordinates (5/24; 21%; in 4/5 cases they remained in the same territory), whereas only one female remained as a helper (in the same territory; 5%). The transition from helper to solitary living was more likely in males than in females (females: 0/22; males: 8/24 Fisher's exact test; $p = 0.02$). The remaining 8 male helpers and 16 female helpers most likely did not survive between years.

DISCUSSION

In cooperative breeders, understanding dispersal decisions and their fitness consequences is essential to explain why individuals continue to stay and help raising offspring of others instead of dispersing early to breed on their own (Clutton-Brock et al., 2002; Koenig and Dickinson, 2016; Suh et al., 2020). Our

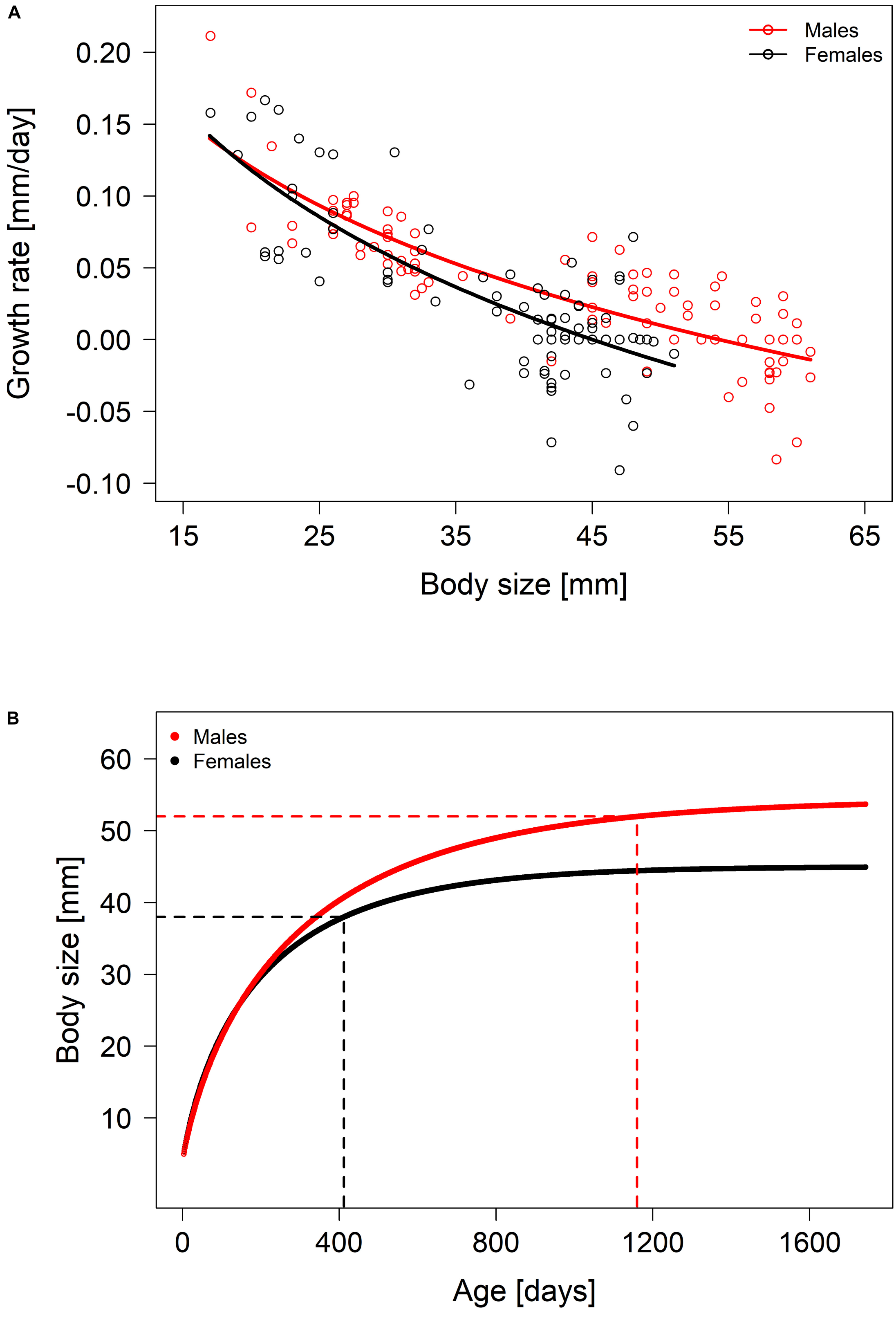

FIGURE 3 | (A) Growth rate in relation to initial body size (SL) of males (red) and females (black). Lines represent the model predicted means. Points represent original data; negative values arise due to measuring errors within and between observers. **(B)** Size-dependent mean predicted ages of males and females. Dashed lines mark the earliest point in time at which individuals became breeders.

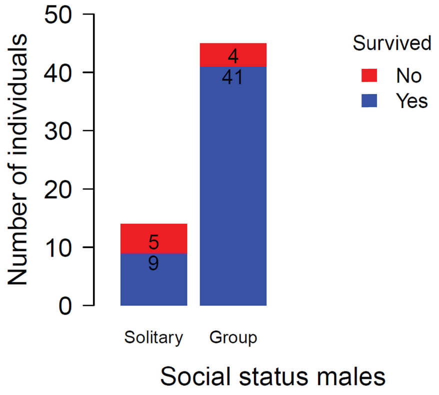

FIGURE 4 | Survival rates of males associated with a group versus those living solitarily over 37 days (from beginning to end of the respective field season). Numbers indicate sample sizes.

results show that in *N. savoryi* different routes to independent breeding exist for males and females, which are associated with divergent growth rates and survival probabilities. Helpers of both sexes grow similarly before reaching sexual maturity and start developing functional gonads at a comparable size (Josi et al., 2021). Most females become breeders right after maturation and concomitantly decelerate growth. Males, in contrast, continue to grow after reaching sexual maturity and either remain as subordinates in the harem and help the dominant breeders, or they disperse or get evicted from the territory and become solitary (**Figure 2**). This was never observed in females and is a risky strategy, exemplified by the reduced survival chances of solitary males.

The first objective of this study was to elucidate how a polygynous mating system relates to sex-specific differences in life-history strategies after reaching sexual maturity. As predicted, females start to reproduce at a younger age than males (**Figure 3B**). Even though a large body size yields a reproductive advantage due to enhanced fecundity, investing in early reproduction seems to pay off for females (Josi et al., 2020b). This is partly because *N. savoryi* females generally lay small clutches (Josi et al., 2019) that are probably not overly constrained by a small body size. Furthermore, like many Lake Tanganyika cichlids, *N. savoryi* has no distinct breeding season (Heg et al., 2005; Josi et al., 2021) and can produce clutches at a high rate (approx. 1 per month under good conditions). These conditions diminish the trade-off between investment in growth versus reproduction (Josi et al., 2019). Therefore, the early attainment of breeding status by females may not be associated with high costs. In contrast, the polygynous mating system involves enhanced competition for breeding territories among males (cf. Limberger, 1983; Tanaka et al., 2015; Jungwirth et al., 2016), which selects for large size in order to gain competitive superiority. This comes at the cost of a delayed onset of reproduction, long after reaching sexual maturity. Indeed, our results show that males reached a breeder position at larger body size and more than twice the age of females.

Overall, our results do not indicate sex-dependent survival differences, but we found that breeder males were more philopatric than females after establishing a territory; 85% of the dispersal events of breeders occurred in females, which dispersed in 91% of these cases to a territory owned by a lager male. Breeder females may benefit from dispersing to a larger territory owner in multiple ways: First, they might gain genetic benefits for their offspring by pairing up with a high-quality male (Andersson, 1994). Second, they might gain increased predator protection from larger male breeders (Wiegmann and Baylis, 1995). These results are corroborated by molecular data suggesting that *N. savoryi* males keep their established breeding territory for longer compared to breeder females (Josi et al., 2021).

Once a breeding position is obtained, the growth rate strongly declines in both sexes, apparently reflecting a trade-off between investing energy in growth or reproduction. This seems to be at odds with some other cooperatively breeding vertebrates, where growth rate was shown to increase after a breeding position was reached (Russell et al., 2004; Heg, 2010; Young and Bennett, 2010), which in turn may increase fertility or reinforce status (Clutton-Brock et al., 2006). In *N. savoryi*, starting to breed independently may be particularly costly, as at this stage helpers are often not present. Hence, costly duties such as digging out a breeding chamber and defending against predators and competitors are carried out exclusively by the breeders when establishing a new territory (Josi et al., 2020a,b). This may require that the available energy resources are primarily invested in reproductive purposes instead of growth, which might explain the outlined difference of growth patterns compared to other cooperatively breeding vertebrates. In summary the polygynous mating pattern of *N. savoryi* can explain important life history parameters such as growth and the age at start of breeding, which relates to sex-specific dispersal decisions.

Our second question concerned the importance of cooperative brood care for sex-specific life history trajectories. The data suggest that in *N. savoryi*, male helpers delay dispersal and provide alloparental care for a longer period than female helpers, which obtain a breeder position earlier. This corresponds with findings in cooperatively breeding birds, where males remain in the safe natal territory to grow until reaching a competitive size, while at the same time providing alloparental care (Pruett-Jones and Lewis, 1990; Cockburn et al., 2017). However, staying for a prolonged time comes with various costs, including the costly help itself (Taborsky and Grantner, 1998), the investment for acquiring a breeder position and the prolonged non-reproductive period. Hence alternative routes to independent breeding may evolve in males. Indeed, our results show a sex difference regarding the transition from helper to solitary status. While 38% of male helpers became solitary between years, this status is apparently never adopted by females. The alternative male trajectories have important implications for survival, as solitary males survived less likely than males living in groups (**Figure 4**). Also in other cooperatively breeding cichlids subordinates leaving their home territory face reduced survival chances due to lacking group protection (Taborsky, 1984; Heg et al., 2004; Tanaka et al., 2016). This prompts the

TABLE 2 | Recaptures and dispersal events recorded within a year.

Status before/after dispersal	*N*	Within harem	Between harems	Comments
Helper to helper	4	2 Males and 2 females	0	All dispersed to a new subgroup within the harem
Subordinate male to breeder	1	1	0	Harem take-over
Helper female to breeder	2	1	1	One[1] dispersed to a solitary male and became breeder. The other established an own subgroup in the same harem where she had been helper
Breeder female to breeder	4	0	4	All females dispersed to a larger dominant male
Subordinate male to solitary	3	0	3	
Solitary male to breeder	1	1	0	In one case a female helper[1] (37 mm) joined the solitary male

[1] Refers to the same individual female.
N refers to the total number of dispersals in the respective class.

TABLE 3 | Re-captures and dispersal events recorded between years.

Status before/after dispersal	*N*	Same location	Different location	Comments
Helper to helper	6	1 Female and 4 males	1 Male	The female and one male remained as helpers with the same breeder male. Three male helpers remained in their territories that were taken over from a neighbouring dominant male (2 cases) or a previously solitary male (1 case). One helper dispersed to another subgroup in the same harem
Helper male to breeder	3	1	2	One inherited the harem and the other two took over new harems
Helper female to breeder	7	3	4	Four helpers dispersed to a new territory, one of which dispersed together with the female breeder. Two helpers became breeders in the same harem where they were helpers. One helper did not disperse. Her partner (breeder male) had been a large subordinate male in the same harem the year before (territory budding-off)
Breeder female to breeder	7	1	6	Two females from different harems dispersed together to a new harem 23m away from the focal colony. Four females dispersed to a new harem. One female did not disperse and remained with the same breeder male over both years
Breeder male to breeder	2	2	0	One became a breeder through territory budding-off
Helper male to solitary	8	0	8	
Solitary male to breeder	1	0	1	

question why male helpers leave their safe natal territory at all. If the solitary phase of subordinate males was a self-chosen strategy, the higher mortality risk should be compensated by future fitness benefits, such as enhanced growth or a higher probability to take over a harem. However, being solitary did not affect growth rate, which was comparable to subordinate males and breeder males. In addition, there was no indication that solitary males became breeders with a higher likelihood than subordinate helpers (**Tables 2**, **3**). Therefore, it seems that leaving the home territory to switch to a solitary state reflects a best-of-a-bad-job strategy. This may result from reproductive competition with the harem owner, which may cause enhanced aggression of the latter, similar to other cooperatively breeding cichlids (Taborsky, 1985; Skubic et al., 2004; Mitchell et al., 2009). For subordinate male helpers, either expulsion from the territory or deliberate dispersal may be the consequence (Dierkes et al., 1999). Similarly, costly sex-specific life history trajectories have been observed also in other cooperative breeders. In pied babblers (*Turdoides bicolor*), for example, solitary males suffer from a continuous body mass loss compared to group members. In consequence, solitary floaters are unable to successfully compete for breeding positions (Ridley et al., 2008). Such sex-specific differences in life-histories and routes to independent breeding suggest that understanding the intrinsic attributes and specific ecology of a species is essential to grasp the evolutionary mechanisms responsible for delayed dispersal and alloparental care. Unfortunately, for many cooperative breeders such data are scarce.

To conclude, this study shows that in *N. savoryi* differences in dispersal decisions between and within the sexes are tightly linked to divergent life history trajectories, including different growth rates, the age at obtaining a breeder status, and survival. Apparently, dispersal is constrained by high levels of predation, and especially males benefit from philopatry through increased survival chances. We suggest that for a better understanding of the evolution of cooperative breeding and dispersal decisions, future studies should investigate the routes to independent breeding by incorporating individual life-history decisions and their associated costs and benefits, as this may elucidate alternative sex- and status-dependent pathways that are of importance for the development of complex social systems (Kingma et al., 2016).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for this study in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

DJ, HT, and JFr planned the study. MT and JFr organized the funding. DJ, HT, JFl, and MR-C collected the data. DJ analysed the data and drafted the manuscript, which was revised by MT and JFr. DJ, JFl, MR-C, MT, and JFr approved the submitted version of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by SNF-grants 31003A_156152 and 31003A_176174 to MT, and 31003A_166470 and 310030_185021 to JFr.

ACKNOWLEDGMENTS

We thank Celestine and the late Augustin Mwewa and their team for their extensive aid at the Tanganyika Science Lodge. We are grateful to the Department of Fisheries, Ministry of Agriculture and Livestock, Republic of Zambia for the permission to conduct this work, and especially Taylor Banda and Lawrence Makasa for their ongoing support. We are grateful to the referees for their thoughtful comments on the first draft of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 03 November 2021
doi: 10.3389/fevo.2021.742588

Sex Role Reversal and High Frequency of Social Polyandry in the Pheasant-Tailed Jacana (*Hydrophasianus chirurgus*)

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *16 July 2021*
Accepted: *06 October 2021*
Published: *03 November 2021*

Citation:
Fresneau N, Lee Y-F, Lee W-C, Kosztolányi A, Székely T and Liker A (2021) Sex Role Reversal and High Frequency of Social Polyandry in the Pheasant-Tailed Jacana (Hydrophasianus chirurgus). Front. Ecol. Evol. 9:742588. doi: 10.3389/fevo.2021.742588

In a few species, males invest more than females in parental care while the females invest in mating competition and producing multiple broods for several mates. Species in the family Jacanidae are commonly used for studying this type of breeding system (called sex-role reversal), and previous studies found discrepancies and variation between species in the expected characteristics of reversed sex roles. Yet, a better understanding of sex role differences in breeding behavior in such species is crucial for disentangling possible evolutionary mechanisms leading to this peculiar breeding system. Sex-role reversal in the pheasant-tailed jacana *Hydrophasianus chirurgus* has been documented long time ago. Since the very early observation of this species, however, there was no attempt to provide a comprehensive and quantitative description of their breeding. This study aims to fill these knowledge gaps by investigating the sex role differences in the breeding behavior of pheasant-tailed jacanas, by observing and monitoring a breeding population in Taiwan. We focused on three main characteristics of sex-role reversal: (1) competition between females for access to males, such as agonistic and courtship behaviors, (2) polyandrous mating, and (3) male-only care. As expected, we found that females provide most of the territory defense toward conspecifics. Males also participated in agonistic behaviors, although less frequently than females. Furthermore, contrary to what was expected, we found that males spent more time than females on courtship behavior. Polyandrous females performed mating and laying sequentially with different mates but maintained the pair bonds simultaneously with multiple males. For the first time for the species, we could estimate that the average number of mates per female (i.e., degree of polyandry) was 2.4 and that at least 81.8% of the females in the population were polyandrous. Finally, our observations corroborated that brood care is predominantly provided by males, nevertheless females were also participating to some degree in brood attendance but never in direct care (i.e., brooding). This study highlights that some aspects of polyandrous breeding might deviate from stereotyped view on sex-role reversal, and stress the importance of further within species and comparative studies in order to fully understand the mechanisms leading to sex-role reversal.

Keywords: polyandry, sex-role reversal, male-only care, Jacanidae, sexual competition

INTRODUCTION

In species providing parental care the sex difference in parental investment, as defined by Trivers (1972), varies widely. In most of the species, on average, females invest more in parental care (Clutton-Brock, 1991; Cockburn, 2006; Székely et al., 2013), which is often termed as conventional sex-roles. However, in a few species, we observe the opposite: males invest more than females in parental care while the females invest in mating competition and breeding with several mates. This is the case in species that have male-only parental care with a polyandrous type of mating system, called sex-role reversal (Trivers, 1985; Andersson, 1994). Occurring only in 1–2% of the species (Cockburn, 2006), sex-role reversal is the rarest type of breeding system in birds.

It was proposed that the sex providing care will be the one limiting the reproduction of the other sex (Emlen and Oring, 1977). In other words, the sex having higher potential reproductive rate will invest more in mating and territoriality (which is defined as a "masculine" role sometimes; Barlow, 2005). In a sex-role reversed species, thus, the females are expected to take upon territoriality and compete with other females for access to mates through aggressiveness. The dominance of females on males in sex-role reversed species is indeed confirmed by their reversed sexual size dimorphism with females being bigger than males and thus expected to be also more aggressive (Székely et al., 2007).

In term of caring for the young, precocial bird species are more flexible than altricial ones as often a single parents can take care of this task while the other parent deserts (Maynard Smith, 1977). This opportunity for uniparental care can lead to sex-role reversal in some cases, where males take most or all care of the young. It is hypothesized that males accept the care provider role because the cost of finding a new mate may be higher than the costs of investing in the current brood, for example due to the shortage of females, thus males may maximize fitness benefits by staying with their mates and provide care for existing offspring (Kokko and Jennions, 2008; Liker et al., 2013). In such populations the opposite is true for females, i.e., they have the opportunity to obtain multiple mates, thus may be selected to invest more in mating and less in care. Although classical polyandrous mating systems and sex-role reversal has been in the scope of many evolutionary studies, yet it is still uncertain from an evolutionary point of view why some lineages have adopted this breeding system (Betts and Jenni, 1991; Andersson, 2005). One reason for the lack of explanation might be the low level of knowledge and inconsistent information that we have about the ecology and behavior of species having such type of breeding.

The Jacanidea is one of the famous group of birds known for their sex-role reversal. All jacana species except the lesser jacana *Microparra capensis* seem to have indeed females breeding with several partners and males taking care of the brood. The polyandrous mating has been confirmed for 6 out of the 8 jacana species. The lesser jacana is monogamous (Tarboton and Fry, 1986; Hustler and Dean, 2002) and the level of information on the Madagascar jacana *Actophilornis albinucha* is insufficient to confirm its supposed polyandrous mating (D'Urban Jackson et al., 2019). However, within the other 6 polyandrous jacana species, the type of copulation pattern (i.e., simultaneous or sequential with different males) and degree of polyandry (i.e., average number of mates per female) vary a lot across the species (see **Table 1** for detailed review). Their territoriality has been described in several species as a super-territory defended by the female which includes several sub-territories defended by her mates. In sex role reversed species, we do expect the females to provide most territorial defense, and thus to be more aggressive than males toward conspecifics. Even though this has been verified in the African jacana *Actophilornis africanus* (Tarboton, 1995), this idea has been challenged with the wattled jacana, the northern jacana and with the bronze winged jacana as in these species males were more likely to be the first one to respond to a conspecific intruder on the territory (Butchart et al., 1999a; Emlen and Wrege, 2004a; Lipshutz, 2017).

In jacanas, male-only care has been confirmed in six species. Yet, the complete absence of females' participation in the care has been challenged: female wattled jacanas and northern jacanas have been observed participating at low level in parental care, even doing some brooding of the chicks, when the male is busy with incubating another clutch (Jenni and Betts, 1978; Emlen and Wrege, 2004a). The authors of the latter study even defined the females as "backup providers of chick care." Yet in other jacanas species the male is readily described as the solely care provider (Tarboton, 1992, 1993; Mace, 2000; Butchart, 2008).

The pheasant-tailed jacana *Hydrophasianus chirurgus* is a classic example of polyandry and sex-role reversal since the very early studies by Hoffmann (1949, 1950), although only a few later studies investigated the breeding biology of the species. Unfortunately, details about their pair bonding is not extensively available: some of the descriptions are anecdotal (Serrao and Shekar, 1962) or based only on the observations of a single female (Thong-aree et al., 1995; Chen et al., 2008a). The fact that this species is polyandrous is well known and observed repeatedly, but the degree of polyandry is yet unknown (**Table 1**). Details about territoriality, female mating competition and male care are also very scarce (Thong-aree et al., 1995; Chen et al., 2008a).

TABLE 1 | Mating system in six jacana species.

		Polyandry					
Species	**Sample size (females)**	**Type (pair bond/copulation)**	**Degree**[a]	**Range**[b]	**% Females**[c]	**% of polygynous males**[d]	**References**
Metopidius indicus	*N* = 8	Simultaneous/Simultaneous	1.6	1–4	50%	0%	Butchart, 1999
Metopidius indicus	*N* = 16	Simultaneous/Simultaneous	1.7	1–4	50%	0%	Butchart et al., 1999a
Jacana spinosa	*N* = 15	Simultaneous/Simultaneous	2.2	1–4	87%	0%	Jenni and Collier, 1972
Jacana spinosa	*N* = 4	Simultaneous/Simultaneous	2.5	1–3	80%	0%	Jenni and Betts, 1978
Jacana jacana	*N* = 12	Simultaneous/Simultaneous	1.2	1–2	18%	0%	Osborne, 1982
Jacana jacana	*N* = 160	Simultaneous/Simultaneous	1.7	1–4	60%	0%	Emlen and Wrege, 2004b
Actophilornis africanus	*N* = 7	Simultaneous/Simultaneous	3.9	2–7	100%	40%	Tarboton, 1992
Actophilornis africanus	*N* = 5	Simultaneous/Simultaneous	1.6	1–2	40%	33%	Tarboton, 1995
Irediparra gallinacea	*N* = 6	Simultaneous/Sequential	2.4	1–3	80%	33%	Mace, 2000
Hydrophasianus chirurgus	*N* = 1	Simultaneous/Sequential	–	3	–	–	Chen et al., 2008a
Hydrophasianus chirurgus	*N* = 1	–	–	4	–	–	Thong-aree et al., 1995
Hydrophasianus chirurgus	*N* = 11	Simultaneous/Sequential	2.4	1–5	82%	4%	This study

[a] *As average number of male per breeding female.*
[b] *Range of number of male per breeding female.*
[c] *% of female having more than one male during the breeding season.*
[d] *% of male having more than one female during the breeding season.*

This study aims at a better understanding of sex role differences in the breeding behavior of pheasant-tailed jacanas, by observing and monitoring a breeding population in Taiwan. We focus on the three parts of the breeding: (1) mate acquisition: first we study the amount of sex differences in agonistic behavior, predicting that females are involved more often than males in territorial defense. Then we investigate possible sex differences in the courtship behavior (i.e., time spent on displays), where we would expect females investing more in courtship when starting a new clutch, since the benefits of producing additional offspring should be higher for the females than for the males with already existing brood. (2) Then we investigate the pair bonding patterns and dynamics in order to calculate the degree of polyandry in this species and proportion of polyandrous females in the population. (3) Finally we study brood care (direct: brooding, and indirect: brood attendance) to understand the extent of the role division between males and females, expecting males to be the solely sex taking care of the brood, whilst females are deserting the male and the brood for creating a new clutch with a new mate.

MATERIALS AND METHODS

Study Site and Population

The study was carried out from late June to early September 2019 in the Pheasant-tailed Jacana Educational Park (Guantian, Tainan City, Taiwan; 23°10′58.0"N 120°18′41.2"E; called Park henceforward). The pheasant-tailed jacana has been classified as an endangered species in Taiwan since 1989. In the last 10 years, the Tainan region has seen an increase in the population as the result of a successful conservation project (from 284 individuals in 2010 to 1024 individuals in 2019; Forestry, 2019), and ca. 80% of this population is found on water chestnut ponds in and around the Park. The Park (i.e., study site) was established in 2007 to promote and educate about this bird as well as providing breeding sites protected from farming activities. Jacanas breed in the reserve from mid-April until the end of September. However, we were able to conduct fieldwork only from mid-June until early September in 2019 due to logistic reasons, so the study period covered the second half of the breeding season. The total area of the study site was about 0.15 km^2, which was divided into 25 ponds (**Supplementary Figure 1**). Although the whole area was studied to monitor the breeding of the birds, due to time restriction and poor visibility of birds on some of the ponds only 7 of the ponds were used for behavioral observation and individual identification (**Supplementary Figure 1**: P-3.1, P-3.2, P-5, P-6, P-7.1, P-7.2, and P-7.3, called hereafter "focal ponds"). The majority of the observations were conducted on pond P-5 as it was the biggest pond with a good visibility to the human observers, which allowed the identification of most resident individuals. Observations in P-3.1, P-3.2, P-5, and P-6 were made from behind wooden hides installed by the Park for visitors. Birds were habituated to the presence of human visitors and observers in these hides that made observations possible, even from short distance, without sign of disturbance. Observations in P-7.1, P-7.2, and P-7.3 were conducted from a handmade mobile bamboo hide. The hide was not moved during the observation and birds were habituated to its presence before the observations.

Individual Identification

Only two males in the focal ponds were ringed so we used detailed drawings of plumage differences (in the black head patch, white wing patch, and tail length, **Supplementary Figures 2, 3**) to ensure reliable identification of the individuals (Byrkjedal et al., 1997; Liker and Székely, 1999). Within-individual consistency of these patterns were checked several times during the season by drawing detailed patterns of identification and re-drawing them blindly again to see the consistency. The drawings were used for identification only in a single season, thus changes in plumage between years was not a problem (the birds molt after the breeding season and the consistency of breeding plumage between years has not been tested). Even if individuals were quite territorial, territory location in itself was not sufficient for identification as change in territory locations was observed through the season. For instance, two different males (M13 and M10, see **Table 2**) were observed nesting at exactly the same spot at different times of the season. The use of plumage differences allowed us to identify 12 females and 23 males. However, one female and one male left the focal ponds shortly after being identified and thus are not appearing in any of the results.

Mating System

Once an individual was identified, its pair bonding, nesting attempts and breeding success were monitored by regular observations through the breeding season. Two individuals were considered to be paired when (1) they were repeatedly observed engaging in courtship behavior (i.e., various ground and aerial displays and vocalizations), copulation or nest building behavior, and (2) they were observed actively feeding next to each other without showing agonistic behavior (see below, these criteria are similar to those used in Butchart et al. (1999a)). Since the bond between the female and male usually persisted for long period (up to several weeks), we were able to infer the pair-bonds from multiple observations for most birds. Extra-pair paternity may occur in jacana (Emlen et al., 1998; Haig et al., 2003), however, in this study we use the term polyandry to describe the social mating system.

TABLE 2 | Breeding performance of pheasant-tailed jacana: details of monitored nests and couples (female – male pairs).

Pond ID	Female ID	Male ID	Nest ID	Number of eggs	No. hatchlings	No. fledglings
P-3.1	F1	M1	N088	2	0	0
P-3.2	F2	M2	N083	4	0	0
		NA	–			
P-5	F3	M3	N066	4	4	3
		M3	N119	4	2	NA
		M4	N093	4	4	2
		M5	N102	4	2	2
	F4	M6	–			
		M7	–			
		M8	–			
	F5	M9	N129	1	0	0
		M9	N132	4	0	0
		M10	N107	2	0	0
		M10	N118	2	1	NA
		M11	N051	4	3	0
		M12	–			
		M13	N080	4	2	0
	F6	M14	N113	4	4	NA
		M15	N124	4	0	0
P-6	F7	NA	–			
		M16	N099	2	0	0
	F8	M17	N086	4	0	0
		NA	–			
P-7.1	F9	M18	N128	4	0	0
		M19	N111	4	1	NA
		NA	N096	3	0	0
P-7.2	F10	M20	N097	4	4	1
		M20	N136	4	0	0
		M21	N120	4	0	0
P-7.3	F11	M22	N060	4	4	NA

NAs for male identification (ID) denote unidentified male but still identified pair bonding (the male left the site before being accurately identified). NAs for number of fledging are from nests where the chicks were younger than 40 days old at the end of the study.

Nest and Brood Monitoring

When a nest was found, we considered the male performing egg care (incubation and shading) as the father. The female that was associated (paired) with that male was considered as the mother. Each nest found in the focal ponds was checked with a scope every day during egg laying, at least every three days during incubation and every day around the expected hatching date (about 23 days after the first egg laying). A nest was considered successful when at least one of the eggs hatched. After hatching, broods were monitored at least every three days and were considered successful when at least one chick reached the age of 40 days. The exact age of fledging has not been determined for this species (Jenni and Kirwan, 2020) but our observations suggest that after 40 days the chicks are quite independent and can fly at least for short distance. We followed a total of 23 nests and 11 broods produced by 19 couples (i.e., female – male pair) on the territories of 11 females (**Table 2**).

Behavioral Observations

We made three types of behavioral observations: agonistic interaction counting, pair bonding behavior observation and brood attendance behavior observation. All behavioral observation were made using a scope (Kowa TSN-601 with a 30x Kowa TSE-14WD eyepiece magnification), behind wooden fence hide or mobile hide (see above) and at least 30 min after arrival to the site for avoiding recording any possible disturbance consequences from the observer's arrival. Agonistic interaction counting was done only in P-5 (see below), pair bonding behavior observations were done in P-3.1, P-3.2, P-5, P-6, P-7.1, P-7.2, and P-7.3 and brood attendance behavior observation were done in P-5, P-7.1, and P-7.2 as the other focused pond did not have brood. All observations were made by one observer (NF) to avoid observer bias. Only identified individuals were targeted for behavioral observation.

During behavioral observation we divided the birds' behaviors into nine categories [some of these postures are also described for the African jacana in Tarboton (1992) and Bonkewitzz (1997)]: (1) agonistic behaviors: threat displays including 'upright threat display,' 'wing spur display,' and ground and aerial attack, (2) courtship behaviors: head down posture (often accompanied by vocalizations), mounting, and copulation, (3) nest-building behaviors: pulling and throwing vegetation toward (potential or existing) nest site and manipulating vegetation on a nest site, (4) egg care behaviors: incubation (i.e., sitting on the eggs) or egg shading (standing above the egg giving them some shade), (5) brooding behaviors: standing or sitting with chicks under the wing, (6) foraging behaviors: walking and pecking at vegetation in water, (7) maintenance behaviors: preening, scratching, stretching or bathing, (8) vigilance behaviors (i.e., alert): standing in an upright posture with the neck extended, without the tail upright and without the nape feather upright (as in agonistic behavior), (9) movement behaviors: walking, running or flying.

Agonistic interactions counting was made only in pond P-5 as it was the pond with a larger number of individuals identified allowing enough interactions with identified participants for statistical analysis. In this pond, the visibility allows to see the majority of the individuals at the same time, so individuals were observed about the same amount of time. It means that when spending time to observe some focal individuals on P-5, any agonistic behavior happening between other known individuals at the same time could also be noticed and counted (number of males on P-5: 11; number of females on P-5: 4). We counted all types of agonistic behavior as described previously. Every agonistic interaction occurrence was recorded throughout the day (even during other behavioral observation, i.e., during pair bonding and brood attendance observations) if it was between two identified individuals. We counted as one occurrence of agonistic interaction from the moment it starts until one or both individuals flew or walk to another area of the pond and the interaction stopped. We divided the agonistic interactions in four categories: Female–Female (FF), Male–Female (MF), whereas Male–Male interactions were split into between males sharing (i.e., had been paired to) the same female (MMP) and between males not sharing the same female (MMN). We did not create two categories in Male–Female interaction as all except one were observed between a female and a male that were not paired.

Pair bonding behavior observations and brood attendance behavior observations consisted of an instantaneous scan sampling of focal individual's behavior for 30 min every 20 s and for 60 min every 30 s, respectively. When an individual was hidden (e.g., behind high grasses) or not visible in the pond we gave a "NA" (i.e., no data) to that record. We calculated the proportion of time spent on each category during the observation (excluding the NA observation). We also noted at every 5 min the approximate distance between the focal individuals. The distances between individuals were estimated using reference objects with known size, e.g., the birds themselves and the surrounding floating lotus and water chestnuts leaves. Individuals were rarely more than 100 m away from the observer. Furthermore, all distance estimations were made by the same observer allowing a good consistency in these estimations. We considered the proximity distance to about 3 m, i.e., if couples were within 3 m distance of each other they were considered in proximity of each other. In the same way if an adult was within 3 m of its brood it was considered in proximity of its brood ("brood attendance" henceforward). We used this distance because, according to our observation, it is the distance where the individuals interact between each other (i.e., vocalization or specific behavioral signal as head-down position or upright threat display) without one flying to the other.

Pair bonding behavior observations were made only on identified pairs observed in proximity of each other on the day of observation. Both male and female behavior were observed at the same time (if both were visible). A total of 44 courtship observations were made, involving 16 different males pair-bonded with 10 different females. Five couples were observed only once, the other 11 were observed at least two times with one couple observed nine times. The reason for the uneven observation time per couple was that some pairs were more often spending time together than others, furthermore, it was also dependent of the observer availability.

Brood attendance behavior observations were made only for identified individuals in the presence of their known chicks, whose number and approximate age were known from earlier observations of the family. Brood identity was assessed from proximity of the chicks to the identified parent and their corresponding age (i.e., size of the chicks). For each observation, we calculated the amount of time that the brood spent in proximity (i.e., within 3 m) of the male only, female only, or both parents, or in the absence of parents. Brood attendance behavior observation were observed for a total of 10 different broods which includes five females and eight males. One brood was observed only once at age of 39 days old (N066), while the rest of the broods were observed at least twice (see **Supplementary Table 1**). In 11 brood observations we did not have data for the female as the females did not appear in sight during the observation. We observed brood attendance (i.e., parent being within 3 m away from the chicks) in 8 out of 22 observations by the female and in 21 out of 22 observations by the male. In one of the observations, behaviors were recorded but neither the male not the female approached the chick close enough to be considered as brood attendance.

Data Analysis

Agonistic interactions counting was analyzed using generalized linear mixed models with a Poisson distribution including individuals' identification as random factor. We first tested if there was a sex difference by constructing a model with the number of interactions as a response variable against the sex of the individual and the type of interaction (intrasexual interaction (FF and MM) or intersexual interaction MF) as well as their two-way interaction as explanatory variables. We then analyzed the males' interactions separately with the number of interactions as response variable against the type of interaction (intersexual interaction: MF, Male–Male interaction sharing same female: MMP and Male–Male interaction not sharing the same female MMN) as explanatory variable.

In order to measure sex difference in time spent on various behavioral categories in the pair bonding behavior observations, we used generalized linear mixed models using a Gaussian distribution, with the proportion of time spent in different behavioral categories (after square-root transformation) as response variable and sex, time of the day (i.e., if the observation was made in the morning or the afternoon) and the time spent in the proximity (i.e., within 3 m) of the mate as well as the two-way interactions between the sex and the two other variable separately as explanatory variables. Couple identification nested in female identification were included as a random intercept. We did this analysis, with separate models, only for the following behavioral categories: (1) agonistic behaviors, (2) courtship behaviors, (3) nest-building behaviors, (4) foraging behaviors, (5) maintenance behaviors, (6) vigilance behaviors, (7) movement behaviors. Egg care behaviors and brooding behaviors were not analyzed as there were not enough occurrence during the pair bonding observations.

In the analyses of brood attendance behavior observations, first, we measured sex difference in time spent on brood attendance (in proximity of the chicks within the 60 min observation) using a generalized linear mixed model with a Gaussian distribution with the proportion of time spent in proximity of the chicks (i.e., brood attendance) as a response variable. We included the sex of the parent (here divides in three categories: male only, female only or both parents are present), the age of the offspring (divided in four categories: less than 10 days old, between 10 and 20 days old, between 20 and 30 days old and more than 30 days old), the time of the day (i.e., if the observation was made in the morning or the afternoon) as well as the two-way interactions between the sex and the two other variables separately as explanatory variables. Couple identification nested in female identification were included as a random intercept. Secondly, to measure sex difference in time spent on different behavior during brood attendance (i.e., only when in proximity of the chicks), we analyzed the proportion of time spent on each behavioral categories separately, as response variable, against the sex of the parents, the age of the offspring and the time of the day of observation as well as the two-way interactions between the sex of the parent and the time of the day as explanatory variables. Interaction between the sex and the age of the offspring could not be included in the models as there was not enough data in each category to be analyzed. We used couple identification nested in female identification as random effects. For this part we focused only on four behavioral categories: (1) agonistic behaviors, (2) foraging behaviors, (3) maintenance behaviors, (4) vigilance behaviors. Courtship behaviors, nest-building behaviors, egg care behaviors, and movement behaviors, were not analyzed here as their low occurrence during brood attendance could not allow it. Sex difference was not analyzed in brooding behavior as it was exclusively performed by the males. Brooding was observed rarely thus we had not enough data to analyze any time or age of the offspring effect.

In all cases we performed stepwise backward model selection procedures starting from the full model. Fixed effects in the models fitted with the maximum likelihood (ML) were tested by comparing a model with and without the fixed effect using likelihood ratio tests (LRTs) against a chi-square distribution (χ^2). Non-significant fixed effects ($P > 0.05$) were removed one by one from the model starting with the least significant. The final model was fitted with restricted maximum likelihood (REML) to obtain the estimates for the fixed effects (Zuur et al., 2009). All statistics were performed in R version 3.5.0 (R Development Core Team, 2018), using the nlme package (Pinheiro et al., 2013) and lme4 package (Bates et al., 2015). Shapiro–Wilk tests were used to analyze normality and Bartlett tests to analyze homogeneity of variances. Tukey method was used for *post hoc* analyses, using emmeans and emtrends from the emmeans R package (Lenth, 2019).

RESULTS

Agonistic Interactions

Agonistic interactions were observed between 15 of the 17 identified individuals that were resident on pond P-5: 4 females and 11 males. Males were significantly less frequently involved in agonistic interaction compared to females [estimate ± SE (male) = –1.84 ± 0.51, χ^2 = 9.64, P = 0.002, **Figure 1**]. Both males and females were involved in more intra-sexual interactions than in inter-sexual interactions [estimate ± SE (intra-sexual) = 0.86 ± 0.17, χ^2 = 27.10, $P < 0.001$, **Figure 1**]. The interaction between sex and type of interaction (intra- or inter- sexual) was not significant (χ^2 = 2.94, P = 0.09). When males were analyzed separately, they tended to be more aggressive toward males pair-bonded with another females (MMN) than toward males pair-bonded to the same female (MMP), and they were significantly more aggressive toward males pair-bonded with a different female (MMN) than toward females (MF) (interaction type: χ^2 = 10.92, P = 0.004; *post hoc* test: MF-MMN: –0.78 ± 0.25, P = 0.006; MF-MMP: –0.24 ± 0.28, P = 0.66; MMN-MMP: 0.54 ± 0.23, P = 0.05; **Figure 1**). All except one male-female interactions were between male and female involved in different couples.

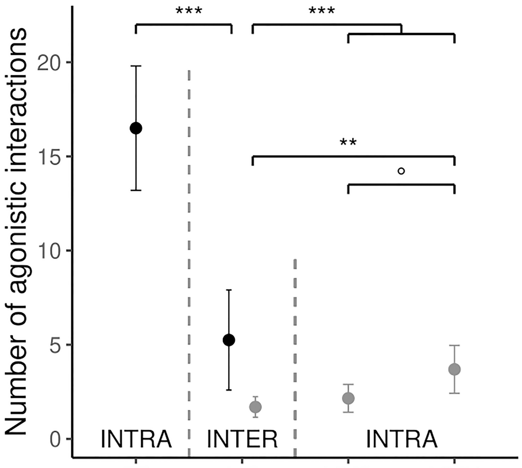

FIGURE 1 | Average number of agonistic interactions for female (in black) and male (in gray) jacanas for intra-sexual (INTRA) and inter-sexual (INTER) interactions. FF, Female–Female; MF, Male–Female; MMP, Male–Male from same Female; MMN, Male–Male from different Female. °P= 0.06, **$P < 0.01$, ***$P < 0.001$. Females: N = 4; Males: N = 11. Error bars denote standard errors.

TABLE 3 | Sex difference in amount of time spent on specific group of behaviors during pair bonding behaviors observation: courtship, agonistic behavior, vigilance, maintenance, foraging, movements, and nest building (see methods for details of these behavioral categories).

	Full model estimates ± SE		Selected model estimates ± SE		χ^2	*P*
Courtship behaviors						
Sex	(Male)	0.08 ± 0.06	(Male)	0.17 ± 0.03	25.41	**<0.001**
Time of the day	(Afternoon)	0.06 ± 0.05	(Afternoon)	0.08 ± 0.03	6.72	**0.01**
Time spent in proximity		– 0.02 ± 0.11		–	1.81	0.18
Sex × Time of the day	(Afternoon × male)	0.01 ± 0.07		–	0.03	0.85
Sex × Time spent in proximity	(Male × proximity)	0.25 ± 0.16		–	3.22	0.07
Agonistic behaviors						
Sex	(Male)	0.01 ± 0.06		–	0.18	0.67
Time of the day	(Afternoon)	– 0.005 ± 0.05		–	0.04	0.84
Time spent in proximity		0.23 ± 0.11		0.23 ± 0.08	8.12	**0.004**
Sex × Time of the day	(Afternoon × male)	– 0.01 ± 0.07		–	0.02	0.9
Sex × Time spent in proximity	(Male × proximity)	0.02 ± 0.16		–	0.01	0.91
Vigilance behaviors						
Sex	(Male)	– 0.04 ± 0.08		–	1.78	0.18
Time of the day	(Afternoon)	0.03 ± 0.06		–	0.05	0.82
Time spent in proximity		– 0.04 ± 0.15		–	<0.001	0.996
Sex × Time of the day	(Afternoon × male)	– 0.07 ± 0.08		–	0.6	0.44
Sex × Time spent in proximity	(Male × proximity)	0.08 ± 0.2		–	0.16	0.68
Maintenance behaviors						
Sex	(Male)	0.14 ± 0.08	(Male)	0.12 ± 0.07	0.12	0.72
Time of the day	(Afternoon)	0.03 ± 0.06		–	0.01	0.9
Time spent in proximity		0.13 ± 0.14		0.16 ± 0.13	0.19	0.66
Sex × Time of the day	(Afternoon × male)	– 0.05 ± 0.08		–	0.38	0.54
Sex × Time spent in proximity	(Male × proximity)	– 0.35 ± 0.19	(Male × proximity)	–0.39 ± 0.18	4.63	**0.03**
Foraging behaviors						
Sex	(Male)	–0.18 ± 0.08		–	3.45	0.06
Time of the day	(Afternoon)	– 0.09 ± 0.07		–	2.27	0.13
Time spent in proximity		–0.17 ± 0.16		–	0.2	0.65
Sex × Time of the day	(Afternoon × male)	0.05 ± 0.09		–	0.31	0.58
Sex × Time spent in proximity	(Male × proximity)	0.22 ± 0.21		–	1.75	0.19
Movement behaviors						
Sex	(Male)	0.06 ± 0.04		0.05 ± 0.02	5.46	**0.02**
Time of the day	(Afternoon)	0.1 ± 0.03		0.05 ± 0.02	3.99	**0.046**
Time spent in proximity		– 0.11 ± 0.08		–	0.13	0.72
Sex × Time of the day	(Afternoon × male)	– 0.11 ± 0.05		–	3.58	0.06
Sex × Time spent in proximity	(Male × proximity)	0.17 ± 0.11		–	2.62	0.11
Nest building behaviors						
Sex	(Male)	0.01 ± 0.04	(Male)	0.003 ± 0.03	6.28	**0.01**
Time of the day	(Afternoon)	– 0.01 ± 0.03	(Afternoon)	–0.01 ± 0.03	2.51	0.11
Time spent in proximity		– 0.004 ± 0.08		–	0.06	0.8
Sex × Time of the day	(Afternoon × male)	0.09 ± 0.05	(Afternoon × male)	0.09 ± 0.04	4.23	**0.04**
Sex × Time spent in proximity	(Male × proximity)	– 0.02 ± 0.11		–	0.03	0.86

*Table shows the estimates and standard error for the full model and for the selected model fitted with restricted maximum likelihood (REML). Factor variables' estimates contrasts term are indicated between parentheses. Stepwise backward model selection done with models fitted with maximum likelihood (ML) are given for each variable estimates (χ^2 and associated P-value). Significant P-values are shown in bold. Proportion of time spent on each behavioral category (used as response variable) was squared root transformed. Number of observations: N = 44; Male: N = 15, Female: N = 10 (**Supplementary Figure 4A**).*

Pair Bonding Behaviors

During pair bonding behavior observations, males spent significantly more time on courtship behaviors than females ($\chi^2 = 25.41$, $P < 0.001$, **Table 3** and **Supplementary Figure 4A**). They also spent more time moving around (movement behaviors) than females ($\chi^2 = 5.46$, $P = 0.02$, **Supplementary Figure 4A**). Analysis showed a significant interaction between sex and proximity on maintenance behaviors: the more time the male and female spend together during the observation the more time the females spent on maintenance while it was the opposite for the males, i.e., the less the males spent time on maintenance (*post hoc* analysis of the interaction: slope estimation for females 0.15 ± 0.13, slope estimation for males –0.24 ± 0.13, $\chi^2 = 4.63$, $P = 0.03$, **Table 3**). We found a significant effect of the interaction between sex and time of the days on the time spent on nest building behaviors ($\chi^2 = 6.46$, $P = 0.01$). *Post hoc* analysis shows that males spend significantly more time on nest building than females during the afternoon only (*post hoc* analysis: female –

male difference during afternoon observations $t_{69} = -3.29$, $P = 0.01$, **Table 3** and **Supplementary Figure 4A**). There was no difference in time spent on foraging, vigilance and agonistic behavior between males and females.

Courtship behavior and movements behavior significantly happened more in the afternoon (Courtship: $\chi^2 = 6.72$, $P = 0.01$; Movement: $\chi^2 = 3.99$, $P = 0.046$ **Table 3** and **Supplementary Figure 3A**). The more time males and females spent together, the more time they both spent on agonistic behavior ($\chi^2 = 8.12$, $P = 0.004$, **Table 3**).

We observed a total of 22 copulations for seven females and nine males. On pond P-5, we observed 17 copulations for three females and five males (details are given in **Figure 2**). Copulations were observed exclusively during the afternoon. Copulations were observed from 4 to 3 days before the first egg laying and the day before each egg laying, except for one couple (F3-M3) where 2 copulations the same day were observed 7 days before the egg laying. However, we cannot exclude the possibility that the female had a nest attempt, but the nest was not found. We never observed more than 2 copulations per couple per day.

Mating System

Out of the 11 focal females, 9 females paired with more than one male (81.8%, **Table 2** and **Figure 3**). Out of the 18 males observed only one was seen changing mate by pair bonding with two different females: M6, who was seen pair bonding with F4 several times without successfully nesting but as soon as the female F4 left the breeding site permanently (i.e., was not seen anymore), the female F3 was seen actively performing displaying behavior toward M6. No nesting attempts were seen from either the couple F4-M6 or F3-M6. Females had 1–5 different males with an average of 2.4 males per females.

Brood Attendance Behaviors

On average, males performed brood guarding (i.e., stayed in proximity of the chicks) during 54.2 ± 6.9% of the observation time whereas females stayed in proximity during 5.7 ± 2.2% of the time. Mixed models analysis showed that male-only brood attendance (i.e., in proximity of the chicks without the female around) was significantly more frequent than female-only brood attendance or both parents brood attendance ($\chi^2 = 70.30$, $P < 0.001$; *post hoc* test: Male-only – Female-only: $t_{56} = -9.41$, $P < 0.001$, Male-only – Both parents: $t_{56} = -8.86$, $P < 0.001$, Female-only – Both parents: $t_{56} = 0.55$, $P = 0.84$; **Figure 4** and **Table 4**). Time spent on brood attendance was significantly higher during the first 10 days of the offspring age compared to when the offspring are more than 20 days old ($\chi^2 = 10.48$, $P = 0.01$; *post hoc* test, age 0–9 – 20–29: $t_{56} = 2.94$, $P = 0.02$; **Figure 4** and **Table 4**). There was no significant difference in time spent on brood attendance according to the time of observation (morning or afternoon) (**Table 4**). Male and female did not differ in the time spent on brood attendance according to the time of the day nor the age of the offspring (i.e., there were no significant sex × time of the day, and sex × age of offspring interactions, respectively; **Table 4**).

Brooding behavior was done exclusively by males (**Supplementary Figure 5**). The behaviors performed during brood attendance were unrelated to the age of chicks, the sex of the parent nor the time of the day (i.e., morning or afternoon) for vigilance, maintenance and foraging behaviors (**Table 4**), however, males performed significantly more agonistic behaviors during brood attendance than females (**Table 4**).

Breeding Success

Out of 23 clutches produced by these couples (1–6 clutches per female and 1–2 clutches per male), 11 clutches (47.8%) hatched successfully, the other clutches failed. Out of 11 broods, we know the fate of six broods: at least four of them successfully fledged at least one chick, so that a clutch has approximately 18% chance of producing a fledged chick (four successful brood out of 18 clutches with known fate). Reason of clutch and brood failure was difficult to determine as it was not directly observed. We suspect that predation by large fishes (which are abundant in the ponds), water snakes and black shouldered kites were the main reasons, and flooding after heavy rain may also has contributed to nest losses.

DISCUSSION

This study highlights several major characteristics of the breeding behavior of the sex-role reversed pheasant-tailed jacanas: (1) We did show as expected that the females participate more than the males in territory defense, but we found that males were also substantially involved in this behavior. (2) Contrary to what is expected males were performing courtship behaviors significantly more often than females. (3) Females were involved in some brood attendance however never performed any direct brood care, for example chick brooding. Finally our study based on observations of the largest number of identified individuals to date provides new data on the frequency and dynamics of polyandrous mating of the pheasant-tailed jacana. We show that this species has a simultaneous pair bonding system with a strict sequential copulation pattern. Although some aspects of breeding behavior of the pheasant-tailed jacana have been investigated by previous studies (e.g., Hoffmann, 1950; Thong-aree et al., 1995; Chen et al., 2008a) our study provides to date the most comprehensive assessment of reproductive roles of the sexes in the species, including sex differences in agonistic behavior, courtship, and parental care. Below we discuss each of these results in detail, and explain their importance within the broader frame of the evolution of sex-role reversal.

Competition for Access to Mates: Agonistic Behaviors

Our study shows that in this species not only females are involved in agonistic behavior toward conspecifics. Even though we indeed see that females were more often involved than males in this kind of interaction, males also showed significant number of agonistic interactions against conspecifics suggesting that both sexes are actively involved in territoriality. This is an interesting finding because in sex-role reversed species it is often thought that only females are territorial (Jenni, 1974; Andersson, 2005). Interestingly, we observed only one agonistic

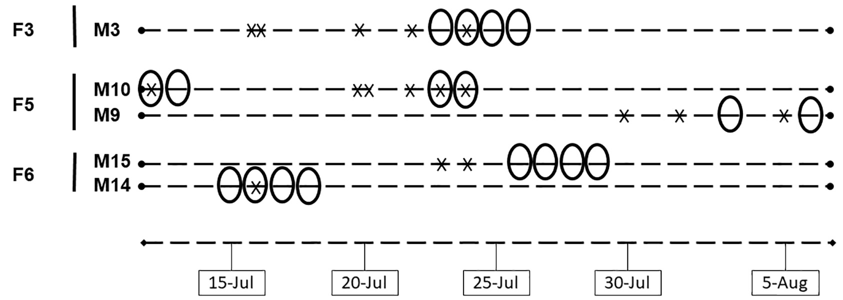

FIGURE 2 | Observed copulations (cross) in relation to egg laying (ovals) for five different couples (three females) during a 26 days period (each dash represent a day). Copulations were not monitored every day.

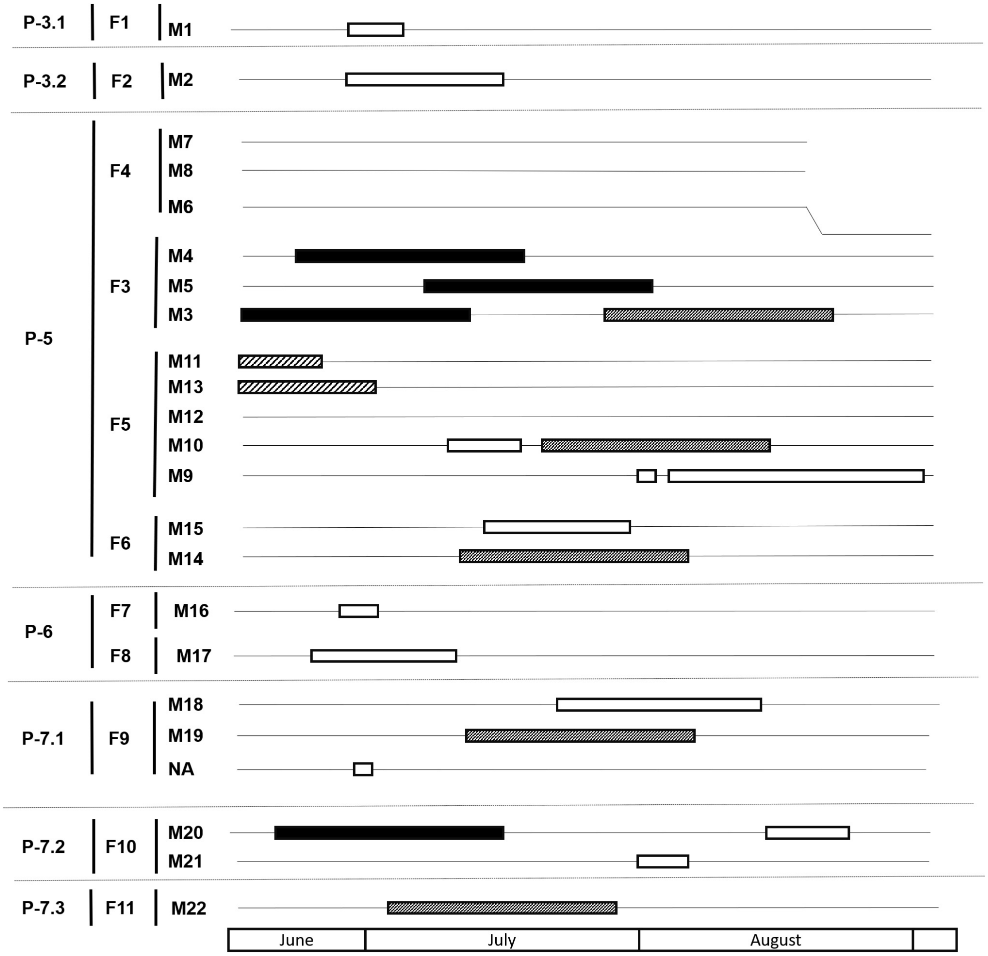

FIGURE 3 | Dynamics of pair bonding and breeding by females (F) and males (M) observed. Horizontal lines represent pair bonding. Each rectangle represents the incubation period for a clutch (performed by the male). White filled rectangles represent clutches that did not hatch. Bold striped filled rectangles represent clutches that hatched but the chicks did not survive until fledging, whereas black filled rectangles represent successful clutches (hatched and fledged at least one chick). Light striped rectangles represent clutches that hatched but chicks did not reach the age of 40 days before the end of the fieldwork, so the success of the brood is unknown. Female F4 formed pair-bonds with three different males but did not make any nest attempt during the studied period, and one of her males (M6) subsequently established a pair-bond with F3. NA denote unidentified male that left before being able to identify him. Details are also given in **Table 2**.

interaction between a female and one of its mates, otherwise all male-female agonistic interaction were between males and females involved in different pairs, which corroborates again that males participate in territorial defense. In their paper Chen et al. (2008b) also reported that male pheasant-tailed jacanas performed some aggressive behaviors, but this study did not

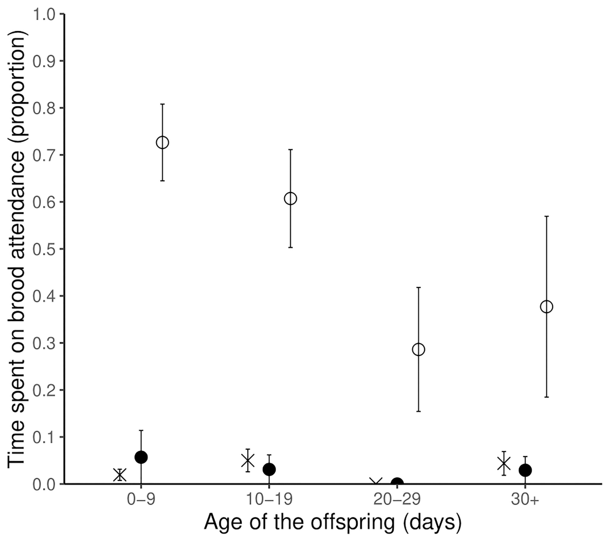

FIGURE 4 | Proportion of time spent on brood attendance (i.e., within 3 m from the chicks) during brood care observation by the male alone (empty circle), female alone (full circle), and both parents (cross). Mean ± SE. Sample size: number of observations: $N = 23$, age 0–9: $N = 6$, age 10–19: $N = 7$, Age 20–29: $N = 6$, Age 30+: $N = 4$. Error bars denote standard errors.

differentiate between conspecific and heterospecific interactions, neither if the conspecific individual included in the interaction was female or male. Involvement of the males in the territorial and thus resources defense is thought to be yet another benefits that the female could gain by mating with multiple males (Fedorka and Mousseau, 2002; Slatyer et al., 2012), because the more mates the female would have the larger territory and more resource could be defended. In the facultative social polyandrous mammals siamang, polyandrous groups have access to larger territory with a higher food density (Lappan et al., 2017).

Furthermore, we found that male-male fights were usually between males not sharing the same female's territory. This latter observation could be explained by several mechanisms. First, once the pair bonding is made with a female, the males of the same female would compete for the female's reproductive investment through other means than fighting (see below). Furthermore, since the males pair bonded with the same female live close to each other, there might be a dominance hierarchy among them based on initial interactions. This might reduce the frequency of fights within the social group and reduce the costs of competition which could ultimately lead to a decline of reproductive success (McDonald et al., 2017). In addition, it has been shown that male cooperation in reproduction could lead to several direct and indirect benefits (Díaz-Muñoz et al., 2014). Finally, the presence of an external male on the territory could not only represent an intruder but also a new potential co-mate and thus competitor for access to the female and resources of the territory, as well as a higher number of nests on the territory attracting more predators. We could then imagine that according to the quality of the habitats of the female, male could tolerate up to only a certain amount of co-mates (Lappan et al., 2017). More studies would be needed in order to measure the frequency and temporal dynamics of such social interactions within the polyandrous groups.

Both males and females increased their aggressiveness (toward other individuals) with the time spent together, the more time they spent together the more they were aggressive. This could be a result of an increase of synchronization in territoriality when they defend together against some intruders. Indeed, we observed several cases when more than two individuals were engaging in aggressive interaction at the same place, perhaps on territory boundaries (NF and AL, personal observations), which may the results of such joint defense by the couples.

Competition for Access to Mates: Courtship Behaviors

Interestingly, males were spending more time in courtship behaviors than females which goes against what we expected for a sex-role reversed species. In polyandry, we would expect the females to invest more into producing a new clutch than the male, since females could maximize their breeding success by obtaining multiple mates whereas the males' success is determined in a large part by the successful rising of the current offspring. One potential explanation for this result is that we did not observe courtship at the beginning of the breeding season when competition between females for mates should be much intensive. Contrary to this explanation, in a previous study Chen et al. (2008b) observed that the time spent by both males and females on breeding behavior increased through the breeding season, which included courtship behavior (although they did not separate it from other activities such as parental care). Alternatively, the courtship behavior we observed may not only serve to establish new or re-establish earlier pair bonds, but may also signal the readiness of males to start a new breeding. Since both nest loss and brood loss were frequent in the study population (see section "Results"), males may compete with each other for the egg laying potential of the female, especially when there are several resident males on the female's territory (as in the case of several females in our study). This high within-male competition for female's attention was observed in the bronze-winged jacana (Butchart et al., 1999b). In such situation the male may benefit from intensively signaling his capacity for a new clutch as well as its quality (Kotiaho, 2002; Pariser et al., 2010), because otherwise he may wait for long periods until female finishes laying for other males. Male–male competition, here shown through courtship can have a strong impact on sexual selection as it has been shown in mammals (e.g., Lührs and Kappeler, 2014), insects (e.g., Russell et al., 2018), and other birds (McDonald et al., 2017). To get a better idea of the relevance of the above explanations, further data on the occurrence of courtship are needed, especially from the earlier part of the breeding season and with precise information on their timing relative to the initiation of the new clutches.

During the courtship behavior observations we found that females spent more time in maintenance behavior than males, which could show that preening might also be used as part

TABLE 4 | Brood attendance duration and behaviors.

	Full model estimates ± SE		Selected model estimates ± SE		χ^2	*P*
Proximity						
Sex of the parents attending	(Female)	0.01 ± 0.12	(Female)	−0.03 ± 0.06	70.30	**<0.001**
	(Male)	0.78 ± 0.12	(Male)	0.55 ± 0.06		
Time of the day	(Afternoon)	0.09 ± 0.11	–	–	0.04	0.83
Age of the offspring	(10–19)	0.02 ± 0.13	(10–19)	−0.03 ± 0.07	10.48	**0.01**
	(20–29)	– 0.12 ± 0.13	(20–29)	−0.21 ± 0.07		
	(30+)	0.07 ± 0.14	(30+)	−0.09 ± 0.08		
Sex × Age of the offspring	(Female × age 10–19)	– 0.06 ± 0.18	–	–	8.44	0.21
	(Male × age 10–19)	– 0.10 ± 0.18	–	–		
	(Female × age 20–29)	0.02 ± 0.18	–	–		
	(Male × age 20–29)	– 0.28 ± 0.18	–	–		
	(Female × age 30+)	– 0.08 ± 0.20	–	–		
	(Male × age 30+)	– 0.40 ± 0.20	–	–		
Sex × Time of the day	(Afternoon × Female)	– 0.06 ± 0.15	–	–	1.5	0.47
	(Afternoon × Male)	– 0.16 ± 0.15	–	–		
Agonistic behavior						
Sex	(Male)	0.14 ± 0.07	(Male)	0.13 ± 0.05	6.11	**0.01**
Time of the day	(Afternoon)	– 0.11 ± 0.11	–	–	2.27	0.13
Age of the offspring	(10–19)	0.11 ± 0.07	–	–	4.45	0.22
	(20-29)	0.02 ± 0.08	–	–		
	(30+)	<0.001 ± 0.06	–	–		
Sex × Time of the day		– 0.03 ± 0.11	–	–	0.09	0.76
Vigilance behavior						
Sex	(Male)	– 0.08 ± 0.12	–	–	0.34	0.57
Time of the day	(Afternoon)	– 0.12 ± 0.22	–	–	0.37	0.54
Age of the offspring	(10–19)	– 0.15 ± 0.15	–	–	3.5	0.32
	(20–29)	– 0.19 ± 0.17	–	–		
	(30+)	−0.24 ± 0.13	–	–		
Sex × Time of the day		0.06 ± 0.20	–	–	0.10	0.75
Maintenance behavior						
Sex	(Male)	0.13 ± 0.17	–	–	0.48	0.49
Time of the day	(Afternoon)	0.27 ± 0.29	–	–	1.61	0.20
Age of the offspring	(10–19)	– 0.02 ± 0.19	–	–	2.21	0.53
	(20–29)	– 0.09 ± 0.21	–	–		
	(30+)	0.18 ± 0.17	–	–		
Sex × Time of the day		– 0.04 ± 0.29	–	–	0.02	0.86
Foraging behavior						
Sex	(Male)	– 0.20 ± 0.15	–	–	2.25	0.13
Time of the day	(Afternoon)	– 0.19 ± 0.26	–	–	0.18	0.67
Age of the offspring	(10–19)	0.14 ± 0.17	–	–	1.00	0.80
	(20–29)	0.14 ± 0.19	–	–		
	(30+)	0.09 ± 0.15	–	–		
Sex × Time of the day		0.09 ± 0.26	–	–	0.20	0.65

*Table shows the results of the full linear mixed model and for the selected models. The models test the difference in time spent on brood attendance or time spent on different behavioral categories (response variables). Table shows the results of the stepwise backward model selection: χ^2 and associated P-value for each variables. Table shows the estimates and standard error for the full model and for the selected model fitted with restricted maximum likelihood (REML). Factor variables' estimates contrasts term are indicated between parentheses. Stepwise backward model selection done with models fitted with maximum likelihood (ML) are given for each variable estimates (χ^2 and associated P-value). Significant P-values are shown in bold. Proportion of time spent on each behavioral category (used as response variable) was squared root transformed. Number of observations: N = 21; Male: N = 8, Female (**Supplementary Table 1** and **Supplementary Figure 4B**).*

of the courtship behaviors. In the same way, we observed the males doing more nest building behaviors than the female, especially in the afternoon, when copulation happens. Nest building by the males may also be used as part of the courtship behaviors, as it has been suggested in other jacana species (e.g., Bonkewitzz, 1997).

Polyandrous Mating

Our study confirmed that most female pheasant-tailed jacanas are polyandrous, although with a great variance in the number of mates (ranging from 1 to 5). The degree of polyandry (mean number of mates per females) was 2.4 and at least 81.8% of the females bred polyandrously.

This put the characteristics of the mating system of the pheasant-tailed jacana (in term of degree and proportion of polyandry) close to the northern jacana. Which, interestingly is one of the closest jacana species to the pheasant-tailed jacana on the phylogenetic tree (Whittingham et al., 2006; D'Urban Jackson et al., 2019).

Pheasant-tailed jacanas were originally classified by Oring (1986) as a classical simultaneous polyandrous species. In more recent papers, the term sequential polyandrous is sometimes used for the species (Thong-aree et al., 1995; Chen et al., 2008b). In a sense, our study inferred the sequential nature of breeding with several males by the females: females produced clutches in a sequential order for different males, having a turnover of about 7 days between the end of laying a clutch for one male and the starting of a new clutch with another male. On the other hand, several females maintained pair bonds with multiple males simultaneously. For example, female F3 had three males simultaneously incubating separate clutches on her territory (**Figure 3**). Our observation suggest that the females regularly visit the territory and nest sites of their mates after laying the clutch, both during incubation and brood care, even during periods when the female is engaged in courtship with another male (NF and AL, personal observations). This suggests that the pair bond is maintained for a longer period between the female and the males resident on her territory, which is also indicated by the laying of new clutches for the same males after clutch or brood losses (see **Figure 3** for examples). It has been observed in northern China that males become receptive to female after hatching of a clutch much sooner than in other species, with some population observed with males having only 2 weeks old brood (Jenni and Kirwan, 2020). This is something that we also witnessed in this population. These observations highlights the particularity of the mating system of pheasant-tailed jacanas as it is different from a strictly sequential polyandry, since in this latter system females usually desert her mate and offspring before initiate a new pair bond with another male (as in the Kentish plover or in the dotterel, Owens et al., 1994; Székely and Williams, 1995). It is nevertheless also different from classical polyandry, which refers to simultaneous pair bonding associated with simultaneous mating. In this paper, we try to clearly differentiate the terms "sequential polyandry" and "sequential polyandrous copulation" which in our opinion describe different level of mating system. We propose that the mating system of this species is closer to the system of other jacanas classified as having classical or simultaneous polyandry, as previously stated by Oring (1986), with however a sequential polyandrous copulation pattern which is different from other jacanas such as bronze-winged jacana (Butchart et al., 1999b). Maintenance of such pair bonding with the different mates through the breeding season could lower the cost of female competition for access to males. Meanwhile, the sequential polyandrous copulation could lower the probability for the male to raise unrelated chick as it was found in the comb-crested jacana (Haig et al., 2003) where pairs were genetically monogamous with only 2.8% of the chicks were unrelated to the father compared to 17% in the wattled jacana (Emlen et al., 1998).

Female birds are able to store sperms for several days, thus sperm competition between the current mate and the previous one of female jacanas is expected to be strong (Dale et al., 1999). In a strong sperm competition context, we would expect a high rate of copulation in order to ensure male's paternity. Our result shows the opposite: few copulations and mainly during egg laying. This observation goes in line with what was observed in the bronze-winged jacana (Butchart, 1999). Potential other strategies might be used by the males to reduce the chance of raising unrelated young. One strategy might be egg removal: indeed a previous paper on the pheasant-tailed jacana shows that males might remove the first egg laid in their clutch in order to reduce the possibility of cuckoldry (Chen et al., 2008a).

Male-Only Care

This study corroborated that parental care in pheasant-tailed jacanas was, as expected, essentially performed by males. This has been indeed already shown in this species (Serrao and Shekar, 1962; Chen et al., 2008b) and other polyandrous Jacanidae species (Jenni and Collier, 1972; Emlen and Wrege, 2004a; Butchart, 2008). Incubation was solely carried out by males and, contrary to what has been observed in wattled jacana (Jenni and Collier, 1972), female pheasant-tailed jacanas do not seem to perform any chick brooding, even when the males are busy with the incubation of a new clutch. Since the pheasant-tailed jacana population presents a strong male biased sex-ratio in the population, females have a more re-mating opportunity than males and are thus expected to invest more in re-mating than in parental care (Liker et al., 2013; Székely et al., 2013). This strong difference of investment could lead to the rigid sex role that we observe. The lack of flexibility by the female in her investment in parental care was observed in a mate removal experiment with polyandrous black coucal, Goymann (2019) showed that the female would not compensate the male absence in brood care.

However, we did observe some participation by the female in brood attendance, with and without the male around. Both males and females lower their time attending the brood with the age of the offspring. In the wattled jacana (Emlen and Wrege, 2004a) the female was observed attending especially young chicks (under 4 weeks). In their paper, Emlen and Wrege (2004a) described the females wattled jacana as "backup providers of chick care." In their study they found indeed that they observed females giving care in only 3% of the observation and each case were exceptional as they occurred in only two types of context: the male was predated, or the male was attending a clutch. This is not what was observed here as we still observed some females attending the brood outside these two contexts, for example when the male was foraging elsewhere. The variance of time spent by the female attending the brood in this study is not enough to see if this investment is related to male quality, pair bonding situation or female quality. Ultimately, the occasional proximity to the broods by the female could also be related to maintaining mating opportunity as it was suggested by several studies in other polyandrous birds (Goymann et al., 2015; Zheng et al., 2021).

We found that the behaviors performed by the females when attending the brood is almost the same as the males when attending the chicks with the exception of brooding behavior and agonistic behaviors. The latter result can be explained by longer time the males spend with the brood thus they may be present more often when the brood needs defense, for example from conspecifics or from other species.

CONCLUSION

In conclusion, our study shows a clear sex-role reversal pattern in the pheasant-tailed jacana with females being highly polyandrous and males taking most of the care of the offspring. However, we highlight some patterns that are important to take in account: (1) males also participated in territorial defense especially toward males that were not paired with the same female and males invested more in courtship behaviors than females. This led us to conclude that male pheasant-tailed jacana might undergo strong male competition for access to female through courtship, but may also cooperate with or tolerate co-mates in order to increase mate and resource defense. (2) Females also participated in brood attendance yet to a low degree confirming that female pheasant-tailed will invest more into mating than providing care, (3) polyandry in this species involves the simultaneous maintenance of bonds with several males by a female which will sequentially copulate with them. These deviations from what is expected in a sex-role reversal species suggest that the mating system shows subtle variations even among closely related species that all exhibit classical polyandry. Our study was conducted on a relatively small number of individuals and only in the second half of the breeding season, that may limit the generality of some our conclusions (e.g., for sex differences in courtship, see above). Thus, a longer study through several years would be useful for further corroboration of our findings. Ultimately, such results on sexual difference in mating and parental investment in a sex-role reversal species can help us to understand better the evolutive mechanisms leading to this unconventional sex-roles.

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 the Forestry Bureau of Taiwan.

AUTHOR CONTRIBUTIONS

AL, TS, AK, and NF conceived and developed the study. W-CL and Y-FL took care of the organization and administration regarding the project and the study site in Taiwan. NF collected the data with the help of Y-FL and W-CL. NF analyzed the data. NF and AL wrote the manuscript with input from TS, AK, and Y-FL. All authors contributed to the final manuscript.

FUNDING

The project was funded by the National Research, Development and Innovation Office of Hungary (NKFIH, grant KH130430). NF was supported by NHKFIH PD134414 grant, AK was supported by NKFIH NN125642 grant, and Y-FL was supported by the Forestry Bureau of Taiwan (108 FD-9.1-C-36 and 109 FD-9.1-C-32). TS was funded by The Royal Society (Wolfson Merit Award WM170050, APEX APX\R1\191045) and by the National Research, Development and Innovation Office of Hungary (ÉLVONAL KKP-126949, K-116310).

ACKNOWLEDGMENTS

We thank the Forestry Bureau of Taiwan, and the City of Tainan for permission to work on their property. We thank the Jacana Ecological and Educational Park for allowing us to work in their park and we are especially grateful to the park employee for kindly welcoming us and helping with the fieldwork handling. We also thank the ÉLVONAL shorebirds community, especially Vojtěch Kubelka for giving extremely good insights and suggestions in the development of this project methodology and fieldwork organization. Two reviewers and the associate editor are thanked for their constructive comments which improved the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.742588/full#supplementary-material

Supplementary Figure S1 | Map of the study site surveyed: The Pheasant-tailed Jacana educational park in Guantian, Tainan, Taiwan. Dark green indicates the ponds used for behavioral observation.

Supplementary Figure S2 | Pictures of different pheasant-tailed jacana individuals from the study population. Pictures **(A,B)** show the sexual size dimorphism between males and females (**A**: male M3 on the left/front and female F3 on the right/back; **B**: male M20 on the left and female F10 on the right). Pictures **(C–F)** illustrate the variation between individuals in their plumage: note the differences in the black patch on the top of the head, in the white wing patch pattern, and in tail length (**C**: male M3 and one of his chicks; **D**: female F10 from another angle; **E**: male M16 and his eggs; **F**: male M17 and his egg).

Supplementary Figure S3 | Examples of individual variability in pheasant-tailed jacana drawn from field sketches made in the study area. **(A)** Male M12. **(B)** Male M1. **(C)** Female F4. **(D)** Female F3.

Supplementary Figure S4 | Proportion of time spent on different focused categories of behaviors by the males (in black) and females (in gray) during **(A)** courtship observation and **(B)** brood attendance (i.e., in proximity of the chicks).

Mean ± SE. Sample size: **(A)** Number of observations: $N = 44$; Male: $N = 15$, Female: $N = 10$ and **(B)** Number of observations: $N = 21$; Male: $N = 8$, Female: $N = 5$. $^{*}P < 0.05$; $^{***}P < 0.00$. Error bars denote standard errors. Details of the results are shown in **Tables 2**, **3**, respectively.

Supplementary Figure S5 | Proportion of time of the observation spent on brooding the offspring by the male according to the age of the offspring (less than 10 days old, between 10 and 20 days old, between 20 and 30 days old, and more than 30 days old). Each dot represents one brood care observation.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors TS.

frontiers
in Ecology and Evolution

REVIEW
published: 05 November 2021
doi: 10.3389/fevo.2021.749495

Mate Choice, Sex Roles and Sexual Cognition in Vertebrates: Mate Choice Turns Cognition or Cognition Turns Mate Choice?

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The idea of "smart is sexy," meaning superior cognition provides competitive benefits in mate choice and, therefore, evolutionary advantages in terms of reproductive fitness, is both exciting and captivating. Cognitively flexible individuals perceive and adapt more dynamically to (unpredictable) environmental changes. The sex roles that females and males adopt within their populations can vary greatly in response to the prevalent mating system. Based on how cognition determines these grossly divergent sex roles, different selection pressures could possibly shape the (progressive) evolution of cognitive abilities, suggesting the potential to induce sexual dimorphisms in superior cognitive abilities. Associations between an individual's mating success, sexual traits and its cognitive abilities have been found consistently across vertebrate species and taxa, providing evidence that sexual selection may well shape the supporting cognitive prerequisites. Yet, while superior cognitive abilities provide benefits such as higher feeding success, improved antipredator behavior, or more favorable mate choice, they also claim costs such as higher energy levels and metabolic rates, which in turn may reduce fecundity, growth, or immune response. There is compelling evidence in a variety of vertebrate taxa that females appear to prefer skilled problem-solver males, i.e., they prefer those that appear to have better cognitive abilities. Consequently, cognition is also likely to have substantial effects on sexual selection processes. How the choosing sex assesses the cognitive abilities of potential mates has not been explored conclusively yet. Do cognitive skills guide an individual's mate choice and does learning change an individual's mate choice decisions? How and to which extent do individuals use their own cognitive skills to assess those of their conspecifics when choosing a mate? How does an individual's role within a mating system influence the choice of the choosing sex in this context? Drawing on several examples from the vertebrate world, this review aims to elucidate various aspects associated with cognitive sex differences, the different roles of males and females in social and sexual interactions, and the potential influence of cognition on mate choice decisions. Finally, future perspectives aim to identify ways to answer the central question of how the triad of sex, cognition, and mate choice interacts.

Keywords: fitness, behavioral flexibility, personality, sexual selection, social cognition, cognitive mate choice, cognitive flexibility, sexual dimophism

Edited by:
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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *29 July 2021*
Accepted: *12 October 2021*
Published: *05 November 2021*

Citation:
Fuss T (2021) Mate Choice, Sex Roles and Sexual Cognition in Vertebrates: Mate Choice Turns Cognition or Cognition Turns Mate Choice? Front. Ecol. Evol. 9:749495. doi: 10.3389/fevo.2021.749495

SEXUAL DIMORPHISMS IN COGNITIVE PERFORMANCE – DO SEXES DIFFER?

'Cognition' is frequently defined as the neuronal processes principally involved in the acquisition, processing, retention, and use of information (Shettleworth, 2001; Dukas, 2004). Another important criterion for cognitive ability is the aptitude to learn and to establish associations between different stimuli (Giurfa et al., 2001). In the context of this review, I would like to describe cognition as cognitive processes that comprise thinking, reasoning, perceiving, imagining, and remembering to the extent that they involve the use of concepts (Bayne et al., 2019). Furthermore, cognition is inherent to the ability of adaptive behavioral plasticity, and is thought to shape and modulate evolutionary dynamics and, possibly, the limits of adaptation profoundly (e.g., Sznajder et al., 2012; Snell-Rood, 2013). For instance, depending on how complex a species' habitat, social environment, or life history is, the more cognitively demanding it is to survive and cope with its challenges. Cognitively flexible individuals perceive and respond more rapidly to (unpredictable) environmental changes. To give a prominent example, a well-developed ability to learn spatially seems to play a prominent role in mate search and mate choice in both vertebrates (e.g., Sherry et al., 1992; Geary, 1995; Kotrschal et al., 2015; Corral-López et al., 2017) and invertebrates (e.g., Papaj and Prokopy, 1989; Dukas, 2005).

Sex roles that females and males play within their population (influenced e.g., by age or social status) can vary tremendously in response to the prevailing mating system. Depending on how cognition determines these highly distinct sex roles, different selection pressures could possibly influence the (progressive) development of cognitive abilities, thereby perhaps inducing sexual dimorphisms in superior cognitive capabilities (Galea et al., 1996; Jacobs, 1996; Johnstone et al., 1996; Lindenfors et al., 2007; Edward and Chapman, 2011). Thus, a species' mating system appears to be a major driver of cognition, with sexual selection being a key determinant of cognitive evolution. It may act directly by promoting superior cognitive abilities during mating competition, for instance, with one sex opting for the other based on a behavioral trait that is strongly influenced by cognition (e.g., solving foraging tasks). For instance, males possibly prefer females with better cognitive abilities in mutual mate choice or sex-reversed species (although this question has only been investigated with females being the choosing sex). Social and sexual interactions as well as the mating system, which attributes distinct responsibilities to each sex ranging from courtship and mate choice to nurturing the offspring, are particularly important for ample cognitive differences (Boogert et al., 2011b; Baur et al., 2019).

Interestingly, sexual dimorphisms in cognitive performance have been observed in a number of different species representing a variety of taxa. Although a number of studies examined cognitive sex differences in various different tasks and primarily independent of a mate choice or reproductive context, their findings have been attributed frequently to result from divergent sex roles. To mention a few examples, females frequently showed greater cognitive flexibility and lower persistence than males in response to new situations in a number of mammal, bird, and fish species (e.g., Ha et al., 2011; Lucon-Xiccato and Bisazza, 2014; Roelofs et al., 2017), while generally better spatial orientation skills were often attributed to males. For instance in humans, males performed better than females in spatial navigation tasks (e.g., Sherry and Hampson, 1997; Moffat et al., 1998), which appears to be the most consistent sex difference in cognition in humans and other mammals (Moffat et al., 1998; Jones et al., 2003; Jones and Healy, 2006). Male guppies (*Poecilia reticulata*) quickly learned to swim through a complex maze, in which guppies had to choose between alternative routes to reach the target, while their conspecific females failed to do so (Lucon-Xiccato and Bisazza, 2017a,b).

However, there are also examples to the contrary. Female guppies were observed to outperform males (a) in a spatial orientation task requiring them to learn to select the correct arm of a T-maze to rejoin a group of conspecifics and (b) in a numerical task requiring them to discriminate between 5 and 10 dots to obtain a food reward (Petrazzini et al., 2017). Although male ravens (*Corvus corax*) were better than females in a color discrimination test, they were inferior in a spatial discrimination task (Range et al., 2006). Female cowbirds (*Molothrus ater*) possess superior spatial learning abilities compared to their male counterparts (Astié et al., 1998; Guigueno et al., 2014) as they need to find and recruit host nests, in which to place their eggs. Furthermore, accumulating evidence point to selection acting on spatial memory in food-caching bird species (Krebs et al., 1989; Roth et al., 2011; Cauchoix and Chaine, 2016; Sonnenberg et al., 2019). Female great tits clearly excelled males in an observational memory task, in which caged great tits were allowed to observe food-caching marsh tits in an indoor aviary before they were allowed to search themselves. Female great tits were as successful at retrieving the cached food as the hoarding marsh tits themselves (Brodin and Urhan, 2015). In summary, the extent of the observed sex differences reported on many species of different taxa appears to depend tightly on the task to be solved and, hence, the cognitive competence involved. While female guppies appeared to be behaviorally more flexible (Laland and Reader, 1999; Lucon-Xiccato and Bisazza, 2014; Lucon-Xiccato and Bisazza, 2017a,b), male Atlantic mollies (*Poecilia mexicana*) clearly outperformed their female conspecifics both in social and asocial trial and error learning of a simple visual color discrimination task followed by a series of reversal learning (Fuss and Witte, 2019; Fuss et al., 2020). Despite the convergence of their learning performance in numerical discrimination experiments, male and female western mosquitofish (*Gambusia affinis*) differed in their cognitive-behavioral responses that could possibly be attributed to different sexual selection pressures (Etheredge et al., 2018).

Additional key determinants comprise cognitive style (i.e., "*the way individuals acquire, process, store, or respond to information regardless of their cognitive ability*", Sih and Del Giudice, 2012) and cognitive performance (i.e., accuracy of behavioral output on a learning task, Shettleworth, 2010). Sex-specific associations between the cognitive style, which is essentially determined by an individual's personality, the training context (i.e., using automated devices such as a Skinner box vs. ecologically more natural training procedures), and the cognitive performance have been observed frequently as well (e.g., Gatto et al., 2020; Griebling et al., 2020; Wallace et al., 2020; Wallace and Hofmann, 2021). In summary, we find strong evidence to support context-dependent differences in cognitive abilities between both sexes. In particular, context-dependency addressing different 'cognitive domains' appears to reflect the respective innate social and/or sexual role, for instance in reproduction.

SOCIAL COGNITION IN A MATE CHOICE CONTEXT

Numerous studies indicate that females select mates based on male cognitive traits. Associations between an individual's mating success and its cognitive abilities (Shohet and Watt, 2009; Keagy et al., 2009, 2011; Cauchard et al., 2013; Preiszner et al., 2017) as well as between its cognitive abilities and sexual traits (Karino et al., 2007; Boogert et al., 2008, 2011b; Mateos-Gonzalez et al., 2011; Keagy et al., 2012; Fabre et al., 2014; Minter et al., 2017) have been reported across species and taxa, suggesting that sexual selection may well shape the supporting cognitive prerequisites (Andersson and Simmons, 2006; Boogert et al., 2011b; Sewall et al., 2013; Isden et al., 2013). Yet, there is, of course, contrary evidence where cognitive ability was not the decisive criterion (e.g., Sewall et al., 2013; Templeton et al., 2014; Anderson et al., 2017). For instance, another study on spotted bowerbirds found no association between mating preference and general cognitive ability or improved performance in obstacle clearance or shape discrimination (Isden et al., 2013). Starlings reared under developmental stress showed a comparable cognitive performance to naturally reared conspecifics in a foraging task, but lower sexual signaling (song performance; Farrell et al., 2012; Peters et al., 2014).

However, superior cognitive abilities do not only provide benefits such as a higher feeding success, a better antipredator behavior, or a superior mate choice, but also demand higher energy levels and metabolic rates (due to well-developed neuronal prerequisites), which possibly decrease fecundity, growth, and immune response. Alongside possible cognitive sex differences, aspects of social cognition have been examined carefully as well. Social cognition implies both social recognition (i.e., acquisition of direct or indirect social information about others) as well as social learning (i.e., retrieving social information from others) from individuals sharing the same sex and, thus, the same social role. Using social information for mate choice to ultimately choose the best possible, maybe 'perfect' mate is particularly important when determining where to look for prospective mates, whom to avoid or pair with, how to distinguish and classify different individuals, and involves integrating and processing multimodal sensory inputs (compare Edward, 2015 for review; Cummings and Ramsey, 2015; Kavaliers and Choleris, 2017). This complex process is complemented by arousal and sexually incentive motivation, and accompanied by preference, responsiveness, and effort of a prospective choice, which includes the conversion of arousal into sexually determined behaviors (Jennions and Petrie, 1997; Kirkpatrick et al., 2006; Ågmo, 2011; Edward, 2015). Choosing females appear to base their mating decisions on multiple male traits (Andersson, 1994; Rosenthal, 2017).

The key features of cognition, i.e., learning and decision-making processes may dynamically change to adapt to new conditions in order to increase their prospects for a high-quality partner. Social information, for instance, can be drawn upon when making decisions about potential partners, which may possibly allow performing 'learned mate choices'. In this context, mate choice may include personal experience with others (i.e., private or personal information) or the observation of conspecifics (i.e., public information) and may have an impact throughout an individual's entire life (reviewed in Hebets and Sullivan-Beckers, 2019). Thereby, social cognition provides a conceptual framework for mate choice or mate choice copying across taxa, including Arachnida, Insecta, Malacostraca, Aves, and Actinopterygii (compare Witte et al., 2015 and Jones and DuVal, 2019 for review). Hence, mate choice copying is another example of the supporting cognitive capability to observe, evaluate, and, if appropriate, imitate other individuals in the same way as individuals do whilst foraging for food or seeking novel habitats (Jones and DuVal, 2019). Interestingly, the prominently hypothesized fitness benefit of superior cognition suggests that smart individuals would be preferred as mates (hypothesis of sexual selection; Darwin, 1871; Jacobs, 1996; Miller and Todd, 1998; Miller, 2000; Boogert et al., 2011a,b; Peters et al., 2014), though it has rarely been examined in non-human individuals (Striedter and Burley, 2019). Moreover, it has not been explored conclusively yet, how the choosing sex judges the cognitive abilities of potential mates, which would be a necessary first step if smarter mates were preferred over their 'not-as-smart' counterparts. Consequently, cognition may also have considerable implications for sexual selection processes (Boogert et al., 2011b; Ryan et al., 2009).

COGNITIVE MATE CHOICE

Mate Choice Based on Cognitive Traits?

Animal courtship displays can be remarkably complex. They may comprise several contiguous steps, involve different modalities (e.g., visual, acoustic, odorous, and/or tactile stimuli), integrate morphological and behavioral aspects and may adapt to and/or depend on different contexts. The ability to perform extensive courtship displays, complex songs or acquire scarce resources through superior foraging and/or spatial orientation skills may be a major contributing factor for the success in outperforming competing conspecifics. Impressive and well-studied examples comprise peacock spiders that use synchronous motion displays (Girard et al., 2011, 2015) or bowerbirds and sage grouse, who adapt their courtship display intentionally in response to female signals (Patricelli et al., 2002, 2006, 2011; Patricelli and Krakauer, 2010). Similarly, in carotenoid-dependent signaling systems such as siskins, brown boobies, house finches, or sticklebacks and guppies, superior foragers produce exaggerated sexual signals to impress courted females (e.g., Endler, 1980, 1984; Kodric-Brown, 1985; Milinski and Bakker, 1990; Senar and Escobar, 2002; Karino et al., 2007; Mateos-Gonzalez et al., 2011; Michael et al., 2018). Therefore, in numerous taxa, males indicate their suitability as potential mates by various elaborate traits. The courted females respond with individual preferences for one or another trait. However, the complexity of the performed courtship behavior seems not to be the only determinant for choosing a suitable mate. Recent findings point to cognitive abilities underlying both 'adaptive' production and evaluation of complex courtship displays (Boogert et al., 2008, 2011a,b; Ryan et al., 2009; Keagy et al., 2012). In this context, cognitive style, i.e., the way an individual processes whatever information about its same-sex or opposite-sex conspecifics, is likely to have a substantial impact on its perception of any sexual signals. Moreover, its cognitive style determines its capability to evaluate these signals in a mate choice situation. For instance, comparisons of individual problem solving capacities within a species, especially while foraging, have shown positive correlations with fecundity (Cole et al., 2012; Wetzel, 2017). There is compelling evidence in a wide variety of taxa that females appear to prefer males who are adroit problem solvers, i.e., they prefer those who presumably have better cognitive abilities (mammals: Spritzer et al., 2005; Prokosch et al., 2009; Kavaliers and Choleris, 2017; Silk and Kappeler, 2017; birds: Keagy et al., 2009, 2011; Boogert et al., 2011b; Peters et al., 2014; Mateos-Gonzalez et al., 2011; fish: Shohet and Watt, 2009; Minter et al., 2017; Keagy et al., 2019). Hence, I anticipate that the females' preference for males demonstrating superior cognitive abilities will increase if females experience either direct and/or indirect benefits through mating with them (Keagy et al., 2009; Boogert et al., 2011b). If an individual shift in preference does indeed yield the expected benefits and confers a reproductive advantage over non-preferring females, this may hypothetically translate into an evolutionary preference change across generations.

Mammals

In humans, superior cognitive abilities and innovativeness are generally associated with affirmative (social, sexual) life outcomes (e.g., Plomin and Deary, 2015). However, the association between innovative problem-solving capabilities and their effects on mate choice, reproduction and fitness in non-human mammals is frequently neglected. Innovativeness facilitates survival in complex or changing environments, and allows individuals to explore and colonize novel habitats (Sol et al., 2005). Despite the obvious ecological and evolutionary benefits of being innovative (Laland and Reader, 1999; Nicolakakis et al., 2003; Reader and Laland, 2003), variation within a species, between the sexes, and across different species is only starting to be studied in more detail. For instance, behavioral studies revealed that primates and hyenas share similar socio-cognitive abilities. Elevated levels of innovativeness compared to other carnivores (Benson-Amram and Holekamp, 2012; Benson-Amram et al., 2016) have been observed in wild vervet monkeys (*Cercopithecus aethiops*) as well as in spotted hyenas (*Crocuta crocuta*). Aiming to determine whether innovativeness might be an adaptive trait in (female) spotted hyenas, Johnson-Ulrich et al. (2019) analyzed innovativeness in problem-solving tests in 29 female individuals and set these alongside long-term data on their fitness, reproduction and survival. They found innovative females to reproduce more cubs; however, their cubs showed a lower probability of survival compared to the fewer cubs of non-innovators. Hence, choosing a mate with superior cognitive abilities seems to pay off initially, but does not translate into an increased likelihood of passing on one's genes to the next generation in the long run. Huebner et al. (2018) examined possible associations between body condition, survival rates, individual cognitive performance on repetitive attempts to solve a food extraction task (removing a sliding cover placed on small boxes to access a food reward), and spatial learning in a four-arm maze in wild gray mouse lemurs (*Microcebus murinus*). The cognitive ability to adopt a newly discovered technique to exploit novel food sources in times of scarcity quickly could provide them with significant fitness advantages. This pronounced ability should impress a female when observing and, finally, choosing a mate, as she could derive better nurturing of her offspring. However, neither the individuals' cognitive performance in both tasks correlated with each other, nor did the performance correlate with the gray mouse lemurs' survival rates (Huebner et al., 2018).

Sociality within a cohabitant community or population is also a relevant dimension in the context of cognition and mate choice. Williams et al. (2020) examined the direct and indirect effects of social position and individual behavioral traits on solving a novel puzzle box in social yellow-bellied marmots (*Marmota flaviventer*). Social relationships, the type of interaction and the individual role (i.e., recipient or initiator) significantly determined the way an individual interacted in cognitive tests. Interestingly, living a very social lifestyle resulted in lower reproductive success, increased hibernation mortality and, consequently, lower survival rates in female but not in male yellow-bellied marmots. Especially young females and their offspring greatly benefited from larger social groups. Again, this effect was not observed in males regardless of age (Montero et al., 2020). Conversely, the strength of affiliation was negatively associated with female annual reproductive success (Wey and Blumstein, 2012). These findings clearly indicate that social behavior, social and/or sex roles, and cognitive abilities not only play an important role during mate choice and reproduction, but also seem to translate in the probability of the own and/or the offspring's survival in a positive or negative way. Male meadow voles defend large home territories against conspecific rivals and their reproductive success is closely linked to finding females to mate with. Indeed, the courting males' spatial learning ability is considerably more pronounced compared to their conspecific females (Gaulin and FitzGerald, 1986, 1989; Galea et al., 1996). As is the case with many rodents, they typically leave their scent on prominent landmarks or along trails to ensure that they are perceived by conspecifics of both sexes. The scent marks provide various social information, such as sex, reproductive state, health condition, or social rank. The odor information targets different recipients (e.g., receptive females, competitors or heterospecifics), who in turn classify its relevance and value according to its phenotype, genotype, and intentions.

Hence, depending on which animal encounters the scent mark, it has to compare the social odor information with memorized details and make various decisions relating to, for instance, mate choice, same-sex competition, social olfactory communication, or sperm distribution/competition. In addition to the availability of mates and resources, the own fitness, age, sex, and social position play a key role to ensure survival and to improve the own fitness. As a result, a complex, associative social memory develops to identify scent marks as being, e.g., from males or females, as known or unknown, as sexually immature or receptive. However, as scent marks of different animals may overlap, complex supporting cognitive capabilities are required to form appropriate associations and adapt behavior accordingly (Ferkin, 2011, 2018).

Birds

Many studies on mate choice in birds focus on physical traits, but aspects of individual personality and social alliances are also receiving increasing attention. In particular, in long-lived avian species, social alliances that form at pre-mature life stages (i.e., prosocial behavior) apparently lead to long-term social bonds (parenthood, long-term cooperative pair and/or group behavior) at a sexually mature age (Kaplan, 2020). Examples of prosocial bird species, who are known for their exceptional cognitive abilities, include jackdaws (*Corvus monedula*) (De Kort et al., 2006; von Bayern et al., 2007; Schwab et al., 2012), Eurasian jays (*Garrulus glandarius*) (Ostojić et al., 2013, 2014), rooks (*Corvus frugilegus*) (Scheid et al., 2008), common ravens (*C. corax*) (Di Lascio et al., 2013; Massen et al., 2015), or even African gray parrots (*Psittacus erithacus*) (Péron et al., 2013). In this context, sociality including its cognitive and affective dimensions, and mate choice might play a key role in birds in terms of a "*pre-sexual attachment to a potential mating partner*" (Kaplan, 2020).

In a wide range of avian species, strong relationships were discovered between the length of parental brood care and brain size, raising suggestions of related enhanced cognitive capabilities. These suggestions were supported by observations, for instance, in several corvids, bowerbirds, albatrosses, and cockatoos, which revealed prolonged parental guidance just until the first own brood to be an essential component in the acquisition of social and cognitive skills (Kaplan, 2020). To give an example, the sexual display of male song is generally considered a sex-specific social behavior that is learned prior to sexual maturity and presumably requires elevated cognitive skills in both sexes (Boogert et al., 2011b; Peters et al., 2014; Anderson et al., 2017). Most bird song research has been performed assuming that songbirds have evolved complex song repertoires due to the pressure of sexual selection on males that arises from both competition between males and female choice (Beecher and Brenowitz, 2005; Byers and Kroodsma, 2009). Interestingly, learned aspects of song have been found to alter female mating preferences in various songbird species (DuBois et al., 2018), although females usually do not sing themselves. It is important to note, however, that females of more than two-thirds of all songbird families also sing in contexts such as tropical dispersal, territoriality, convergent sex roles, and sexual dichromatism. In many species, however, females have lost song secondarily (compare Odom et al., 2014 for review).

Several studies on the relationship between a male's vocal repertoire and various cognitive processes try to determine whether the male's song may provide conspecific females with information on his cognitive abilities (Boogert et al., 2011b; Sewall et al., 2013; Anderson et al., 2017). So, sex roles appear to be clearly assigned in many songbird species: while males sing as part of their courtship display, females listen to and evaluate the quality of the males' song. Although the neuronal prerequisites that guide the complex process of singing and song composition in male and female songbirds are well understood (Nowicki et al., 2002; Gobes and Bolhuis, 2007; Mooney, 2009; Jin, 2013; Sewall et al., 2013; Geberzahn and Aubin, 2014; Odom et al., 2014), there is still a lack of understanding of the relationship between song learning and other cognitive processes (Anderson et al., 2017) in the context of mate choice. Female songbirds prefer males presenting a larger vocal repertoire (Searcy, 1984; Lampe and Saetre, 1995) and song is deemed an honest signal of male quality and fitness measures. In male song sparrows (*Melospiza melodia*), song repertoire size correlates positively with an array of fitness traits comprising territory ownership, heterozygosity, immune system quality, longevity, and lifetime reproductive success (Reid et al., 2005; Boogert et al., 2011a). In a 20-year-long-term study, Reid et al. (2005) analyzed data from free-living male song sparrows (*M. melodia*) in terms of song repertoire size and the number of independent and recruited offspring and grandoffspring. The larger the song repertoire was, the longer-lived the males were and the greater was their reproductive success extending into the next and even the succeeding generation. Wild female song sparrows (*M. melodia*) prefer males with a rich vocal repertoire, which also correlates with various fitness measures (e.g., song repertoire size and different motor, color association, reversal learning or detour-reaching tasks tested in captivity, Boogert et al., 2011a,b; DuBois et al., 2018). Moreover, song complexity as a sexual signal has been linked to male zebra finch performance in a novel foraging task (*Taeniopygia guttata castanotis*, Boogert et al., 2008). Conversely, Sewall et al. (2013) challenged song sparrow males (*M. melodia*) in a spatial memory task and discovered an inverse relationship between spatial memory performance and male vocal repertoire.

Associations between song repertoire size and cognitive performance were examined in wild song sparrows (*M. melodia*). Initially, males' song repertoires were recorded in the field. Subsequently, these males were tested in motor, color association and reversal learning, and detour-reaching tasks (Boogert et al., 2011a,b). The individuals' color association performance was positively correlated with their performance in the subsequent reversal task. Interestingly, their performance did not correlate with the other learning tasks they were challenged with. Nevertheless, males having a wider song repertoire were able to solve difficult tasks in a shorter period of time, but performed poorly in the reversal task compared to males having a more limited song repertoire (Boogert et al., 2011a,b). Yet, results remain inconclusive as no correlations were observed in laboratory-raised song sparrows following the same experimental agenda (Anderson et al., 2017). In a recent study, a group of 49 swamp sparrows (*Melospiza giorgiana*) was examined for their song quality (i.e., repertoire size, vocal performance, song learning; DuBois et al., 2018). Subsequently, song quality was compared to the performance in five different cognitive tests, comprising a novel foraging task, a color association and a reversal task, a spatial learning task, and a detour-reaching task. Unexpectedly in the context of the previous study by Boogert et al. (2011b), none of the song parameters were considered indicative of any cognitive performance level. Hence, these findings contradict the hypothesis on song properties indicating overall cognitive abilities in swamp sparrows (DuBois et al., 2018). Likewise, no associations between song repertoire and cognitive performance (detour reaching, spatial memory) were obtained when testing wild North Island robins (*Petroica longipes*), which are a foraging species, admittedly with a quite different ecology than sparrows (MacKinlay and Shaw, 2019). In summary, there remains controversy about the phenomenon of bird song and its implications for cognitive mate choice (compare Searcy and Nowicki, 2019 for review).

However, several studies on different bird species attempted to investigate associations between cognition and mate choice from different perspectives. In food caching New Zealand robins (*P. longipes*), males' memory performance in a spatial task during winter severely influenced their subsequent breeding success. Individuals with higher performance levels produced more fledglings and, subsequently, more independent offspring per nesting attempt. Males with superior memory performance spent more time in flight while foraging and provisioning, and, additionally, provided their chicks with an increased share of large prey items. These effects were absent in female robins (Shaw et al., 2019). Male bowerbirds spend a considerable amount of time building their bowers to attract females and convince them to mate. Several studies revealed that the male satin bowerbirds' mating success was positively associated with their problem—solving performance and aggregate measures of their cognitive ability (Keagy et al., 2009, 2011). The males were challenged with six different cognitively challenging tasks, including two problem-solving tasks, one mimetic repertoire task and three bower-rebuilding tasks. Although no correlations between the males' performances in different tasks were observed, females chose the overall well-performing males, thereby apparently considering information about several behavioral display traits (Keagy et al., 2012). However, Isden et al. (2013) reported conflicting observations by finding no relationship between performance on cognitive and problem-solving tasks and mating success in male spotted bowerbirds.

Two studies, one using zebra finches (*T. guttata castanotis*; Chantal et al., 2016) and the other using budgerigars (*Melopsittacus undulatus*; Chen et al., 2019) explored whether females would modify their mating preference after having observed the cognitive performance of males in a problem-solving task:

Initially, zebra finch females were challenged with a set of two males to assess their spontaneous preference for one or another (Chantal et al., 2016). Then, both zebra finch males (i.e., the preferred and the unpreferred one) were trained to open a tube by removing the lid to access a food reward (Chantal et al., 2016). In order to manipulate their success, one male (the previously unpreferred male, now assigned to be the 'solver') was challenged with a tube the lid of which was pressed only halfway and which could be easily opened. The other male (the previously preferred male, now assigned to be the 'non-solver') was challenged with a tube the lid of which was fully pressed and, thus, was impossible to open. Subsequently, females were allowed to observe both males being challenged with the tube-opening task. In contrast to the first preference test, females preferred the solvers, i.e., the previously unpreferred males in the final second preference test. To determine (a) whether females were able to discriminate between both males and (b) whether the males' problem-solving abilities had in fact triggered the shift in female preference, all birds took part in a color association task. Females were well able to discriminate visually between the presented males, and their preference was found to be independent of the males' learning rate in this task. Considering the results of both tasks in a shared context, zebra finch females were found to significantly prefer the most skillful (i.e., the initially unpreferred) male in both tasks. As males differed in their feeding rates in both treatments, females appeared to use the males' foraging efficiency as an important criterion when choosing a mate (Chantal et al., 2016).

The other study (Chen et al., 2019) followed a similar experimental design, in which budgerigar females were challenged initially with a set of two males to assess their spontaneous preference for one or another as well. Then, non-preferred male budgerigars were trained to open transparent boxes containing seeds. Meanwhile, the preferred males and females were exposed to already-opened containers, so they could not attempt to solve the following foraging task. Subsequently, each female was allowed to observe the trained (but initially unpreferred) males repetitively opening the boxes, while the untrained (but initially preferred) males failed. In consecutive second preference test trials, females changed their social preferences in favor of the successful, formerly unpreferred males. Control tests suggested that the females' preference shift did not only reflect the observation of trained males feeding on seeds, i.e., the males' ability to provide food. Furthermore, females showed no preference for other females trained to open the seed boxes, indicating that the main finding related to an intersexual context (Chen et al., 2019).

However, even with these two carefully designed studies, which have yielded impressive results, we cannot be entirely convinced that cognition was the main factor in the females' choice. First, neither zebra finch nor budgerigar females were allowed to attempt the problem-solving tasks (i.e., zebra finches opening tube lids or budgerigars opening boxes) themselves. Yet, in order to be able to evaluate and, subsequently, rank the males' individual abilities in solving a particular task, the judge herself needs to be aware of the complexity of the task she is now meant to assess. It is therefore difficult to predict the extent to which the zebra finch or budgerigar females were able to assess the cognitive aptitudes of the males performing the test. Second, neither zebra finch nor budgerigar females were allowed to observe the males while learning and, thus, examine differences in the males' learning processes. Hence, females exclusively witnessed the output of the preceding training, which was attended only by a selection of the males. Third, the zebra finch and the budgerigar males' success in problem solving was manipulated in both studies, either by closing the tubes at varying degrees of tightness (Chantal et al., 2016) or by training only the unpreferred males (Chen et al., 2019). Instead of assessing cognitive problem-solving abilities, the observing females may have interpreted the differences in males' ability to access the containers as differences in the males' physical strength (Striedter and Burley, 2019). Indeed, the training itself could have had an impact on the males' behavior. Different degrees of training might have also been a reflection of different levels of male self-confidence or audacity in approaching the task in the female's presence, which, potentially, could have influenced her choice. For instance, the trained (initially unpreferred) males may have acted more keenly when handling the tubes or containers since they were already acquainted with the experimental setting, while the inexperienced (but initially preferred) males were not. Hence, this raises the question of the extent to which personality traits such as boldness, shyness, retentiveness, or self-confidence, i.e., an individual's cognitive style play an important role in (a) approaching cognitive tasks, and in (b) assessing cognitive abilities by potential mates. Camacho-Alpízar et al. (2020) added another perspective by questioning whether successful problem-solving can be linked to cognitive abilities at all, as "*non-cognitive factors (e.g., persistence) are often correlated with problem-solving success*".

Fish

Beyond mammals and birds, an increasing number of studies addresses the role of cognition in mate choice of various fish species. Relative to all vertebrate taxa, fish do not only represent the greatest species diversity, but also inhabit the most diverse physical and social environments. They are characterized by the greatest variation in brain anatomy of all vertebrates. This gives them the neuronal basis for different levels of behavioral plasticity in response to their environment. In turn, this suggests, hypothetically, an equivalently wide variety of cognitive traits relating to social interactivity and mate choice. Many fish species can flexibly adapt their physiology and behavior to cope better with challenging environmental conditions. In fact, a major component of this flexibility is supported and influenced by both cognition and neuronal plasticity (Ebbesson and Braithwaite, 2012; Herczeg et al., 2019). Novel foraging information propagates considerably faster between female guppies than between males, possibly because the reproductive success of female guppies is inherently more strongly linked to resource availability than it is the case for male guppies (Reader and Laland, 2000). Male guppies (*P. reticulata*) were trained to solve two different mazes to obtain a food reward (Shohet and Watt, 2009). Subsequently, female guppies were allowed to repeatedly observe several different trained males orienting within the maze. Consecutive mating preference tests as well as the time it took a male to learn both mazes were used to determine a possible association between the females' preferences and the males' learning ability. Indeed, the observing females preferred the faster-learning males, who they judged to be more attractive than the slow learners in subsequent mating preference tests. Furthermore, the females' preference was not related to the males' body size or coloring, although males of a stronger orange color solved the tasks faster than their less colorful peers. Similar to the bird studies (Chantal et al., 2016; Chen et al., 2019), guppy females were unfortunately not allowed to observe the males while learning to pass through the mazes, nor were the females themselves challenged with any maze in order to be able to judge the level of difficulty and/or the males' performance. Consequently, we cannot fully reject the objection that females may have taken into account any other, unmeasured traits such as the males' display rates (Matthews et al., 1997; Kodric-Brown and Nicoletto, 2001) or the males' general mobility (Van Oosterhout et al., 2003) to base their preference on. Results were supported by findings in sailfin tetras (*Crenuchus spilurus*) using a comparable test paradigm. At the same time, the highly ornamented males were considerably more neophobic than their less ornamented rivals, presumably because they experience greater predation pressure while also having a higher predicted fitness payoff (da Silva Pinto et al., 2021). Accordingly, sexual selection by means of female preference seems to result in greater cognitive abilities of smart, beautiful males, whereby cognition is becoming a vital part of their attractiveness to females.

A study on male threespine sticklebacks (*Gasterosteus aculeatus*) examined a very different aspect of cognition, namely inhibitory control, which may possibly be related to male sexual signals (Minter et al., 2017). Inhibitory control describes a cognitive process, which enables an individual to inhibit its natural, habitual, or dominant behavioral response to certain stimuli for adopting a more appropriate behavior to meet its intended goals (Hauser, 1999; Boogert et al., 2011b; Bray et al., 2014; Rystrom et al., 2019). In threespine sticklebacks, males provide all parental brood care, but at the same time, they need to avoid eating their own fry that closely resemble their prey. Hence, males with better inhibitory control would be more successful in rearing their offspring, resulting in higher fitness levels. Initially, male sticklebacks were challenged with a detour-reaching task. Subsequently, the males were assessed for their sexual signals (coloration, nest area and courtship vigor) to determine whether this visual information would reveal the males' cognitive abilities, which proved not to be the case. Females preferred to mate with males that showed better initial inhibitory control, suggesting that females possibly consider this male trait as a crucial trait for mate choice (Minter et al., 2017). Keagy et al. (2019) reported similar results, revealing that neophobia differences between both sexes allowed male threespine sticklebacks to consistently outperform females in a detour task. However, unlike female sticklebacks, who preferred cognitively superior males (Minter et al., 2017), male sticklebacks did not express this preference when choosing females (Keagy et al., 2019). Hence, we may derive two possible lessons: either males merely disregarded the females' cognitive performance in the present task when courting a female to mate with or the chosen detour task did not adequately reflect their preference for aspirational female cognitive skills.

In answering the first key question *'Do cognitive skills guide an individual's mate choice and, ultimately, does learning change an individual's decisions?'*, we can conclude that (a) sexes differ in their cognitive abilities depending on the given challenge, presumably because of the different roles they play within their social community. This suggests that the cognitive processes governing their mate choice decisions will also differ. Moreover, we can deduce that (b) cognitive abilities indeed have a considerable influence on individual mate choice decisions in species across different taxa, and (c) the males' displays of learned behavioral patterns alter the females' mate choice decisions. We can draw these conclusions for three large vertebrate taxa – i.e., mammals, birds and fish. Further research should aim to expand our knowledge to amphibians and reptiles, which have been less extensively examined in this field so far.

Preference for Mates Demonstrating Superior Cognitive Skills

Given the current state of research as discussed above using numerous mammal, bird and fish examples, we still struggle to answer the second key question: '*How and to which extent do individuals use their own cognitive skills to assess those of their conspecifics when choosing a partner?'*. Compared to their inferior conspecifics, cognitively superior individuals are frequently preferred as mates in various species across different taxa (e.g., crossbills, guppies, or humans; Keagy et al., 2009; Shohet and Watt, 2009; Snowberg and Benkman, 2009; Miller, 2011). Yet, it has rarely been investigated whether the cognitive ability *per se* increases the potential mate's attractiveness or whether the candidate appears to be in a better (physical) condition on account of his or her cognitive abilities, e.g., to be able to access better food sources (Riebel, 2011; Boogert et al., 2011b). Moreover, we cannot safely predict whether cognitive abilities are reflected in observable (e.g., visible) physical attributes, as seems to be the case, for instance, with nutritional status, parasite prevalence, immune competence, or social rank. Mate choice plays a key role in sexual selection, with significant fitness consequences and, presumably, profound cognitive challenges. Remarkably, however, only a limited number of studies has determined the importance of the cognitive abilities of the choosing individual to date. For instance, in food-caching wild mountain chickadees, males with superior spatial learning and memory abilities had larger clutches and greater numbers of fledged young. At the same time, superior female spatial learning and memory capabilities resulted in fledglings with greater body mass. These effects were not observed reciprocally. The disparity in reproductive investment among females appeared to reflect individual variation in spatial memory abilities on the one hand, and to integrate both their own and their mate's superior cognitive abilities on the other (Branch et al., 2019). When challenging female threespine sticklebacks with a spatial learning task and its reversal, cognitively more flexible females were observed to devote more time to assess prospective male partners in a dichotomous mate choice task. However, it were these highly motivated females, who made more mistakes at the beginning of a reversal phase, which may be due to them developing faster or more robust problem-solving routines and, subsequently, adapting more slowly to new conditions. Nevertheless, they were ultimately faster in relearning the task (Rystrom et al., 2019). Another study examined the learning accuracy of male and female rose bitterlings (*Rhodeus ocellatus*) in a spatial learning task in terms of the males' reproductive success (Smith et al., 2015). Following the spatial orientation task, males participated in competitive mating trials, in which they either played the role of a guardian or of a sneaker male. When evaluating the males' reproductive success via paternity analysis in association with their learning rates, high-performing sneaker males produced the most offspring. Subsequently, this learning ability was revealed hereditary to the offspring, which suggests that cognitive acuity may be subject to intra-sexual selection (Smith et al., 2015). Interestingly, superior cognitive abilities in spatial memory of male lekking long-billed hermits (*Phaethornis longirostris*) were favored by female choice and, consequently, played a crucial role in male mating success. Superior males were more likely territorial and the structure of their mating vocal signals was more consistent compared to their inferior male conspecifics. In summary, enhanced spatial memory as a measure of male superior cognitive ability is as important to female lekking hummingbirds as weapon (i.e., beak tip length) and body size and strength (i.e., weight lifting during vertical flight) are to territory ownership when choosing a mate to pair with (Araya-Salas et al., 2018).

The level of an individual's cognitive ability is frequently associated with its brain size (e.g., larger brains provide enhanced cognitive abilities). Aiming to examine the cognitive capabilities of guppies (*P. reticulata*) involved in mate assessment, Corral-López et al. (2017) hypothesized that guppy females with smaller brains would have lower cognitive capabilities than their larger-brained conspecifics, causing their mate choice to differ. Therefore, both small- and large-brained females were subjected to cognitive tests for color discrimination, condition, swimming ability and optomotor response, in which no differences were observed. However, the two groups differed significantly in their mate choice decisions regarding mate quality assessment. The authors concluded that limited cognitive abilities could be among the reasons why an individual may be either able or limited in its ability to assess the quality of a prospective mate. Although no direct association between male brain size and their overall sexual behavior was observed (Corral-López et al., 2015), males with comparatively larger brains were considerably better at discriminating differently sized females in the context of mate choice (Corral-López et al., 2018).

MATE CHOICE, COGNITION AND PERSONALITY

Adding to the complexity of the picture, an individual's cognitive abilities and mate choice decisions decisively determine its personality and vice versa. However, this review does not aim to recount studies and arguments on this broad topic in detail. Therefore, I will address this exciting topic only briefly by describing a few fish examples. Shortly, the concept of ’personality’ covers at least three domains, comprising (1) ”*contextual generality*” at a particular age or moment of time, (2) ”*temporal consistency*” in and between the assessed personality traits, and (3) the impact of ”*genes and experience*” on personality development throughout an individual’s life-history (Stamps and Groothuis, 2010; Kaiser and Müller, 2021). Concisely, ‘personality’ is generally considered a ”*consistent between-individual variation in clusters of behavioral traits independent of factors such as age or sex*” (Mather and Carere, 2019). The growing body of evidence suggests that an animal’s personality contours both its cognitive style and performance as a function of the relevant ‘cognitive domain’. For instance, an animal can approach a given task quickly and boldly, or, alternatively, slowly and cautiously, while possibly acting more precisely. These behavioral patterns have been and are still used to anticipate an individual’s performance. The nature of the task, i.e., which cognitive domain should be addressed (e.g., spatial learning or memory, color discrimination, counting) also plays a pivotal role. An increasing number of studies attempted to establish a link between these facets (e.g., Carere and Locurto, 2011; Sih and Del Giudice, 2012; Guillette et al., 2017; Dougherty and Guillette, 2018; Wallace et al., 2020). Yet, animal personality traits have been reported in a variety of both vertebrates (mammals: Malmkvist and Hansen, 2002; Sih and Bell, 2008; Réale et al., 2009; birds: Groothuis and Carere, 2005; Portugal et al., 2017; reptiles and amphibians: Cote et al., 2008; Kelleher et al., 2018; Sih et al., 2018; fish: Toms et al., 2010 for review; Kareklas et al., 2016; Jolles et al., 2019) and invertebrates (ground beetles: Labaude et al., 2018; cuttlefish: Zoratto et al., 2018, bees: Walton and Toth, 2016; cockroaches: Stanley et al., 2017; compare also Mather and Logue, 2013 for review). In addition to influencing an individual’s cognitive style and performance, the courting and the choosing individuals’ personalities frequently affect mate choice decisions. Thereby, different personality types and, possibly, different social and/or sex roles may persist within populations (e.g., paternal, maternal or joint brood care and feeding of the young, paternal defense of the nest and/or the caring partner, territory marking, averting of predators). Darwin (1871) already noted that *“when we behold two males fighting for the possession of the female, or several male birds displaying their gorgeous plumage, and performing strange antics before an assembled body of females, we cannot doubt that, though led by instinct, they know what they are about, and consciously exert their mental and bodily powers. [. . .] Our difficulty in regard to sexual selection lies in understanding how it is that the males which conquer other males, or those which prove the most attractive to the females, leave a greater number of offspring to inherit their superiority than their beaten and less attractive rivals”.*

There is still very little information on how the triad of mate choice, cognition and personality interacts in the light of the sexes’ roles yet. Considering the key questions of this review, suffice it to say that the personality phenotypes of the interacting individuals and, by implication, assortative or disassortative mate choice strategies appear to play an

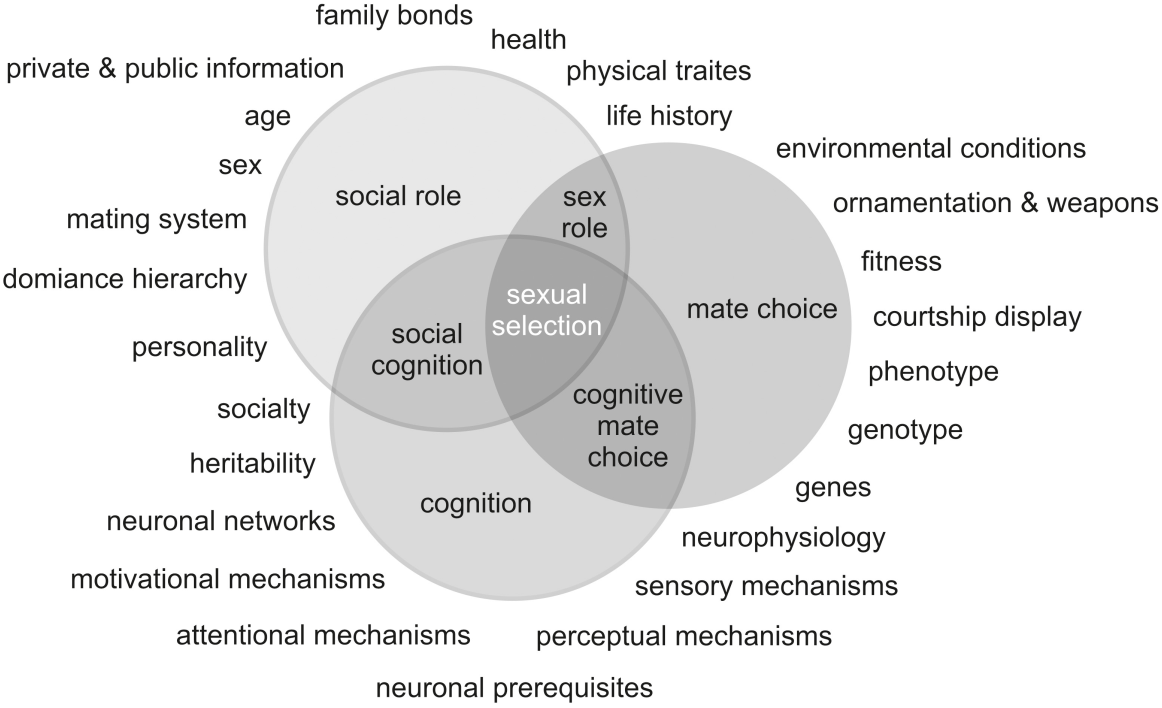

FIGURE 1 | Mate choice, cognition, and social roles converge on social cognition, cognitive mate choice, and sex roles, which are mutually dependent on one another as well as on various powerful determinants, and represent important determinants of sexual selection.

intriguing role in many taxa. Depending on a species' respective environmental conditions, assortative mate choice of similarly behaving individuals or disassortative mate choice of apparently antagonistic, but complementary behaving individuals may be favored (mammals: e.g., Ihara and Feldman, 2003; Massen and Koski, 2014; Rangassamy et al., 2015; Martin-Wintle et al., 2017; birds: e.g., Both et al., 2005; van Oers et al., 2008; Schuett et al., 2011; Gabriel and Black, 2012; Horton et al., 2012; Fox and Millam, 2014; Pogány et al., 2018; Clermont et al., 2019; Collins et al., 2019; fish: e.g., Ariyomo and Watt, 2013; Laubu et al., 2017; Scherer et al., 2017; Schweitzer et al., 2017; Chen et al., 2018; invertebrates: e.g., Kralj-Fišer et al., 2013; Montiglio et al., 2016; Baur et al., 2019). This could be a decisive competitive advantage both genotypically and phenotypically in the context of bi-parental brood care. From this brief glimpse into the complex world of cognition, personality and mate choice, we can imagine the extent to which these three dimensions (a) influence each other, (b) influence sex roles within a mating system and, in turn, (c) are influenced by sex roles depending on the (social/sexual) context (but compare Munson et al., 2020 for an comprehensive review on mate choice and behavioral types).

CONCLUSION AND FUTURE PERSPECTIVES

This review sought to answer a number of key questions. Aiming to answer the first question, *'Do cognitive abilities guide an individual's mate choice and, ultimately, does learning alter an individual's mate choice decisions?'*, evidence was provided to support the strong influence of an individual's role, determined by its sex, social status, mating system, and cognitive performance on mate choice decisions. Additionally, the males' displays of learned courtship seem to alter the females' mate choice decisions. Subsequently, findings gave rise to further questions: *'How and to which extent do individuals use their own cognitive skills to assess those of their conspecifics when choosing a partner?'*, and *'How does an individual's role within the mating system influence the choice of the choosing sex in this context?'*. Sexual selection and mate choice take place within a complex framework of an animal's social interactions. Several determinants such as environmental conditions, cognitive abilities, dominance hierarchies, family bonds, age, or sex of the individuals involved markedly affect these interactions. The determinants, in turn, depend on an individual's social role and, more precisely, the distinct role of its sex in its social surroundings. Additionally, attentional, motivational, sensory and perceptual mechanisms depend on the corresponding neuronal prerequisites inherent to every individual. These mechanisms are known to exhibit substantial differences between sex and species, but are vital (a) to survival, cooperation and reproduction, and (b) to social interaction (**Figure 1**). The role of learning in the acquisition of traits that are targets of mate choice and the consequences of superior cognitive capabilities on this central evolutionary process were carefully elucidated. In this context, males and females may possibly resort to divergent optima in their (domain-specific) cognitive traits, which are shaped by different life history strategies and different experiences at different life stages. However, any individual regardless of sex and social role could potentially be limited in achieving its best cognitive performance due to social constraints and/or sexual conflicts within its mating system.

Sex-role inversed species constitute another yet understudied dimension to this review's topic. In role-inversed species such as pipefish or seahorses, males provide the higher investment in the offspring by carrying eggs internally. Similar to the conventional mating systems that have been discussed so far, males choose depending on the female's body size and the intensity of her courtship display (Berglund et al., 1986a,b; Vincent et al., 1992; Berglund and Rosenqvist, 2003; Barlow, 2005; Berglund et al., 2005). In these species, females are likely to be the brighter, more colorful sex. However, the social framework and the physiological prerequisites underlying the development of these role reversals have been and remain widely discussed (e.g., Beal et al., 2018; Mobley et al., 2018; Anderson et al., 2020; Lipshutz and Rosvall, 2020). Possible interactions between their mate choice and the impact of superior or inferior cognitive abilities of the choosing or the chosen sex constitute fascinating future research topics.

Benefiting from many examples mostly from three major vertebrate groups, this review summarizes a large number of studies that attempt to elucidate many different aspects relating to cognitive sex differences, the different roles of males and females in social and sexual interactions, and the potential influence of cognition on mate choice decisions. Nevertheless, the central question of how this triad interacts remains partially unanswered. Most studies commence with a first mate choice test to discriminate preferred and unpreferred potential mates. Subsequently, the unpreferred individuals of the sex to be chosen are trained in any type of problem-solving task. Following the presentation of their acquired skills, a second mate choice test is performed to reveal a potential preference shift in the choosing sex. There is virtually never an equal training of both potential mates (i.e., preferred and non-preferred individuals) or of the individuals of the choosing sex in the assigned task. However, this approach does not take into account any possible reflection of cognitive abilities in distinct physical, physiological, or morphological characteristics or in specific behavioral patterns that are not known to us yet but may well be perceived by the selecting sex of the observed test species. Thus, if only a subset of individuals performing a particular role receive training, this may inadvertently but disturbingly bias test results caused by an inadequate testing paradigm. Having said that, are animals even capable of accurately judging the cognitive abilities of potential mates in solving a particular task if they themselves have never learned the task they now assess? How should they judge the degree of difficulty and assess the problem-solving skills of a potential mate they do not even know themselves? Therefore, prior to the second mate choice test, all participants, regardless of the role assigned to their sex (i.e., choosing or courting sex in their respective mating system), should be trained to test their performance and assess their cognitive abilities. Accordingly, the choosing sex should be trained to solve the task (e.g., opening a feed box or navigating in a maze) to allow them to determine the difficulty of the given task. Only then should the choosing sex observe the learning progress of the courting sex to assess the learning ability of the prospective mates. Finally, a second mate choice test should determine a possible shift in preference. Additionally, carefully designed neurobiological experiments should help to unravel the neuronal involvement, processes, mechanisms as well as the molecular basis for cognitive mate choice, taking into account the different roles males and females play in social and sexual contexts (e.g., male/ male-, female/ female-, female/ male-, parent/offspring-interactions). Brain development, cognitive plasticity, and the plasticity of social and (sexual) incentive cooperation could have a crucial influence, as not all mate choice decisions are driven by competition exclusively.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

I thank Klaudia Witte for the opportunity to write the manuscript and her helpful comments to improve it. I thank Nils Krützfeldt for proofreading the manuscript. I also thank the University of Siegen for the financial support provided through its Open Access Publication Fund. Last but not least, I thank the guest editor for inviting me to write about this research topic, as well as both reviewers for their time and support.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

REVIEW
published: 11 November 2021
doi: 10.3389/fevo.2021.749499

Mate Choice, Sex Roles and Sexual Cognition: Neuronal Prerequisites Supporting Cognitive Mate Choice

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Across taxa, mate choice is a highly selective process involving both intra- and intersexual selection processes aiming to pass on one's genes, making mate choice a pivotal tool of sexual selection. Individuals adapt mate choice behavior dynamically in response to environmental and social changes. These changes are perceived sensorily and integrated on a neuronal level, which ultimately leads to an adequate behavioral response. Along with perception and prior to an appropriate behavioral response, the choosing sex has (1) to recognize and discriminate between the prospective mates and (2) to be able to assess and compare their performance in order to make an informed decision. To do so, cognitive processes allow for the simultaneous processing of multiple information from the (in-) animate environment as well as from a variety of both sexual and social (but non-sexual) conspecific cues. Although many behavioral aspects of cognition on one side and of mate choice displays on the other are well understood, the interplay of neuronal mechanisms governing both determinants, i.e., governing cognitive mate choice have been described only vaguely. This review aimed to throw a spotlight on neuronal prerequisites, networks and processes supporting the interaction between mate choice, sex roles and sexual cognition, hence, supporting cognitive mate choice. How does neuronal activity differ between males and females regarding social cognition? Does sex or the respective sex role within the prevailing mating system mirror at a neuronal level? How does cognitive competence affect mate choice? Conversely, how does mate choice affect the cognitive abilities of both sexes? Benefitting from studies using different neuroanatomical techniques such as neuronal activity markers, differential coexpression or candidate gene analyses, modulatory effects of neurotransmitters and hormones, or imaging techniques such as fMRI, there is ample evidence pointing to a reflection of sex and the respective sex role at the neuronal level, at least in individual brain regions. Moreover, this review aims to summarize evidence for cognitive abilities influencing mate choice and vice versa. At the same time, new questions arise centering the complex relationship between neurobiology, cognition and mate choice, which we will perhaps be able to answer with new experimental techniques.

Keywords: sex role, plasticity, immediate early gene (IEG), neurotransmitters and hormones, personality, neural integration, sex difference, brain size

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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *29 July 2021*
Accepted: *21 October 2021*
Published: *11 November 2021*

Citation:
Fuss T (2021) Mate Choice, Sex Roles and Sexual Cognition: Neuronal Prerequisites Supporting Cognitive Mate Choice.
Front. Ecol. Evol. 9:749499.
doi: 10.3389/fevo.2021.749499

INTRODUCTION

An individual perceives, acquires and stores private and public (social) information available in its environment, responds to it, and, finally, draws an appropriate decision. Various sensory and cognitive processes associate, integrate and prioritize a wide variety of (social and/or non-social) environmental stimuli within an individual's perceptual range, thereby controlling important behavioral responses and life history decisions. For instance, odor perception is an important determinant of different aspects of zebra finch social behavior (e.g., kin/parent recognition: Krause et al., 2012; Golüke et al., 2016; Caspers et al., 2017; mate choice: Caspers et al., 2015) and is mirrored sex-specifically in brain activity (Golüke et al., 2019). Daylight length is one of the factors that migratory birds use to decide when to start their fall or spring migration. Various factors determine which information to prioritize for a decision: how does an individual perceive the information? Is it capable of drawing an (proximate) association between the stimulus, the likely consequences, and its own fitness benefits? Has natural and/or sexual selection adequately shaped an individual's abilities to assess the costs and benefits of appropriate versus inappropriate decisions? Does it aptly possess the cognitive skills for any of these complex learning, memory, and evaluation processes? Hence, depending on the context, different forms of learning prevail. These include rapid, very robust and irreversible learning processes such as imprinting shortly after birth and slower, more complex but flexible processes such as trial-and-error learning (e.g., access to a nutritious food source). For instance, in some birds, horses, goats, or bovids, newborn young have to be able to keep up with their parents shortly after birth. To them it is crucial to grasp this need quickly, because slow learners may not be given a second chance (e.g., Ewer, 1956; Hess, 1959, 1964; Immelmann, 1975; Salva et al., 2015; Mayer et al., 2016). On the other hand, Meerkat adults teach their young in several successive steps and over a period of several weeks how to handle highly venomous scorpions as a particularly tasty and nutritious food source (Thornton and McAuliffe, 2006; Thornton, 2008; Thornton and Raihani, 2010). For non-learners, any contact with live venomous prey may be the last.

'Cognition' is commonly described in terms of the neuronal processes that are principally engaged in the reception, processing, storage, and retrieval of information (Shettleworth, 2001; Dukas, 2004). Yet the capacity to learn and to draw associations between different stimuli is another important determinant of cognitive ability (Giurfa et al., 2001). More specifically, 'cognition' as all processes that involve thinking, reasoning, perceiving, imagining, and remembering to constitute concepts that "*can be systematically recombined with each other*", are "*stimulus-independent*" and, thus, can be transferred to new contexts (Bayne et al., 2019). For instance, is an animal able to recognize familiar individuals in a group of conspecifics regardless of context? Is it able to distinguish and categorize these familiar individuals as potentially having different consequences for itself (e.g., companion, rival, potential mate)? Is its response to a particular individual characterized by its ability to remember previous interactions?

Moreno and Mossio (2015) added the idea of "*neurodynamic autonomy*" to the discussion, which suggests that once the neurodynamic organization has reached a certain level of autonomy and is subject to a set of higher-level monitoring and integration mechanisms, the self-organizing dynamic character develops. Drawing on their notion of "*neurodynamic autonomy*", interactive experiences such as affective interactions contribute significantly to the development and shaping of socio-cognitive mechanisms. Accordingly, cognition also involves the effective control of attention and emotions, which drives the need to perceive, interpret, and respond to the emotions (and, thus, behavior) of other organisms. Thereby, "*neurodynamic autonomy*" contributes to communicative, competitive, and cooperative aspects of social behavior (e.g., inter- and intraspecific, inter- and intrasexual) (Moreno and Mossio, 2015).

In many species across different taxa, mate choice is a highly selective process that involves both, intra- as well as intersexual selection processes aiming to pass on one's genes, making the underlying behavioral mechanisms a significant factor in sexual selection. Although many aspects of mate choice behavior, coloration and ornamentation are well understood, the underlying neuronal mechanisms of female mate choice have been fairly neglected. In vertebrates, this sexual behavior is likely to be guided and adapted by an interplay of the same neuronal circuits as social, non-sexual behavior (e.g., territorial aggression, parental care). Mate choice behavior is modified permanently in response to ecological and social changes, which are initially perceived sensorily, undergo neuronal analysis and interpretation in a subsequent step, and, finally, elicit to an adequate behavioral response (compare DeAngelis and Hofmann, 2020 for review). Cognitive processes allow the synchronous processing of multiple sources of information from the (in-) animate environment and from a variety of social cues. However, the exact neuronal mechanism, the cognitive prerequisites and effectiveness inducing the display recipient to finally select a particular courting partner are not yet fully understood. Across all vertebrate taxa, the prevalence of more or less well-developed cognitive capabilities is closely linked to brain development and, to some extent, to overall brain size (brain to body mass ratio, encephalization quotient) or the prominence of single brain regions such as the telencephalon. Moreover, connectivity and the number of neurons in a given brain region or neuronal network are considered appropriate determinants of cognitive performance in an individual, sex, or species (Iwaniuk, 2017). Information an individual receives from the environment can trigger complex neurophysiological mechanisms such as neuronal signal transduction, structural and

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Abbreviations: *ApoD*, apolipoprotein D; *BDNF*, brain-derived neurotrophic factor; CMM, caudomedial mesopallium; Dm, Dl, medial and lateral telencephalon; *egr-1*, early growth response gene 1; fMRI, functional magnetic resonance imaging; GnRH, gonadotropin releasing hormone; HV, ventral zone of periventricular hypothalamus; IEG, immediate early gene (e.g., *c-fos, egr-1*); LNH, lateral neo- and hyperstriatum; MNH, medial neo- and hyperstriatum; NCC, caudocentral nidopallium; NCM, caudomedial nidopallium; NI, *Nucleus incertus*; POA, preoptic area; SBN, social behavior network; SDMN, social decision-making network; SPG, synaptic plasticity gene; VTA, ventral tegmental area; Vs, supracommissural nucleus of the ventral telencephalon; Vv, ventral zone of the ventral telencephalon.

synaptic modifications, or molecular regulations, ranging from altered gene expression to epigenetic changes that ultimately lead to changes in brain function, phenotypic variations, and adaptive behaviors (Maruska et al., 2014).

However, the development of higher cognitive skills provides true selective benefits only if they cover the (fitness) costs. At a behavioral level, costs may involve time-consuming trial-and-error learning processes until a behavioral pattern is appropriately adapted. Both (social) imprinting and complex (social) learning processes ensure that the respective brain regions involved are cross-linked. Different nuclei are recruited to support different aspects of learning, depending on the dominant learning process in progress. The same applies to social cognition, i.e., cognitive skills in sexual and non-sexual contexts and interactions, respectively (e.g., Bolhuis and Honey, 1998; Reader, 2003; Di Giorgio et al., 2017; Joiner et al., 2017). To give an example, the cognitive traits of innovativeness and problem-solving skills are considered particularly attractive to potential mates. They were found to be correlated positively to the individual learning ability and to the size of the corresponding associative brain regions in birds (hyperstriatum ventrale, neostriatum) and primates (neocortex, striatum) (Reader, 2003). At the neuronal level, the development and maintenance of the adequate neuronal circuits demand an increased energy supply. Many neuronal mechanisms, molecular pathways, and neuronal networks known to mediate sexual and social behavior and, in particular, to be involved in social cognition in vertebrates appear to be highly conserved evolutionarily across taxa. Altogether, they appear to govern cognition in mate preference and, therefore, to modulate important aspects of sex-specific mating behavior (Weitekamp and Hofmann, 2014). Although a number of exciting examples of social cognition have been observed in various species at a behavioral level, there is still astonishingly limited understanding of the underlying neuronal prerequisites and the neuronal plasticity of adaptive and context-dependent sexual behaviors in different vertebrate groups. More specifically, a surprisingly high level of uncertainty remains about neuronal mechanisms involved particularly in the process of mate choice, which is why this review covers social (non-sexual) cognitive issues, but aims to highlight the context of mate choice whenever available. Moreover, differently pronounced sexual dimorphisms in brain structure, brain size, and in various neuronal mechanisms have been found in different species. However, how does the neuronal activity of males and females differ with respect to sexual cognition? Hence, is the sex and/or the respective sex role within the prevalent mating system mirrored at the neuronal level? How does cognitive competence affect mate choice and, conversely, how does mate choice affect cognitive competence of both sexes?

SEX-DRIVEN BEHAVIOR IS REFLECTED IN THE BRAIN

Genes, neuronal prerequisites, and their ensuing processes and mechanisms are shaped, modified, and adapted throughout an individual's life, depending on the environmental conditions (e.g., con- and heterospecifics, predation, and food availability), its life and learning experiences, sexual status, social rank within its group etc., to name just a few examples. The same applies in reverse, implying a reciprocal interaction between all the aforementioned determinants.

Different social contexts showed different covariance patterns of 11 genes and their associated behavior (Ramsey et al., 2012). The important role of plasticity in courtship behavior was also evident upon blocking NMDA (*N*-methyl-D-aspartate) receptors, which play a critical role in learning-induced synaptic plasticity, while significantly affecting female preference behavior (Ramsey et al., 2014). Learning-induced synaptic plasticity can be observed also in various vertebrate taxa in various social (e.g., Sockman, 2007; Wang et al., 2014; Cummings, 2015; Cummings and Ramsey, 2015; Delclos et al., 2020) contexts and (non-social) learning tasks (e.g., Alcock, 2001; Bozon et al., 2002; Davis et al., 2003; Fuss and Schluessel, 2018).

There are several facets of reproductive behavior in most sexually reproducing species that differ between males and females. The term 'sex' in the context of this review refers to biologically defined and genetically acquired differences between males and females that are evident in their physiology and reproductive abilities or potentials. Also included are biological factors such as internal or external sex organs, gonadal differentiation (testis and ovary), gamete production, sex hormones (e.g., androgens, estrogens, and progestogens), or sex chromosomes (e.g., XY male, XX female in most mammals, or ZZ male, ZW female in birds) that are considered to determine a sex. For instance, sex chromosomes and, particularly, sex chromosome genes (X- or Y-linked and Z- or W-linked genes) are contributory to sex differences in the brain of both mammals and birds ("*neuronal sex chromosome genotype*", Arnold, 2004; Scholz et al., 2006; Jazin and Cahill, 2010; Maekawa et al., 2014; Loke et al., 2015). Several studies propose genetic and non-genetic factors, such as social incentives or other environmental influences, may interact (e.g., Ristori et al., 2020). Environmental influences can be highly complex and may be driving forces for plastic changes in brain morphology (Maguire et al., 2000; Driemeyer et al., 2008; Quallo et al., 2009; Scholz et al., 2009; Blumenfeld-Katzir et al., 2011; Lerch et al., 2011; Fong et al., 2019). Consequently, sex roles (or their reversal), ecology, or sex differences respond plastically to environmental drivers with regard to their effects on female or male reproductive fitness (e.g., Amundsen, 2018; Hare and Simmons, 2020, 2021). In this context, sex roles are intuitively associated with both stereotypically female and male sexual behaviors, which comprise biological phenomena such as, for instance, mating competition, mate choice, or nature, extent and/or duration of parental care (e.g., Ah-King and Ahnesjö, 2013). The nature and the behavioral expression of sex differences vary greatly between species, populations, or cultures. Consequently, sex roles refer to socially encoded behaviors, traits, and/or attributes associated with being (genetically) male or female.

Several neuronal processes supposedly interact with each other to coordinate sensory perception, memory, cognitive and emotional responsiveness in a complex neuronal network to allow for a coherent perception and decision-making framework (e.g., Skov and Nadal, 2020). For instance, based on whole-brain functional maps, socially deprived zebrafish were observed to experience significantly weaker activity in brain regions associated with social behavior and social stimulus processing, but significantly higher activity in regions involved in anxiety or stress when back in contact with zebrafish raised in social populations. These early isolation impairments were reversed by modulating serotonin levels in formerly isolated fish (Tunbak et al., 2020). When analyzing swimming movements in a variety of different contexts ranging from hunting and predator avoidance to social interactions, socially deprived larvae where observed to exhibit significantly more social avoidance responses compared to their group-raised conspecifics (Marques et al., 2018). Studies in human and non-human primates identified a key moderating involvement of the amygdala, the ventromedial frontal cortices and the right somatosensory cortex, which are crucial to view conspecifics, retrieve knowledge, or trigger an appropriate behavior (i.e., perceptual representation of socially relevant stimuli) (Adolphs, 1999). Together with instinctive knowledge, perception and processing of socially relevant information (as examples of acquired knowledge) appear to promote social cognition, with a strong dependence on the own sex role (Geary, 2002; Proverbio, 2017, 2021; Pearce et al., 2019). Sexual and social challenges and (unpredictable) ecological events initiate adaptive physiological and behavioral responses that may provide an animal with either a selective advantage or disadvantage.

Cognitive Sex Differences and Social Information Trigger Multiple Regulatory Neuronal Processes

Social information triggers various neuromodulatory mechanisms. These mechanisms contribute to the adaptive plasticity of social support (e.g., Snell-Rood and Snell-Rood, 2020). In addition, they enable adaptive plasticity in social learning including, but not limited to copying mate choice (e.g., Gouda-Vossos et al., 2018; Zhuang et al., 2021), vocal learning, tutoring, and preference in early sensory periods (e.g., Hauber et al., 2021), dealing with nutritional stress (and its consequences, e.g., on offspring growth, brain development, and learning; e.g., Nowicki et al., 2002), or disgust (e.g., to enable pathogen and/or toxin avoidance; Kavaliers et al., 2019 for a review). Likewise, acquiring sexual information triggers numerous regulatory neuronal processes to allow social individuals to assess and respond quickly and appropriately to a potential mate's courtship display, but taking into account their own social and/or sexual role, motivation, and cognitive abilities (Kavaliers and Choleris, 2017). Accordingly, a central focus of neuroscience research is to elucidate (a) how mate choice processes emerge and are managed in the brain, (b) how the brain perceives and integrates sexual interaction and, subsequently, (c) replies to changes in the sexual context by designing a well-adapted behavioral response. In many conventional mating systems, females provide a greater investment into the offspring than males. Although there are species in which males participate in brood care or even provide it completely, it is initially to the females to expend the greater amount of energy for gamete production. Therefore, females frequently perform the pivotal role of choosing between competing males. There is a rich body of studies examining many behavioral details of the female decision-making process. Across taxa, there is evidence that neuronal substrates and networks supporting competitive male–male aggression, temporal or lifelong pair bonding, or (shared) parental brood care are presumably also engaged in (female) mate choice. Supporting neuronal prerequisites include, for instance, the *Nucleus accumbens*, the amygdala, the preoptic area (POA), or a number of cortical areas (DeAngelis and Hofmann, 2020). In this context, it is likely that these circuits are modulated over a lifetime, and in response to an individual's (speciesspecific and cross-species) social and ecological environment (e.g., predation pressure, food availability, and defense of territory). This so-called neuronal plasticity presumably serves as the basis for behavioral plasticity and vice versa. In the context of mate choice, the choosing sex (mostly the females) has to be equipped with an elaborate and adequate set of higher cognitive abilities in order to (a) discriminate between and (b) to classify all available information such as the prospective mates' social rank, body condition (e.g., body size and coloration), health condition (e.g., nutritional status, infections, and parasitism), personality traits (e.g., aggressiveness, boldness, explorativeness, sociality, and risk-taking), and problem-solving abilities (e.g., spatial orientation, finding new resources such as food or shelters).

Sex Differences: Cognitive Processes

Sex-specific differences in behavior, brain activity, and brain anatomy have been found repeatedly in a wide variety of animal species in virtually all major vertebrate taxa. Numerous studies have provided wide ranging evidence to map the cognitive processes involved in social competence in the vertebrate brain. There is increasing support of the idea that complex cognitive functions are associated with a general pattern of activation of multiple brain networks, rather than with individual brain regions (McIntosh, 2000; Sporns, 2010; Fuss and Schluessel, 2018). Cognitive sex dimorphisms at a behavioral level along with their prospective neuronal mirroring help to understand the neuronal integration of cognitively demanding mate-choice cues. However, despite years of research, we can still only vaguely imagine the concrete neuronal 'blueprint' or 'wiring scheme', its diversity and composition for an (in-) dependent mate choice of one and against the other conspecific (Phelps et al., 2006; DeAngelis and Hofmann, 2020). Basically, mating behavior as an example of a complex social, cognitive behavior is accompanied by a change in neuronal brain activity and, conversely, triggers corresponding neuronal adaptations in response to, for instance, courtship, comparison of different prospective mates, or recognition and choice of the 'perfect mate'. Complex neuronal gene expression (e.g., IEGs, emission of secondary, retro- or anterograde neurotransmitters etc.) is involved frequently in the integration of cognitive mate-choice cues in the respective brain regions, aiming to serve as an initial and rapid neuronal response and, consequently, to allow context-depended behavioral adaptations. It triggers plastic adaptations in the neuronal circuits, the synaptic activity of the targeted neuronal network, its metabolic processes, or the recruitment of further transcriptional pathways (Robinson et al., 2008; Zayed et al., 2012; Whitney et al., 2014) responsible for controlling and consolidating the intended (socio-) cognitive behavior (Dragunow, 1996; Davis et al., 2003; Plath et al., 2006).

The nature of the task, i.e., which 'cognitive domain' is addressed (e.g., spatial learning or memory, color discrimination, and counting) also plays a vital role in cognitive processes. In an interesting review, Yagi and Galea (2019) summarized a number of early and recent studies reporting pronounced sex differences in the hippocampus of humans and rodents in the context of spatial orientation. In addition to different sex-specific strategies in coping with given orientation tasks, testosterone or ovarian hormones, respectively, play a crucial role and could, for instance, increase or attenuate the preference for a particular strategy. Since these different strategies are processed in different neuronal ways, these results also pointed to the involvement of different regions in the male and female brain in spatial navigation. Additionally, age- and environment-dependent sex-specific differences in hippocampal morphology, cell signaling, synaptic plasticity, and activity in performing memory tasks have been reported in numerous mammalian species including humans and rodents (compare Koss and Frick, 2016 for review). Likewise, sex-specific differences were found in the age-dependent altered neuronal gene activity in the hippocampus of different-aged zebra finches (Kosarussavadi et al., 2017). In analogy to the domain-specific cognitive involvement of different brain nuclei, different patterns of gene activity are also reflected in the respective recruitment of multiple neuronal circuits depending on a social context (O'Connell and Hofmann, 2011, 2012). Taking fish in various social contexts as an example, locally increased brain activity [indicated by increased *c-fos*, *egr-1/Aptegr-1*, brain-derived neurotrophic factor (*BDNF*) IEG expression levels] has been revealed in brain areas associated with social behavior. These include, for instance, the anterior POA, the nuclei of the 'social behavior network' (SBN) within the basal forebrain and midbrain, or the dorsolateral telencephalon (dorsal, central, and lateral subdivisions) (Burmeister et al., 2005; Harvey-Girard et al., 2010; Wood et al., 2011; Maruska et al., 2013). For instance, sex-specific differences were found in the age-dependent altered gene activity of *apolipoprotein D* (*ApoD*) and the immediate early gene *egr-1* in the hippocampus of different-aged zebra finches when solving a spatial task in a four-arm maze. Males performed better than their female conspecifics of the same age, and younger birds learned slightly better than older ones. The latter effect was particularly evident when comparing females of different ages involved in memory events. Females showed also a higher *egr-1* expression than their male counterparts. The same was observed with respect to *ApoD* expression levels in young zebra finches, possibly indicating neurobiological compensation of older birds (Kosarussavadi et al., 2017). At the same time, there is the advantage of being able to respond rapidly and dynamically to new contexts and adjust decisions via novel, flexible connections between the brain regions involved ('functional reconfiguration of connectivity', Sporns, 2010). The involvement of neuronal prerequisites supporting cognitive mate choice also echoes the different sex roles in mate choice. In both sexes, the respective brain network takes the lead, whose nuclei mainly store the (sexual) information on a particular situation (domain-specific social encoding).

Neuronal Integration of Different Stimuli in the Context of Mate Choice

It is well accepted that at least two processes of intersexual selection developed in parallel and, to some extent, mutually dependent. On the one hand, the promoting sex (i.e., the 'display producer') will possibly develop unique courtship displays to appear more attractive to the choosing sex than competing rivals. On the other hand, it would only be worth to develop sophisticated courtship displays, if the choosing sex (i.e., the 'display recipient') was able to perceive the spectacle and to compare between the contenders. Hence, it depends on the particular role a sex is assigned to in its respective mating system (e.g., monogamy, polygamy, promiscuity, conservative or sex-reversed role models, among others). For instance, sexually dimorphic characteristics include the vocal repertoire, the sound-generation, and the sound-perceiving morphology. Thresholds for the recognition of sounds, and the neuronal key domains involved in the processing contribute to our understanding and interpretation of a particular species' communication processes. Any display beyond the perceptive range of the (choosing) recipient will remain undetectable (e.g., invisible, odorless, and inaudible) (Rosenthal, 2017). The brain regions and neuronal networks involved in courtship and mate choice, as well as the neuronal integration of different stimuli (targeting well-studied senses such as olfaction, vision, or audition) have been studied across taxa, for instance via altered gene expression as neuronal activity markers or via the modulatory effects of neurotransmitters and hormones.

To give an example, in mammals there is parallel processing of 'sexual odors' (e.g., pheromones) by the vomeronasal system (e.g., flehmen behavior, Takigami et al., 2000; Vedin et al., 2010; Smith et al., 2015) in contrast to 'asocial odors' (e.g., food), which are processed by the main olfactory system (Bressler and Baum, 1996; Døving and Trotier, 1998; Kondo et al., 2003). Immunohistochemical studies used IEGs followed by brain lesions to reveal potentially altered activity and involvement of different brain regions in female mice after smelling males of different sex status (intact, castrated). IEG findings suggested a preference for intact males in the POA or medial amygdala. Moreover, lesions of these regions suggested that they were not involved in odor discrimination *per se*, but rather in a coordinated adjustment of female choice behavior in mice and rats (Bressler and Baum, 1996; Kondo et al., 2003; Sakuma, 2008; DiBenedictis et al., 2012).

With respect to visual processing of mate choice information, synaptic plasticity genes (SPGs) have been identified in both the optic nerve (*grin1, march8, BDNF, thoc6, cant1*, and *thap6*) and telencephalon (*inhba, neurod2, smarcc1, c-fos, egr2b*, and *thap6*) of female guppies that differ in their expression patterns in view of differently colored males engaged in 10-min courtship displays (Bloch et al., 2018). These genes were flexibly coordinated by different transcription factors. Hence, they served as distinct "*neuromolecular switches*", inducing different neurogenomic conditions underlying mating decisions and social behaviors (Bloch et al., 2018). They observed a clear difference in SPG expression levels between females that showed a preference for specific males in contrast to their indifferent conspecifics, which allowed them to pinpoint a brain region devoted to sensory processing (optic tectum) and a brain region devoted to a decision-making purpose (telencephalon). Particularly the SPGs *grin1* and *glul* are well known to play critical regulatory roles in fish learning and memory processes. Consequently, Bloch et al. (2021) applied differential coexpression analysis on *grin1* and *glul* to unravel the supportive and dynamic neurogenomic network that is involved in mate choice in female guppies during different mating conditions (evaluation of attractive and unattractive males) and social contexts (familiar conspecific females). Depending on the context, a remarkable degree of neuronal network recoding was revealed in the choosing female brain in different social situations. Supplementary analyses suggested, depending on the social, that these changes particularly affected learning, memory and other cognitive functions. For instance, some neuronal networks were found to be exclusively active during mate choice, while others only started to operate during non-specific social interactions (Bloch et al., 2021). Across taxons, face recognition in human, macaque and sheep brains involves respective specialized and social domain-specific neuronal networks, which are recruited context-dependent (Kendrick and Baldwin, 1987; Kanwisher and Yovel, 2006; Tsao et al., 2006, 2008). Visually guided mate preferences resulting in an altered expression pattern of several neuronal processing genes in the central brain, optic nerves, and ommatidia have also been reported from invertebrates (e.g., *Heliconius melpomene, Heliconius cydno*; Rossi et al., 2020). Besides a social context, the 'mode' of visual perception used to perceive a courtship display (left eye, right eye, or both eyes) seems to play an important role in mate preference and, consequently, in mate choice. For instance, zebra finch males displayed more pronounced courtship behavior when only the right eye was available compared to only the left eye. Additionally, right-eyed as well as binocular males preferred females with distinct orange beaks over females with gray beaks, which was not observed in left-eyed males (Templeton et al., 2014). Thus, an altered gene expression of *zenk* and *c-fos* during early courtship, song production but also sexual imprinting appears to be closely associated with the ability to evaluate prospective mates, male attractiveness, and reproductive success in various brain regions [including the optic tectum, the caudomedial mesopallium (CMM), the lateral neo- and hyperstriatum (LNH), the medial neo- and hyperstriatum (MNH)] (Lieshoff et al., 2004; Avey et al., 2005; George et al., 2006).

Relating to auditory mate choice signals, using functional magnetic resonance imaging (fMRI), immediate early gene expression patterns (*egr-1*), along with behavioral tests, Van Ruijssevelt et al. (2018) discovered another brain region involved in social decision making, particularly in the context of mate choice. Hitherto, these tasks have largely been attributed to the well-known sensory integrative regions of the central nidopallium in the avian forebrain, which are known to be involved in executive functions, but also in the processing of other higher cognitive tasks such as the perception and evaluation of male courtship songs. In addition to the well-known involvement of the central nidopallium, they were able to reveal increased activity in the caudocentral nidopallium (NCC), a brain region assigned to the evaluation of acoustic signals underlying mate choice. The CMM responded to male songs of comparable temporal-acoustic patterning. In female starlings (*Sturnus vulgaris*), for instance, their preference for long or short songs elicited increased activity in the CMM depending on their previous social experience with conspecifics and the prevailing environmental conditions (Sockman et al., 2002; Sockman, 2007; Sockman and Ball, 2009). In túngara frogs (*Physalaemus pustulosus*), hearing calls from their own or a closely related frog species induced a sex-insensitive response of the IEG *egr-1* in the *Nucleus olivaris superior*, which is responsible for processing acoustic stimuli in the brainstem. At the same time, the male torus semicircularis (laminar nucleus) responded with an increased *egr-1* expression to con- and heterospecific calls, while females responded only to conspecific calls (Hoke et al., 2008). Hence, a preselection of the stimuli to be integrated in the decision-making process in the telencephalon appears to depend on the sex and the sex role, respectively, aiming to decide more effectively between rivals (males) or potential mates (females) (Wilczynski and Ryan, 2010).

Conclusively, it is not only an individual's genotype playing an influential role in mate choice. Instead, genotype together with neuronal gene expression patterns that are presumably evolutionarily conserved across different vertebrate taxa, constitute the basis of a decision in favor of or against a potential mate.

Neurotransmitters and Hormones

Other contributing determinants of a sex-specific neuronal integration of cognitive mate choice cues include the modulatory effects of many neurotransmitters and hormones. Many of these pathways are thought to be evolutionary conserved and operate similarly in many vertebrate taxa. Various neurotransmitters (e.g., dopamine and serotonin), opioid peptides, (sex) steroid hormones (e.g., testosterone and estrogens), corticosteroids, neurosteroids, and neuropeptides (e.g., oxytocin or arginine-vasopressin) precisely modulate the interplay of different neuronal networks, including the mesolimbic reward system, the SBN, the social salience network and other brain regions involved in social and/or sexual recognition as well as learning in the contexts of mate choice (Choleris et al., 2009, 2012; O'Connell and Hofmann, 2011; Gabor et al., 2012; Goodson, 2013; Petrulis, 2013; Ervin et al., 2015; Dumais and Veenema, 2016; Ashley and Demas, 2017; but see Kavaliers and Choleris, 2017; Froemke and Young, 2021 for review). For instance, in adult and larval zebrafish, the neuro-endocrine system, namely the fish ortholog of oxytocin (i.e., isotocin or 'zebrafish oxytocin') and, possibly, the fish ortholog of mammalian vasopressin (i.e., vasotocin or 'zebrafish vasopressin') appeared to support their social behavior and social preference, but not anxiety-related behavior (Landin et al., 2020). In humans, sex hormones were observed to regulate sexual behavior, memory capabilities and, consequently, hippocampal neurogenesis (Choleris et al., 2018). In humans and non-human animals, oxytocin reinforces sex differences in mate choice, suggesting its release during courtship reinforces sex-dependent priorities in both attractiveness and mate choice initially and, thereafter, supports pair bonding (Xu et al., 2020; reviewed in Froemke and Young, 2021). Via fMRI, a highly specific activation in the right ventral tegmental area (VTA) and in the right caudate nucleus in response to pictures of dearly loved ones was identified in 17 human volunteers. Both are areas associated with the dopaminergic reward system in mammals, including humans, and are associated with both reward and motivation. The subcortical dopaminergic pathways are part of a ‘universal arousal complex’ that initiates romantic love, which is considered a motivational system that triggers not a specific but a variety of emotions, resulting in a prominent activation of the VTA and the caudate nucleus (Fisher et al., 2005). Sockman and Lyons (2017) focused on telencephalic regions of Lincoln’s Sparrows (*Melospiza lincolnii*), i.e., the CMM and caudomedial nidopallium (NCM), which are known to mediate attention and (vocal) perception. In these brain regions, they examined neuromodulatory changes in monoaminergic activity in the context of female song preferences during mate choice. Reflecting the females’ assessment of the song scenery as attractive or pleasant, the monoaminergic response of the CMM and NCM changed. For instance, moving between a very pleasant to a less pleasant song scenery, the pleasant scenery increased the threshold for an adaptive behavioral response (i.e., turning toward male song), thereby mirroring behavioral plasticity in response to changing signaling environments in the sexual context.

SEX-SPECIFIC COGNITIVE PERFORMANCE AND PERSONALITY

In view of as well as beyond sexual and/or social contexts, individuals may differ in numerous ways when dealing with cognitive tasks. For instance, they may differ in their speed and/or accuracy in solving the task (‘performance level’, Shettleworth, 2010). Moreover, they may differ in the way they evaluate and respond to a new, previously unknown task (personality and ‘cognitive style’, e.g., Sih and Del Giudice, 2012; Thornton and Lukas, 2012; Mazza et al., 2018). Hence, all (cognitive) processes related to mate choice are determined substantially by an individual’s personality as well. To name only two outmost examples, an individual can proceed a given task quickly, boldly, and maybe impetuously. Alternatively, it can approach the same task very slowly and cautiously, but perhaps act more precisely. However, the mere existence of inter-individual differences does not imply the existence of personality, as personality presupposes stable inter-individual differences. Consequently, behavioral differences cannot equate with personality. Nevertheless, both dimensions mutually interact. Morphological, physiological, and plastic behavioral sex differences have been described consistently in many animal groups across taxa (e.g., Iwaniuk, 2017; Vallortigara and Versace, 2017; Cummings, 2018; Darda et al., 2018; Turano et al., 2018; Luders and Kurth, 2020; Kurth et al., 2020). These could derive from the different roles both sexes perform in their social environment (e.g., brood/nest care, social training by mothers, defense and food provisioning by fathers), which are frequently instrumental in shaping and being shaped by personality traits. The growing body of evidence suggests that an individual’s personality shapes both its cognitive style and its cognitive performance. Both are determined by the particular cognitive domain the cognitive test is designed to examine. Therefore, a growing number of studies are now attempting to establish a link between the determinants of an individual’s cognitive performance, its cognitive style, and the respective cognitive domain (Carere and Locurto, 2011; Sih and Del Giudice, 2012; Guillette et al., 2017; Dougherty and Guillette, 2018; Wallace et al., 2020). Dougherty and Guillette (2018) provided a comprehensive meta-analysis on 19 species ranging from mammals and birds to reptiles, fish, and even insects to explore the interactions between animal personality in terms of an individual’s exploration, boldness, activity, aggression and sociability patterns and an individual’s cognitive skills in terms of cognitive flexibility and error ratios in initial and reversal learning tasks. Sex was determined one of the major variables influencing the observed associations across all taxa (Dougherty and Guillette, 2018).

Sex-Specific Cognitive Performance

For instance, female guppies (*Poecilia reticulata*) showed greater behavioral flexibility than their male conspecifics in a visual discrimination task (Laland and Reader, 1999; Lucon-Xiccato and Bisazza, 2014, 2017a,b). In another study, two closely related molly species (*Poecilia latipinna* and *Poecilia mexicana*) and their more distant relative, the guppy (*P. reticulata*) participated in an individual trial-and-error learning paradigm. Females of all three species were successful in all training phases of a visual dichotomous color discrimination task, followed by a series of reversal learnings. In contrast to the successful females, guppy males failed to learn even the general test paradigm. While no sex differences were observed in sailfin mollies (*P. latipinna*), closely related Atlantic molly males (*P. mexicana*) were clearly superior to females in all tasks they were assigned to, with some of them even reaching the one-trial learning level (Fuss and Witte, 2019). Moreover, Atlantic molly males performed significantly better in a socially learned visual dichotomous color discrimination task accompanied by serial reversals by inhibiting their prior response faster than their respective conspecific females (Fuss et al., 2021). Thus, what seemed to indicate an universal mechanism across different taxa with females responding clearly more flexible (e.g., observed in primates, rodents, domestic poultry, and teleosts) appears to be reversed in this fish species. Putatively, the observed sex differences in performance level, cognitive style and personality may account for the different sex roles in mating competition, mate choice, or complex sexual and social interactions in general, resulting from different selection pressures on both sexes during sexual selection. However, although cognitive sex differences appear to be present in many species across all taxonomic groups (e.g., fish: Lucon-Xiccato and Bisazza, 2017b; Cummings, 2018; birds: Kosarussavadi et al., 2017; mammals: Koss and Frick, 2016;

Mazza et al., 2018, 2019), the range of excellent to rather poor cognitive abilities between and within taxa appear to fluctuate greatly (Shaw and Schmelz, 2017). The same applies to individuals of the same species and even domain-specifically to a single individual (Titulaer et al., 2012; Carazo et al., 2014; Mamuneas et al., 2015; Lucon-Xiccato and Bisazza, 2016, 2017b; Etheredge et al., 2018; Wallace et al., 2020). Beyond influencing determinants such as, for instance, ecology, life history, or social context, an alternative explanation for the enormous variability in cognitive capabilities observed across individuals, species, or taxa is merely methodological. Frequently, an array of different cognitive tests is used assessing both the same but also different cognitive traits. Indeed, this sometimes confounds the comparison of different results severely.

Reflection in the Brain

By now, many studies suggest a reflection of sex-specific along with associated personality-driven behaviors in the brain. Examples include different levels of and different responses to steroid hormones (e.g., Manson, 2008; Carroll et al., 2010; Lenz and McCarthy, 2010; McEwen and Milner, 2017), distinct expression profiles of neuronal genes [e.g., immediate early genes (IEGs); e.g., Banerjee et al., 2013; Chow et al., 2013; Yagi et al., 2016, 2017; Kosarussavadi et al., 2017; Gegenhuber and Tollkuhn, 2019, 2020] indicating increased or decreased activity in specific brain regions, or even different brain sizes (e.g., Kotrschal et al., 2013; Chen et al., 2015; Corral-López et al., 2017a,b). Thus, a change in cellular processes regularly induces a change in gene expression. Characteristic changes in gene expression reside in brain regions specific to sexual behavior such as mate competition, mate choice, or social behavior such as aggression between males or females of the same or a different species. Any form of learning, whether long-term learning driven by life-history experiences or short-term learning driven by learned mate choice behavior or innovativeness in an (acute) problem-solving task, is accompanied by a series of tightly coordinated changes in gene activity in the relevant brain microstructure. This also changes the coordinated processes of a single or multiple neuronal circuits, which in turn induce situational changes and adaptations in behavior (compare Baker et al., 2017 for review). Consequently, complex gene expression (e.g., IEGs, neurohistochemistry, emission of secondary, retro- or anterograde messengers in the brain, etc.) and neuronal stimulus processing, which constitute the basis of an adapted reaction, are interconnected closely. Thus, combining information from genetics such as candidate gene analysis, bioinformatics and behavioral biology, e.g., on (cognitive) behavioral flexibility will help to reveal genome mapping, neuronal and neurohistochemical mechanisms of complex behavior particularly in the context of mate choice and, more broadly, in adaptive evolutionary contexts (Baker et al., 2017). Delclos et al. (2020) observed general, although not brain region-specific alterations in neuronal gene expression in response to different social and sexual contexts using transcriptomic profiles of both the sensory periphery and whole brains of female swordtail fish (*Xiphophorus birchmanni*). Along a shy-bold personality axis, conspecific encounters triggered an increased expression of immune-associated genes, olfactory and visual genes, and genes associated with fear, learning, and memory in visually and chemically exposed individuals. Also, visually and chemically heterospecific encounters led to an increased expression of genes associated with neurogenesis, synaptic plasticity, and social decision making, possibly indicating a stress coping strategy. For instance, neuroligins such as *nlgn2b* or *npy8ar* reflected a pivotal role in distinguishing between both transcriptomic profiles (i.e., conspecific or heterospecific profile), as did stress-coping genes and social decision-making network signaling pathway genes. The social decision-making network (SDMN) is "*a highly conserved network of forebrain and midbrain regions that evaluates the salience and rewarding properties of a social stimulus by integrating sensory information about the (social) environment with an individual's own condition and prior experience, eventually resulting in a behavioral choice. Evolutionarily ancient signaling pathways – such as steroid hormones, neuropeptides and biogenic amines – regulate SDMN function in the context of social behavior*" (DeAngelis and Hofmann, 2020). Similar results were obtained in zebrafish (*Danio rerio*) tested for proactive or reactive stress coping styles in contextual fear learning to chemical alarm substance from donor conspecifics. They showed neural plasticity in activity-dependent expression patterns of neurotransmission-related genes (*npas4a* and *gabbr1a*) in the medial and lateral zones of the dorsal telencephalon (Dm, Dl) and in the supracommissural nucleus of the ventral telencephalon (Vs) (Baker and Wong, 2019, 2021). Accordingly, many personality traits are supported by basic neuronal and neuroendocrine circuits that are plastically organized in the same or very similar ways in most vertebrates (e.g., Hofmann et al., 2014) and invertebrates (e.g., Hartenstein, 2006). These circuits can be drawn upon in comparable pathways across species to regulate the individual developmental stages in response to extrinsic and intrinsic events. In male green anoles, an altered gene expression of calcium channels, *integrin alpha-10*, and androgen and secretin receptors in the ventromedial hypothalamus was observed in close association with boldness during social agonistic and sexual interactions with conspecifics (Kabelik et al., 2021). Kelly and Goodson (2014) elucidated the sex-specific roles of oxytocin- and vasopressin-expressing neurons in the paraventricular hypothalamus of male and female zebra finches (*Taeniopygia guttata castanotis*) in social stress, aggression, sociability, individual preference for either larger or smaller groups, and pair bonding. While decreased vasopressin expression resulted in decreased sociability in all finches, it resulted in higher levels of aggression in males but lower levels in females. Conversely, decreased oxytocin availability in female zebra finches elicited lower sociability, weaker pair bonding, and weaker stress coping. Interestingly, sex-specific changes were observed in opposite-sex aggression levels but not in same-sex aggression levels (Kelly and Goodson, 2014). Comparable sex-specific interactions between social behavioral traits (aggression, dominance) and vasopressin were reported in male and female Syrian hamsters, prairie voles and other mammals as well (compare Dumais and Veenema, 2016; Terranova et al., 2017 for review). Depending on the social system and the prevailing sex roles, males and females may prefer to mate with a similarly behaving mate (assortative mating preference) or, conversely, a differently behaving one (disassortative mating preference).

FEMALE MATE CHOICE AND NEURONAL RESPONSE

The neuronal involvement and the interplay of the prefrontal cortex, septum, hippocampus, amygdala, and hypothalamus (or the corresponding brain areas in non-mammals, respectively) in social interactions were observed in different species across different taxa.

Sexual Information Influence Neuronal Activity

In mammals, these brain areas receive projections from the pontine tegmentum (*Nucleus incertus*, NI). The NI network is involved in social recognition and has been found to modulate the activity of sensory, emotional and executive brain regions. In studies of same-sex or opposite-sex mating preferences and the role of learning in their development, the medial preoptic area (mPOA) was determined to be a key region in particular for olfactory mediated sexual preferences, which goes beyond the mere control of copulatory behavior (rats and mice: Pfaus et al., 2012; Xiao et al., 2012; Graham and Pfaus, 2013; Zhong et al., 2014; ferrets: Paredes and Baum, 1995; hamsters: Martinez and Petrulis, 2013). It receives and responds to sensory information pertaining the physiological state of a potential partner (e.g., its health condition, fitness, or stage in the reproductive cycle), precisely integrates rewarding aspects of mating, exhibits context-dependent motor control and, hence, seems essential for mate assessment. While excitotoxic NI lesions impaired the recognition of conspecifics, a concurrent *egr-1* activation in the amygdala, septum and hypothalamus and *egr-1* inhibition in the hippocampus appeared to support the modulating properties of the NI network (García-Díaz et al., 2019).

Another example of how sexual information on a preferred male can influence the neuronal activity of choosing females used IEGs (*egr-1*, cellular homolog of *fos*) as neuronal markers for brain activity in gravid cichlid females (*Astatotilapia burtoni*). Initially, females were allowed to choose between two phenotypically equivalent males, who then had to fight with one another. Seeing her preferred male win caused a significant activation of the social behavioral network (SBN) nuclei known to be associated with reproduction. However, if the preferred male lost the fight, the activation of fear-associated nuclei in the lateral septum was induced. Hence, sexual information in the context of mate choice and its (anticipated) consequences powerfully activated specific parts of the female brain, independent of the actual social interaction (Desjardins et al., 2010). When exploring mate preference, aversion and sexual cognition in female northern swordtail brains (*Xiphophorus nigrensis*), nonapeptide gene expression (*isotocin, vasotocin*) was observed to differ depending on the sexual context associated with affiliation. Conversely, synaptic plasticity genes such as *neuroserpin, neuroligin-3, tyrosine hydroxylase (TH1),* and *NMDAR* responded merely to sexual contexts with distinct expression patterns. Females subjected to different mate choice contexts (large courting males, small coercive males or both) showed significantly higher *neuroligin-3* expression levels in the medial and lateral telencephalon (Dm, Dl), the ventral zone of periventricular hypothalamus (HV), the POA and the ventral zone of the ventral telencephalon (Vv), which are associated with sexual and social behavior. While no context- or behavior-related changes in *TH1* mRNA expression patterns were observed in any brain region linked to female preference, *neuroligin-3* levels were closely linked to mate choice contexts involving many courting male phenotypes. Thus, the aforementioned forebrain regions were predominantly involved in the processing of information on potential mates, being selectively supported by large parts of the brain with regard to the respective mate choice context (Wong et al., 2012; Wong and Cummings, 2014).

Genes, Pathways, and Brain Regions Associated With Mate Choice and Learning

Moreover, genes, pathways, and brain regions, which are considered to be closely associated with mate choice and learning appeared to be involved. Following preference tests of female mosquitofish (*Gambusia affinis*) choosing between female or coercive male conspecifics as well as between coercive or courting male heterospecifics (*P. latipinna*), the expression levels of *neuroserpin, egr-1,* and *early B* were examined by whole-brain gene expression analyses. Surprisingly, there was a positive association between the upregulation of the genes under investigation in view of courting heterospecies, which was absent in coercive heterospecies. The observed neuronal response was consistent with female mate preference, respectively. At the same time, older choice-experienced females chose courting males more quickly and ignored the coercive ones than younger females, suggesting previous learning experiences (Wang et al., 2014). Moreover, *neuroserpin* and *neuroligin-3* were observed to be strongly expressed in female mosquitofish (*G. affinis*) in mate choice events, but the same candidate genes were downregulated in female swordtails (*X. nigrensis*) in a similar but social context. Conversely, *neuroserpin* and *neuroligin-3* were expressed progressively in asocial and movement situations. This divergent gene response seems to perfectly mirror the different mate choice behavior of both species, with mosquitofish females choosing large, colorful males, while swordtail females try to avoid coercive males (Lynch et al., 2012). In the three poeciliid species under investigation, previously acquired mate choice experiences as well as memory retention of these experiences appear to play a determining role in future encounters and, therefore, appear to require some degree of cognitive ability.

Genes associated with prosociality were found to have higher predictive power for social contexts that differed by social vigilance. Similarly, genes associated with synaptic plasticity and learning were strongly tied to mate choice contexts. Blocking these important synaptic plasticity processes in the brain caused female preference to be suppressed. Gene expression and pharmacological manipulations in female northern swordtails suggest that selecting a mate involves sexual cognition and, hence, neuromolecular processes associated with learning at cellular (i.e., synaptic plasticity genes) and local (i.e., amygdala and hippocampus) levels (compare Cummings, 2015 for a review).

Acoustic Communication as an Example of Neuromolecular Interaction

In frogs (*Spea bombifrons, P. pustulosus*), the processing of acoustic signals in the midbrain plays an important role in mate choice (phonotaxis). For instance, acoustic communication signals from a wide range of animals occupy a large part of the biologically relevant frequency spectrum and require sophisticated midbrain integration and processing. They are frequently subject to (e.g., seasonal or diurnal) temporal variations, as some species of a wide array of taxa ranging from birds, insects, amphibians, mammals to fish, tend to be nocturnal, while others are diurnal, resulting in a constantly changing frequency and sequence composition of the soundscape (e.g., Ruppé et al., 2015; Ferreira et al., 2018; Gottesman et al., 2020). Female túngara frogs (*P. pustulosus*) expressed comparable preferences for courtship calls at low and medium noise densities, but no preference at high noise densities, which diminished their decision-making accuracy (Coss et al., 2021). However, mating preference can be predicted more accurately by the activity of the estradiol-gated, acoustically sensitive POA (as part of a sensory-endocrine circuit), which integrates forebrain inputs to the midbrain auditory response and, subsequently, influences motor responses directly via descending projections to the medulla and spinal cord. In addition, the basal forebrain including the POA, septum and *Nucleus accumbens* (Chakraborty and Burmeister, 2015) responds to mating calls, with the POA providing an increased production of Gonadotropin Releasing Hormone (GnRH; Burmeister and Wilczynski, 2005). Thus, many different regions of the SBN appear to be involved in evaluating mating calls, whereby the sensory system filters for relevant signals to which the POA attributes a context-dependent level of importance (Burmeister, 2017; Taylor et al., 2019). In summary, the involvement of the POA in mate choice and mate preference has been demonstrated across several taxa, including primate and non-primate mammals, amphibians and fish and, thus, appears to be a highly preserved mechanism.

BRAIN SIZE, COGNITIVE ABILITY AND MATE CHOICE

Present literature appears to support the 'social intelligence hypothesis', according to which relatively big brains and higher cognitive abilities have developed to cope with the variable, sometimes apparently erratic behavior of potential mates or other group members in general. Larger brain size appears to be evident primarily in species that (a) live in larger social groups, (b) experience a greater reliance on social learning in response to a variable environment, and (c) exhibit a prolonged reproductive period (Holekamp and Benson-Amram, 2017).

Brain Size Predicts Mate Assessment and Behavioral Plasticity in Guppies

Kotrschal et al. (2014) linked brain size as a proxy for cognitive ability to personality and behavioral plasticity of guppies (*P. reticulata*) by artificially selecting for large and small brain size in the laboratory. The guppy females' brain size apparently greatly impacts the assessment of male attractiveness during mate choice. In turn, guppy male brain size appears to strongly interfere with the judgment of female quality during male mate choice as well. Likewise, brain size along with environmental complexity seem to play a pivotal role in the mating behavior of both male and female guppies (Corral-López, 2017; Corral-López et al., 2017a,b). In another study (Herczeg et al., 2019), guppies were initially kept in social groups in enriched aquaria. Subsequently, they were separated in empty aquaria where they were exposed to visual predator stimuli. Prior to experiments, the researchers expected a stronger response to stress (indicated by lowered behavioral activity), to be followed by faster habituation (indicated by rapid increases in behavioral activity) of the large-brained individuals over time relative to the small-brained ones. Both selection lines (i.e., large-brained and small-brained fish) showed progressive sensitization (i.e., increasing risk aversion) to predator stimuli, thus, providing support for the hypothesis on the relationship between brain size and behavioral plasticity. The extent of individual or sex-specific personality differences remained unaffected by the selected brain size (Herczeg et al., 2019). Likewise, using two lineages selected for their relative brain sizes and different cognitive abilities, Corral-López et al. (2020) examined the mate choice behavior of guppies in the context of predation threat and different sex ratios. While female guppies with relatively larger brains became increasingly willing to copulate as predation threat decreased (female biased sex ratio), so did individuals with smaller brains in male guppies, which also appeared more aggressive at the same time. However, females did not show a preference for large-brained males, which may indicate an influence of brain size on the mating propensity of male guppies.

Linking Brain Size and Cognitive Ability to Social-Sex Selection Pressures

It has been widely assumed that relatively larger brains comprise a higher number of neurons and connections between them, hence, leading to better cognitive abilities. However, critical of the link between relative brain size and cognitive ability is that there are virtually no studies actually examining this phenomenon. In order to assess cognitive ability in association with the relevant neuronal substrates properly, important parameters such as interconnectivity and the number of neurons involved [as a measure of (higher) neuron density] need to be determined along with the (relative) brain size. Analyses of the two large- and small-brained guppy strains, respectively, showed that breeding favoring one brain size or the other did indeed result in shifts in the number of neurons both throughout the brain and, particularly, in the telencephalon. At the same time, neuronal density correlated negatively with individual brain size (Marhounová et al., 2019). Hence, this study appears to confirm a close association of individual brain size with neuron number, and thus putatively, with cognition – at least within the fish species under investigation. An effect of brain size on female learning performance has been reported in swordtails (*Xiphophorus multilineatus*) subjected to a classical conditioning paradigm as well (Griebling et al., 2020). In the context of mating and pair bonding, the brains of seven closely related cichlid species with different sexual behaviors from Lake Tanganyika located between Tanzania, Zambia, Burundi, and Congo were studied volumetrically. Significantly greater telencephalon volumes were found in species with predominantly monogamous mating systems compared to polygamous cichlid species (Pollen et al., 2007). These results were probably attributable less to mating behavior than to shared parental brood care and living in complex habitats, the latter requiring concurrently considerably higher cognitive abilities, for instance, in spatial orientation. Similar results have also been reported in different stickleback species, where the cognitive challenges of parenting resulted in significant sexual dimorphism in brain size, particularly in the brain of the respective brood-caring parent (Gonzalez-Voyer et al., 2009; Kotrschal et al., 2012; Samuk et al., 2014; Toli et al., 2017). In more than thirty cichlid species, the mating system (polygamous and monogamous) was observed to significantly influence sex differences in telencephalon size, with sexual dimorphisms being present only in polygamous species (male telencephalon > female telencephalon) (Gonzalez-Voyer and Kolm, 2010). Likewise, Holekamp and Benson-Amram (2017) sought to link brain size in spotted hyaenas to their enhanced cognitive abilities and strikingly pronounced social skills. As predicted by the social intelligence hypothesis, spotted hyaenas have considerably larger brains and enlarged frontal cortices compared to less social hyaena species. Frontal cortex volume was significantly greater in male sexually mature spotted hyenas than in females despite equal endocranial volume, although males and females of this species face similar requirements regarding success level and size of hunting territory when pursuing vertebrate prey (e.g., Boydston et al., 2005; Holekamp and Benson-Amram, 2017). Even in humans, the capacity for behavioral flexibility in response to environmental changes is considered an important determinant in the evolution of the human brain (Holekamp and Benson-Amram, 2017).

The Cognitive Buffer Hypothesis

These findings seem to support the social intelligence hypothesis at first glance. However, other studies revealed that domain-general cognitive abilities did not develop in response to social-sex selection pressures, but predominantly in response to permanently changing and, therefore, novel environmental conditions in general, which are followed by alterations in the associated social context ('cognitive buffer hypothesis'; Holekamp and Benson-Amram, 2017). The cognitive buffer hypothesis derives from observations particularly in primates by means of positive correlations between brain size and longevity, taking into account other factors such as sex, social structure, and life history (Allman et al., 1993; Hofman, 1993; Reader and Laland, 2002). In this context, a larger brain seems to be associated with both longer life and a slower pace of life, thus also influencing important life history traits such as development or sexual or reproductive traits.

Therefore, in summary, the cognitive buffer hypothesis may provide a framework for explaining the evolution of vertebrate brain size (Jiménez-Ortega et al., 2020). A longer lifespan would allow individuals to exploit their costly investment in brain size to the maximum by, for instance, devoting more time to finding innovative solutions to problems that would otherwise jeopardize their survival and reproductive success (Sol and Lefebvre, 2000; Shultz et al., 2005; Sol et al., 2005, 2008). Moreover, learning allows for a certain degree of behavioral flexibility to deal with challenges in the animate and inanimate environment, for instance in the context of reduced food availability, increased predation risk, or a mating system with frequent coercive copulations (Richerson and Boyd, 2000; Reader and MacDonald, 2003; Sol, 2009a,b; Sol et al., 2016). However, individual, species- or sex-specific brain size, its consequences for cognitive ability and mate choice are under intense discussion and far from being clear-cut. Nevertheless, the examples presented here show that there appears to be a coherency, at least in fish and mammals, which is worth continuing to illuminate. Hence, future studies should try to elucidate further the evolution of vertebrate brain size along with its consequences for cognitive mate choice.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This review aimed to throw a spotlight on the neuronal prerequisites, networks and processes supporting the interaction between mate choice, sex roles and social cognition, hence, supporting cognitive mate choice. Considering the numerous exciting studies, mainly covering the three largest vertebrate groups of mammals, birds and fish, there is support that the neuronal activity of males and females indeed differs with respect to social cognition. In terms of results derived from gene-based neuronal activity markers (IEGs and SPGs), modulatory effects of neurotransmitters and hormones or even fMRI studies, there are ample indications supporting the idea that the sex as well as the respective sex role within the prevalent mating system is mirrored at a neuronal level, at least in individual brain regions. The same can be assumed for the cognitive capabilities affecting mate choice, just as mate choice affects cognitive abilities in both sexes. An appropriate behavioral response is supported and triggered by the associated neuronal and molecular prerequisites. Hence, carefully designed behavioral studies together with state-of-the-art neuroanatomical techniques will allow for testing cause, effect, and (modulatory) interactions of an observed behavioral pattern and the substrates decisively coordinating an appropriate response.

So far, however, our knowledge largely relies on the observation of a confined set of networks (e.g., SBN and SDNM) or distinct recruited brain regions (in particular the associative areas of the telencephalon, the sensory areas of the telencephalon and the midbrain as well as their respective correlates across taxa). Many crucial intermediary steps, associations, and, accordingly, many cause-and-effect relationships along with the truly relevant level of brain organization remain obscure to us so far. For instance, we do not know how precisely females assess the cognitive performance of males, if it is perhaps reflected in any physical attributes, or how the brain responds to merely observed mate choice situations. When observing sexual social interactions, the brain and/or the neuronal substrates involved could hypothetically respond in the same way as when drawing own decisions. In analogy to the mirror neurons known, for instance, from mammals, the neuronal substrates of an individual could adaptively modulate their activity and revert to the observed, and, hence, learned and memorized information, when an individual actually decides and effects its own decision. By comparing many observed mate choice situations, a kind of 'blueprint' may develop, possibly allowing an individual's mate choice decisions to progress significantly faster, more efficiently, and more accurately. In order to bridge this gap in knowledge, future studies should strive to integrate the behavioral and neurobiological dimensions in the context of cognitive mate choice. In view of the technical progress and novel methods in behavioral studies and neurobiology, we may anticipate an array of fascinating lessons, discoveries, and insights to shed light on the complex relationship of mate choice and cognition.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

I thank Klaudia Witte for the opportunity to write the manuscript and her helpful comments to improve it. I thank Nils Krützfeldt for proofreading the manuscript. I also thank the University of Siegen for the financial support provided through its Open Access Publication Fund. Last but not least, I thank the guest editor for inviting me to write about this research topic, as well as both reviewers for their time and support.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 17 November 2021
doi: 10.3389/fevo.2021.745752

Sex Reversal and Performance in Fitness-Related Traits During Early Life in Agile Frogs

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *22 July 2021*
Accepted: *27 October 2021*
Published: *17 November 2021*

Citation:
Bókony V, Ujhegyi N, Mikó Z, Erös R, Hettyey A, Vili N, Gál Z, Hoffmann OI and Nemesházi E (2021) Sex Reversal and Performance in Fitness-Related Traits During Early Life in Agile Frogs. Front. Ecol. Evol. 9:745752. doi: 10.3389/fevo.2021.745752

Sex reversal is a mismatch between genetic sex (sex chromosomes) and phenotypic sex (reproductive organs and secondary sexual traits). It can be induced in various ectothermic vertebrates by environmental perturbations, such as extreme temperatures or chemical pollution, experienced during embryonic or larval development. Theoretical studies and recent empirical evidence suggest that sex reversal may be widespread in nature and may impact individual fitness and population dynamics. So far, however, little is known about the performance of sex-reversed individuals in fitness-related traits compared to conspecifics whose phenotypic sex is concordant with their genetic sex. Using a novel molecular marker set for diagnosing genetic sex in agile frogs (*Rana dalmatina*), we investigated fitness-related traits in larvae and juveniles that underwent spontaneous female-to-male sex reversal in the laboratory. We found only a few differences in early life growth, development, and larval behavior between sex-reversed and sex-concordant individuals, and altogether these differences did not clearly support either higher or lower fitness prospects for sex-reversed individuals. Putting these results together with earlier findings suggesting that sex reversal triggered by heat stress may be associated with low fitness in agile frogs, we propose the hypothesis that the fitness consequences of sex reversal may depend on its etiology.

Keywords: sex reversal, sex determination, life history, tadpole behavior, carbamazepine, terbuthylazine, chlorpyrifos

INTRODUCTION

Sex is a fundamental aspect of individual state in all sexually reproducing organisms. Having testes *versus* ovaries often comes with a diverse set of differences in physiology, morphology, life history, and behavior, including mating and parental strategies often labeled as "sex roles" (Schärer et al., 2012; Immonen et al., 2018). In species with genetic sex determination, where the process of gonad development is triggered by genomic elements, males and females also often differ in their genetic make-up. For example, the chromosome restricted to one sex (e.g., Y in male-heterogametic systems) is inclined to undergo degeneration, which may lead to sex differences in mortality rates and senescence (Marais et al., 2018). In species with environmental sex determination, where the fate of the gonads is decided by external factors such as temperature during early ontogeny, sex ratios and hence population viability may be particularly vulnerable to environmental changes (Mitchell and Janzen, 2010). Thus, the way males and females come to be has crucial implications for population dynamics and thereby biodiversity conservation.

In vertebrate animals, genetic and environmental sex determination have traditionally been thought of as mutually exclusive systems, but research on ectotherms, especially in the past decade, has revealed a growing number of species in which the two systems naturally coexist (Baroiller and D'Cotta, 2016; Holleley et al., 2016). In such species, environmental influences can override the effect of genes during sex determination in early life, resulting in sex reversal whereby individuals develop phenotypic sex discordant with their genetic sex (Alho et al., 2010; Baroiller and D'Cotta, 2016; Holleley et al., 2016; Lambert et al., 2019; Nemesházi et al., 2020). Theoretical studies predict that sex reversal has far-reaching consequences for demography, population persistence, sex chromosome loss and evolutionary transitions between sex-determination systems (Grossen et al., 2011; Quinn et al., 2011; Bókony et al., 2017; Wedekind, 2017; Schwanz et al., 2020; Nemesházi et al., 2021). Studying these consequences empirically is especially important in light of the ongoing rapid human-induced environmental alterations, including climate change and chemical pollution, which may increase sex-reversal frequency in nature (Nemesházi et al., 2020).

One of the most urgent questions regarding sex reversal is how sex-reversed individuals compare to concordant males and females in terms of performance in fitness-related traits. Understanding this issue would be highly valuable for at least three reasons. First, it would facilitate forecasting the effects of sex reversal on demography and evolution, because many of these theoretically predicted effects critically depend on the viability and reproductive success of sex-reversed individuals (Grossen et al., 2011; Bókony et al., 2017; Nemesházi et al., 2021). Second, it would provide insight into the ultimate and/or proximate drivers of sex reversal. On the one hand, sex reversal may be an adaptive sex-allocation strategy that allows individuals to develop the sex that is most beneficial under the prevailing environmental conditions (Geffroy and Douhard, 2019), similarly to temperature-dependent sex determination (Schwanz et al., 2016). In this case, sex-reversed individuals may perform at least as well or even better than concordant individuals, as found for fecundity in a reptile (Holleley et al., 2015). On the other hand, sex reversal may be a mechanistic consequence of endocrine disruption due to early life stress, as demonstrated in fish (Senior et al., 2012; Baroiller and D'Cotta, 2016). In this case, sex-reversed individuals may display reduced performance in important life-history traits due to early life stress, as seems to be the case in an anuran amphibian (Nemesházi et al., 2020; Mikó et al., 2021b). Yet another alternative is that sex reversal may arise by random sex determination (Perrin, 2016): in absence of strong genetic and environmental triggers, sex may be determined by stochastic variability in gene expression levels. This process may coexist with genetic and environmental sex determination, and can explain considerable proportion of phenotypic variance (Perrin, 2016). Thus, random variation (i.e., no systematic difference) between sex-reversed and concordant individuals in fitness-related performance might indicate random sex determination. Thirdly, because sex reversal de-couples genetic and phenotypic sex, it allows for evaluating the relative importance of sex-linked genes *versus* gonadal effects (sex hormones and other sex-specific modifiers that orchestrate sex-biased gene expression) in the development of sex-specific life histories and behaviors. Despite all these reasons for studying the consequences of sex reversal, we have very little empirical information on the fitness of sex-reversed individuals, apart from fish in aquaculture (Senior et al., 2012), where sex reversal is artificially induced and thus may not necessarily be ecologically relevant. Researchers have only just begun to investigate the relationship between ecologically relevant sex reversal and individual performance, and most of the little existing knowledge comes from a single reptilian species, where high incubation temperatures produce male-to-female sex-reversed individuals that display a complex combination of male-like, female-like, "supermale" and "superfemale" traits (Holleley et al., 2015; Li et al., 2016; Jones et al., 2020).

Here we address the fitness-related performance of sex-reversed individuals by using data from two previous experiments (Bókony et al., 2020; Mikó et al., 2021a) on agile frogs (*Rana dalmatina*), a European species whose natural populations contain a considerable number of female-to-male sex-reversed adults (Nemesházi et al., 2020). Both experiments were designed to test sub-lethal effects of larval exposure to environmentally relevant concentrations of chemical pollutants on early life traits related to fitness. Neither of the chemical treatments affected phenotypic sex ratios significantly (Bókony et al., 2020; Mikó et al., 2021a), but as we report here, sex-reversed individuals occurred in both experiments, indicating that some agile frog tadpoles spontaneously undergo sex reversal even in the absence of any sex-reversing chemical or thermal treatment, in accordance with another study (Mikó et al., 2021b). Therefore, these data offered us the opportunity to examine whether genetic and phenotypic sex and the combination thereof (i.e., reversed vs. concordant sex) are associated with differences in life history and behavior during early ontogeny. The larval phase is of critical importance in amphibian life history because mortality during this stage can be extremely high (Riis, 1991), mainly due to predation, pond desiccation, and limited food availability. Thus, larval survival depends to a large extent on the behavioral strategies (predator avoidance, foraging activity) adopted by tadpoles (Skelly, 1994) and the speed of their development (Griffiths, 1997). Also, the rates of development and growth until metamorphosis can have life-long effects on fitness in amphibians (Smith, 1987; Berven, 1990; Altwegg and Reyer, 2003). However, sex differences in larvae are very rarely investigated due to the difficulties of phenotypic sex identification in immature animals (Ujhegyi and Bókony, 2020) and genetic sexing in amphibians overall (Nemesházi et al., 2020). In the agile frog, males typically start reproducing 1 year earlier than females (Riis, 1991; Sarasola-Puente et al., 2011), and

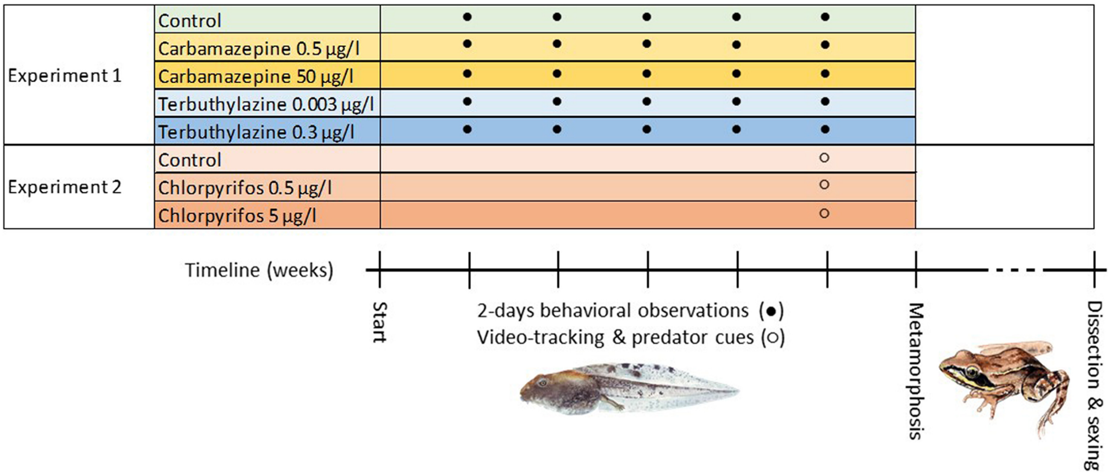

FIGURE 1 | Schematic illustration of the experimental design (paintings by Bálint Bombay).

larger males are more successful in male-male competition (Vági and Hettyey, 2016). Therefore, we predicted to observe higher growth rate and faster larval development in males. Fast growth and development require high food intake, so we predicted that male tadpoles would spend more time feeding and, in trade-off, take higher predation risk (Urszán et al., 2015). At least some of these sex differences might be sex-chromosome-linked, since the agile frog has an XX/XY sex-chromosome system (Jeffries et al., 2018); this would make the female-to-male sex-reversed individuals resemble concordant females. However, the agile frog sex chromosomes are homomorphic (Jeffries et al., 2018), suggesting limited genetic differentiation between the sexes. By this latter logic, female-to-male sex-reversed individuals may be more likely to resemble concordant males, due to the presence of testes which produce androgen hormones that stimulate the expression of male phenotypic traits (Guarino and Bellini, 1993). We evaluated these predictions by comparing early life development, growth, and behavior among three groups: males and females with concordant genetic and phenotypic sex, and female-to-male sex-reversed individuals.

MATERIALS AND METHODS

Experimental Procedures

The detailed methods of the two experiments have been published in two open-access papers (Bókony et al., 2020; Mikó et al., 2021a). Here we provide a brief description of each experiment, and present detailed methods only for those aspects that are directly relevant for the current study. All experimental procedures were approved by the Ethical Commission of the Plant Protection Institute and carried out according to the permits issued by the Government Agency of Pest County (Department of Environmental Protection and Nature Conservation) and the Budapest Metropolitan Municipality (Department of City Administration, FPH061/2472-4/2017).

The study design is illustrated in **Figure 1**. For both experiments, we collected freshly spawned agile frog eggs in March 2018 from ponds in woodland habitats in north-central Hungary. We sampled 8 egg masses from each of three ponds for experiment 1, and further 10 egg masses from one of these ponds for experiment 2 (**Supplementary Table 1**). The eggs were taken into our laboratory, where the two experiments were conducted simultaneously in the same room under artificial photoperiod that mimicked the outdoors dark-light cycles. We raised eggs and tadpoles in reconstituted soft water (RSW) at 19°C water temperature. We started both experiments when the hatchlings reached development stage 25 (Gosner, 1960) by placing haphazardly selected tadpoles into white plastic boxes (14 × 9.5 cm base area) filled with 1 L RSW. Each animal was kept in a separate, individually labeled box throughout the entire experiment. The boxes were arranged in a randomized block design to ensure that all treatments were homogeneously distributed across the shelves in the laboratory.

In both experiments, we exposed the tadpoles to environmentally relevant concentrations of water-polluting chemicals for which sex-related endocrine-disrupting effects had been reported. In experiment 1, we applied two concentrations each of carbamazepine (0.5 and 50 µg/L), a pharmaceutical drug (Galus et al., 2013, 2014), and terbuthylazine (0.003 and 0.3 µg/L), a herbicide frequently used in Europe (Kjeldsen et al., 2013). In experiment 2, we applied two concentrations of chlorpyrifos (0.5 and 5 µg/L), a broad-spectrum insecticide (Bernabò et al., 2011). The applied two concentrations, respectively, for each chemical correspond to the mean (or median) and close-to-maximum values reported from surface waters (Bókony et al., 2020; Mikó et al., 2021a). In both experiments, the control group of tadpoles was kept in clean RSW to which we added ethanol as solvent control (1 µL 96% ethanol to 1 L RSW); all other treatment groups also contained this amount of ethanol as vehicle. In experiment 1, we distributed 480 tadpoles (20 from each family) evenly across five treatment groups with four replicates in each treatment × family combination (4 tadpoles × 24 families × 5 treatments). In experiment 2, we distributed 144 tadpoles evenly across three treatment groups (48 tadpoles per treatment, with 4–6 tadpoles in each treatment × family combination).

We exposed tadpoles to the treatments over the entire duration of their larval development. Twice a week, we renewed each treatment by changing the rearing water and fed the tadpoles *ad libitum* with chopped, slightly boiled commercial spinach. We collected different kinds of data on tadpole behavior in the two studies (see below), with our focus being on foraging activity in experiment 1 and anti-predatory response in experiment 2. When the tadpoles reached the start of metamorphic climax (appearance of forelimbs at development stage 42), we recorded this date and measured their body mass (±0.1 mg). We raised the post-metamorphic animals in individual rearing boxes, feeding them *ad libitum* with small crickets until they reached the minimum age that allows reliable identification of phenotypic sex by the gross anatomy of the gonads (Ogielska and Kotusz, 2004; Bernabò et al., 2011). On average 15 weeks (96–136 days) after starting the experiment, we weighed the animals to the nearest 0.01 g and dissected them for phenotypic sexing (see below). During dissection, we removed the entire digestive tract and measured its mass; this value was subtracted from froglet body mass to remove any variance due to food remains in the gut.

Behavioral Observations and Video Analysis

In experiment 1, we observed the behavior of each tadpole in their rearing boxes containing *ad libitum* food, using the "instantaneous sampling" method (Altmann, 1974) four times a week during the larval period, totaling 20 observations per tadpole. Four researchers conducted the observations on 2 days each week, in one morning session and one afternoon session (each lasting ca. one hour) each day. During each session, we scanned all tadpoles once in a fixed order, recorded their instantaneous behavior as inactive, feeding, or swimming, and we also categorized the location of the tadpole within the box as on the bottom, next to the wall (i.e., within one tadpole distance from the wall, but not on the bottom), or in the open (i.e., not near the bottom or wall). In each session, a single observer scanned all tadpoles, and the identity of observers was rotated between sessions.

In experiment 2, we video-recorded tadpole behavior 1 month after starting the experiment and 3 days thereafter. As we had a limited number of cameras, we video-recorded only a subsample (83%) of individuals (40 from each chemical treatment group). On each occasion, we transferred the tadpoles into new containers identical to their rearing boxes, but with no food (to facilitate automatic tracking). On the first occasion, the box contained RSW with the same chemical treatment the individual was reared in, whereas on the second occasion all tadpoles were moved into clean RSW for video recording. Each time, we recorded the tadpoles' behavior for 20 min, and then exposed them to a startling stimulus by abruptly pouring 40 ml RSW into their water. Typically, the tadpoles reacted by a short burst of swimming ("escape"), followed by a period of motionlessness ("freezing"). To allow for measuring these responses we continued recording for another 20 min after the stimulus. As a main objective of experiment 2 was to assess anti-predator responses, half of the tadpoles within each chemical treatment group received clean RSW as startling stimulus on both occasions, whereas the other half received chemical cues indicating predation risk. The chemical cues were prepared as described in Hettyey et al. (2016), using water from the tank of European perch (*Perca fluviatilis*) that had been feeding on agile frog tadpoles. As the European perch is a native predator in our region, agile frog tadpoles respond to chemical cues indicating its presence by decreasing their activity, even if they are predator-naïve (Hettyey et al., 2016).

From the video recordings, we collected data on tadpole activity using the automatic tracking software ToxTrac (Rodriguez et al., 2018). All tracking results were manually checked for quality; all tracking data used in the current paper were error-free. We calculated the following variables from the first 20 min of each recording (i.e., before the addition of predator cues): total distance moved as a measure of locomotor activity, proportion of area used as a measure of exploration rate, and proportion of time spent near the wall (within a 50-pixel wide stretch from the side of the box; the tadpoles had an average snout-to-vent length of ca. 40 pixels in the videos) as a measure of risk aversion. For quantifying the startle response, the second 20 min of each video recording (i.e., after the addition of predator cues) were analyzed manually by a single observer, who recorded the following variables: the intensity of immediate reaction to the startle ("startle response"), categorized on a 0–3 scale (0: no movement, 1: slight movement, 2: swimming away apparently calmly, 3: swimming around fervently); the duration of escape, measured as the time spent moving continuously from the startle stimulus until the first stop; and the duration of freezing, measured as the time spent motionless after the escape until the first movement thereafter. Note that the duration of escape and freezing were quantifiable only in those individuals whose startle response had a non-zero intensity score.

Phenotypic and Genetic Sexing

We euthanized the animals by immersion into 6.66 g/L MS-222 solution buffered to neutral pH. After dissection, we examined the gonads under an Olympus SZX12 stereomicroscope at 16 × magnification, and categorized phenotypic sex as male (testes), female (ovaries), or uncertain. There were only two individuals in the latter category, and one of them also had uncertain genetic sex (see below); we excluded this animal from all statistics presented in this paper. Due to a low level of mortality in both studies, we had data on phenotypic sex for 439 individuals in experiment 1 and 135 individuals in experiment 2 (**Table 1**).

Throughout both experiments we monitored survival daily, and stored a tissue sample from each animal that died before dissection, as well as from all dissected animals, in 96% ethanol. We extracted DNA using Geneaid Genomic DNA Extraction Kit for animal tissue (Thermo Fisher Scientific), following the manufacturer's protocol, except that digestion time was 2 h. We identified genetic sex of all animals, and sex reversal in

TABLE 1 | Sample sizes by sex in each treatment group, and odds ratios (OR) with 95% confidence intervals (CI) for sex-reversal rate in genetic females (XX genotype) between the control group and each treatment group.

Experiment	Treatment	Sex-reversed (XX male)	Concordant		Died*		Other[†]	OR (95% CI)
			XX female	XY male	XX	XY		
1	Control	2	45	35	5	6	0	–
	Carbamazepine 0.5 μg/l	0	55	34	2	1	1	0 (0, 4.53)[‡]
	Carbamazepine 50 μg/l	0	53	37	2	1	0	0 (0, 4.70)[‡]
	Terbuthylazine 0.003 μg/l	1	38	48	4	3	1	0.46 (0.03, 6.22)
	Terbuthylazine 0.3 μg/l	2	50	36	2	3	1	0.41 (0.04, 4.20)
2	Control	5	23	19	1	0	0	–
	Chlorpyrifos 0.5 μg/l	6	21	20	0	0	0	1.61 (0.30, 10.69)
	Chlorpyrifos 5 μg/l	5	20	16	2	2	0	1.80 (0.21, 8.95)

The table excludes 15 individuals for which we had no data on sex (7 escaped before dissection and DNA sampling; 8 died before dissection and could not be sexed genetically).

**Phenotypic sex could not be diagnosed for the individuals that died before dissection.*

[†] *Three phenotypic males had unknown genetic sex due to marker disagreement (Rds3 genotype was female whereas Rds1 genotype was male).*

[‡] *These two odds ratios were taken from Fisher's exact tests; the rest from a binomial mixed model (overall effect of treatment: Wald test, $\chi^2 = 1.24$, df = 4, P = 0.871).*

phenotypically sexed individuals, using a recently developed molecular marker set (Nemesházi et al., 2020) which has been validated for agile frog populations in our study area, including the three populations sampled for the present study. In short, first we tested all froglets for marker Rds3 (≥95% sex linkage; primers: Rds3-HRM-F and Rds3-HRM-R) using high-resolution melting (HRM), and we accepted an individual to be concordant male or concordant female if its Rds3 genotype was in accordance with its phenotypic sex. Those individuals that appeared sex-reversed by Rds3 were also tested for marker Rds1 (≥89% sex linkage; primers: Rds1-F, Rds1-R and Rds1-Y-R) using PCR, and were accepted to be sex-reversed only if both markers confirmed sex reversal. Those individuals that were not phenotypically sexed due to early mortality were screened for both Rds1 and Rds3. Individuals with discrepant genotyping results (i.e., contradiction between Rds1 and Rds3) were considered to be of unknown genetic sex. This approach yielded data on genetic sex for 465 individuals in experiment 1 and 140 individuals in experiment 2 (**Table 1**).

For those individuals that we identified as sex-reversed, we also examined the gonads histologically to make sure that the mismatch was not due to incorrect categorization of phenotypic sex. We also investigated gonad histology in the individual for which phenotypic sex was uncertain based on gross anatomy but the genetic sex was unambiguous. We fixed the dissected gonads (not separated from the kidneys, because of small gonad size) in neutral-buffered 10% formalin, and prepared histological sections as described in our earlier papers (Nemesházi et al., 2020; Mikó et al., 2021b). For one individual, the sections failed to include gonadal tissue. For all other individuals examined, the gonads were clearly identifiable by histology either as testes (n = 14) or ovotestes (testes containing a few oogonia, see **Supplementary Figure 1**; n = 6, including the individual whose phenotypic sex had been uncertain based on gross anatomy). Therefore, in the analyses we treated these individuals as phenotypic males (female-to-male sex-reversed individuals).

Statistical Analyses

An overview of all our analyses is given in **Supplementary Table 2**. First, we tested whether sex-reversal rate was independent of chemical treatment by analyzing the phenotypic sex of genetic females in a generalized linear mixed-effects model with binomial error and logit link, including treatment type as fixed factor, and family nested in experiment as random factors. Two treatment groups lacking sex-reversed individuals had to be excluded from this analysis because such separation in logistic models results in unreliable estimates; thus, we used Fisher's exact tests to compare sex-reversal rate in these two groups with the respective control group.

We tested whether survival rate differed between genetic males and genetic females using a Cox's proportional hazards model, including family nested in experiment as random effects, and treating the dissected individuals as censored observations (i.e., these animals survived until the end of the study). In all remaining models (see below), we included sex as a three-category factor (concordant male, concordant female, or sex-reversed), thus individuals with missing data on either genetic or phenotypic sex were excluded.

We analyzed the duration of larval development (number of days from starting the experiment at stage 25 until the start of metamorphosis at stage 42), body mass at metamorphosis, and juvenile body mass (measured at dissection ca. 2 months after metamorphosis; excluding gut mass) by pooling the data of the two experiments, taking into account chemical treatment as a fixed factor and family nested in experiment as random factors. Duration of larval development was analyzed with a Cox's proportional hazards model, whereas both mass variables were analyzed with linear mixed-effects models, allowing for heteroscedasticity among the three sex categories. In the model of juvenile body mass, we also included age (number of days from starting the experiment until dissection) as a covariate. To investigate the trade-off between development and growth in tadpoles in the three sex categories, we added the interaction between the duration of larval development and sex into the model of mass at metamorphosis; in this model we allowed for heteroscedasticity also among families because residual diagnostics indicated that a few families exhibited outliers in the relationship between development and growth.

We analyzed tadpole behavior separately for the two experiments. For experiment 1, we analyzed two variables. We compared the proportion of observations in the open among the three sex categories with a Fisher's exact test, because separation in the data precluded the use of logistic models (thus, in this statistical test we could not control for potential effects of other predictors such as chemical treatment). To analyze the proportion of individuals feeding, we used a generalized linear mixed-effects model with binomial error and logit link. For experiment 2, we analyzed the pre-startle distance moved, exploration rate, and time spent near the wall with linear-mixed effects models, allowing for heteroscedasticity among the three sex categories. We analyzed the intensity of startle response using a cumulative-link mixed model with logit link function, and the durations of escape and of freezing with Cox's proportional hazards models, excluding those tadpoles that did not react with movement. All models of behavioral variables included individual nested in family as random factors, chemical treatment as a fixed factor, and the fixed effects of date (a covariate in experiment 1, expressing the number of days from starting the experiment; and a two-category factor in experiment 2) and time of day (a two-category factor in experiment 1, and a covariate in experiment 2, expressing the order of video recordings which were done in 7 consecutive bouts). Additionally, the model of experiment 1 included shelf height and observer identity as fixed factors and water temperature as a covariate (for detailed explanation on these covariates, see Bókony et al., 2020). To investigate the change of feeding rate over time in the three sex categories, we added the interaction between date and sex into the model of feeding rate (we expected that young tadpoles with undifferentiated gonads would behave similarly, whereas feeding rate may diverge between sexes in later stages as the gonads become differentiated). For experiment 2, the model of exploration rate included the total distance moved as a covariate, because we aimed to investigate the percentage of area used independently of locomotor activity. The three models of post-startle variables also included the total distance moved as a covariate, because post-startle activity may depend on pre-startle activity. Furthermore, these latter three models included stimulus type (i.e., whether the stimulus water contained predator cues or not) as a fixed factor, and its interaction with sex to test whether the effect of predator cues on behavior differed between sex categories.

In each model of development, growth, and behavior, we tested the effect of sex by pre-planned comparisons (Ruxton and Beauchamp, 2008). Specifically, we extracted three linear contrasts from each model: genetic males *versus* genetic females (the latter including sex-reversed individuals), phenotypic females *versus* phenotypic males (the latter including sex-reversed individuals), and sex-reversed *versus* concordant individuals (the latter including males and females). We provide these contrast estimates with 95% confidence intervals (CI) as non-standardized measures of effect size (Nakagawa, 2004; Nakagawa and Cuthill, 2007); we interpret CIs excluding zero (or one, in case of odds ratios and hazard ratios) as statistically significant. Further, to test whether the sexes differed in the trade-off between development and growth, we estimated the slope of relationship between the duration of larval development and body mass at metamorphosis for each sex category, and then applied the above three linear contrasts to the slopes. We used the same approach to compare the slope of change over time in feeding rate among the sexes. To test whether the sexes differed in the effect of predator cues on post-startle behaviors, for each sex category we estimated the difference in each behavioral variable between animals startled with *versus* without predator cues, and again we applied the above three linear contrasts to these predator-effect estimates.

All analyses were performed in the R computing environment v4.0.3 (R Core Team., 2020). Although our sample sizes are unbalanced due to the small number of sex-reversed individuals, we used mixed models throughout, which provide a flexible and powerful tool for appropriately analyzing unbalanced and heteroscedastic data (Pinheiro and Bates, 2000). For each analysis, we checked that the statistical requirements of the model were met by visually inspecting relevant graphs of residuals. Our data with a detailed, annotated R script are available as given in the Data Availability Statement.

RESULTS

Sex Ratios and Sex Reversal

In the total sample, there was significant female bias in genetic sex ratio (344/605 = 56.9% females, 95% CI = 0.53–0.61) but not in phenotypic sex ratio (305/574 = 53.1% females, 95% CI = 0.49–0.57). Out of the 571 individuals for which both genetic and phenotypic sex was identifiable, 21 were female-to-male sex-reversed (**Table 1**), 16 of which originated from the pond sampled for both experiments (9.8% sex-reversal rate), and 5 from the two ponds sampled only for experiment 1 (2.4 and 3.1% sex-reversal rate, respectively). The sex-reversed individuals came from 8 (out of 34) different families (5–75% of individuals sex-reversed per family; **Supplementary Table 1**), which does not conform to a homogeneous distribution of sex reversal among families ($\chi^2 = 233$, df = 33, $p < 0.001$). In 3 out of the 34 families we found no genetic males at all; two of these families exhibited 30 and 75% sex-reversal rate, respectively (**Supplementary Table 1**). The frequency of sex reversal among genetic females was independent of chemical treatments (6.4% overall; **Table 1**). Survival rate until dissection did not depend on genetic sex (18 females and 16 males died; hazard ratio: 0.84, 95% CI: 0.60–2.38).

Development and Growth

We found no significant difference between any combination of genetic and phenotypic sex in the length of larval development and body mass at metamorphosis or at dissection (**Table 2** and **Figure 2**). However, the relationship between the length of larval development and mass at metamorphosis varied significantly with sex (**Table 2** and **Figure 3A**): animals that metamorphosed later had higher body mass in both concordant

TABLE 2 | Differences in life-history and behavioral traits by genetic and/or phenotypic sex, shown as non-standardized effect sizes with 95% confidence intervals (CI).

Trait	Sex-reversed vs. concordant individuals[§]	Genetic females vs. genetic males[†]	Phenotypic females vs. phenotypic males[‡]
Duration of larval development (hazard ratio)	1.30 (0.75, 2.26)	1.07 (0.79, 1.47)	0.82 (0.61, 1.12)
Body mass at metamorphosis (mg)	7.24 (−16.40, 30.90)	9.16 (−5.09, 23.4)	1.92 (−12.00, 15.80)
Trade-off between larval development and growth (mg) *	**−7.79 (−14.4,−1.19)**	**−4.02 (−7.74,−0.30)**	**3.76 (0.15, 7.37)**
Juvenile body mass (g)	−0.05 (−0.11, 0.02)	−0.02 (−0.06, 0.02)	0.03 (−0.01, 0.07)
Frequency of feeding (odds ratio)	1.63 (0.71, 3.74)	1.31 (0.84, 2.04)	0.81 (0.52, 1.25)
Change of feeding rate with age (odds ratio) *	1.08 (0.98, 1.18)	1.05 (1.00, 1.10)	0.97 (0.93, 1.02)
Total distance moved (kilopixels)	**−5.73 (−11.10,−0.40)**	−2.90 (−7.86, 2.06)	2.83 (−1.66, 7.32)
Exploration rate (% of area visited)	−2.30 (−12.10, 7.47)	−4.43 (−12.3, 3.40)	−2.13 (−9.27, 5.01)
Time spent near wall (%)	−4.93 (−17.3, 7.47)	0.75 (−8.42, 9.92)	5.68 (−2.02, 13.4)
Intensity of startle response (cumulative odds ratio)	2.42 (0.61, 9.51)	1.02 (0.36, 2.90)	0.42 (0.17, 1.05)
Effect of predator cues on intensity of startle response (cumulative odds ratio) *	0.23 (0.01, 3.93)	0.97 (0.11, 8.41)	4.22 (0.66, 27.08)
Duration of escape (hazard ratio)	0.74 (0.36, 1.52)	1.15 (0.63, 2.11)	1.56 (0.90, 2.70)
Effect of predator cues on escape duration (hazard ratio) *	**0.25 (0.07, 0.89)**	0.66 (0.21, 2.12)	2.68 (0.97, 7.46)
Duration of freezing (hazard ratio)	1.69 (0.86, 3.29)	0.99 (0.54, 1.84)	0.59 (0.34, 1.03)
Effect of predator cues on freezing time (hazard ratio) *	0.91 (0.27, 3.05)	2.06 (0.66, 6.45)	2.26 (0.82, 6.22)

*Significant differences (i.e., CI excluding 0 or, in case of odds ratios and hazard ratios, 1) are marked with bold text. The comparisons marked with an asterisk are calculated from the effect sizes given in **Table 3**.*

[§] *Concordant individuals include XX females and XY males.*

[†] *Genetic females include sex-reversed individuals and concordant females.*

[‡] *Phenotypic males include sex-reversed individuals and concordant males.*

males and concordant females (**Table 3**), whereas in sex-reversed individuals the slope of this relationship did not differ significantly from zero (**Table 3**). Some sex-reversed individuals metamorphosed relatively early and with large mass (**Figure 3A**); most of them came from those families where we detected no genetic males at all (**Supplementary Figure 2**). Other sex-reversed individuals metamorphosed relatively late and with small mass (**Figure 3A**); most of them had testicular oogonia (**Supplementary Figure 2**).

Tadpole Behavior

In experiment 1, none of the 5 sex-reversed individuals were ever observed in the open; 0.37 and 0.39% of observations of concordant females and concordant males, respectively, were in the open. This difference was not significant (Fisher's exact test: $p = 0.900$). Also, feeding frequency did not vary significantly with sex (**Table 2**). The proportion of tadpoles feeding increased slightly as the tadpoles aged and this increase appeared greatest in sex-reversed individuals and smallest in concordant males (**Figure 3B**); however, the slopes had wide confidence intervals (**Table 3**) and did not differ significantly between any combination of genetic and phenotypic sex (**Table 2**).

In experiment 2, the total distance moved was significantly shorter in sex-reversed tadpoles than in concordant individuals (**Table 2** and **Figure 2**), but exploration rate and time spent near the wall did not vary significantly with sex (**Table 2** and **Figure 2**). Similarly, we found no significant differences in the intensity of startle response and the durations of escape and freezing between any combination of genetic and phenotypic sex (**Table 2**). However, the presence of predator cues modified these behaviors in sex-dependent ways (**Table 3**). Among concordant females, those that received predator cues responded less intensely to the disturbance than those that received clean water (**Table 3** and **Figure 4**); there was no such difference in concordant males or in sex-reversed individuals (**Table 3** and **Figure 4**). The duration of escape was shorter in sex-reversed individuals if they received predator cues than when they did not (**Table 3** and **Figure 5**); there was no such difference in concordant individuals (**Table 3** and **Figure 5**). The duration of freezing after the escape reaction was longer in concordant females in the presence of predator cues than in clean water (**Table 3** and **Figure 5**); the similar trends in concordant males and sex-reversed individuals were not statistically significant (**Table 3** and **Figure 5**). Out of all these sex differences in the effects of predator cues, only the one for escape duration was statistically significant (**Table 2**).

DISCUSSION

In this study, we confirmed that female-to-male sex reversal occurs in agile frogs at a relatively low frequency (6.4%) in the absence of thermal stress, and demonstrated that it was independent of chemical treatments representing ecologically relevant concentrations of carbamazepine, terbuthylazine, and chlorpyrifos. We are confident that these mismatches between genetic and phenotypic sex were indeed sex reversals, because our phenotypic sexing was backed up by histological analysis, and our genetic sexing method is based on two sex-linked markers that are located relatively far from each other on the sex chromosomes. The latter makes it highly unlikely that we would misdiagnose rare mutation or recombination events as sex reversal, which can

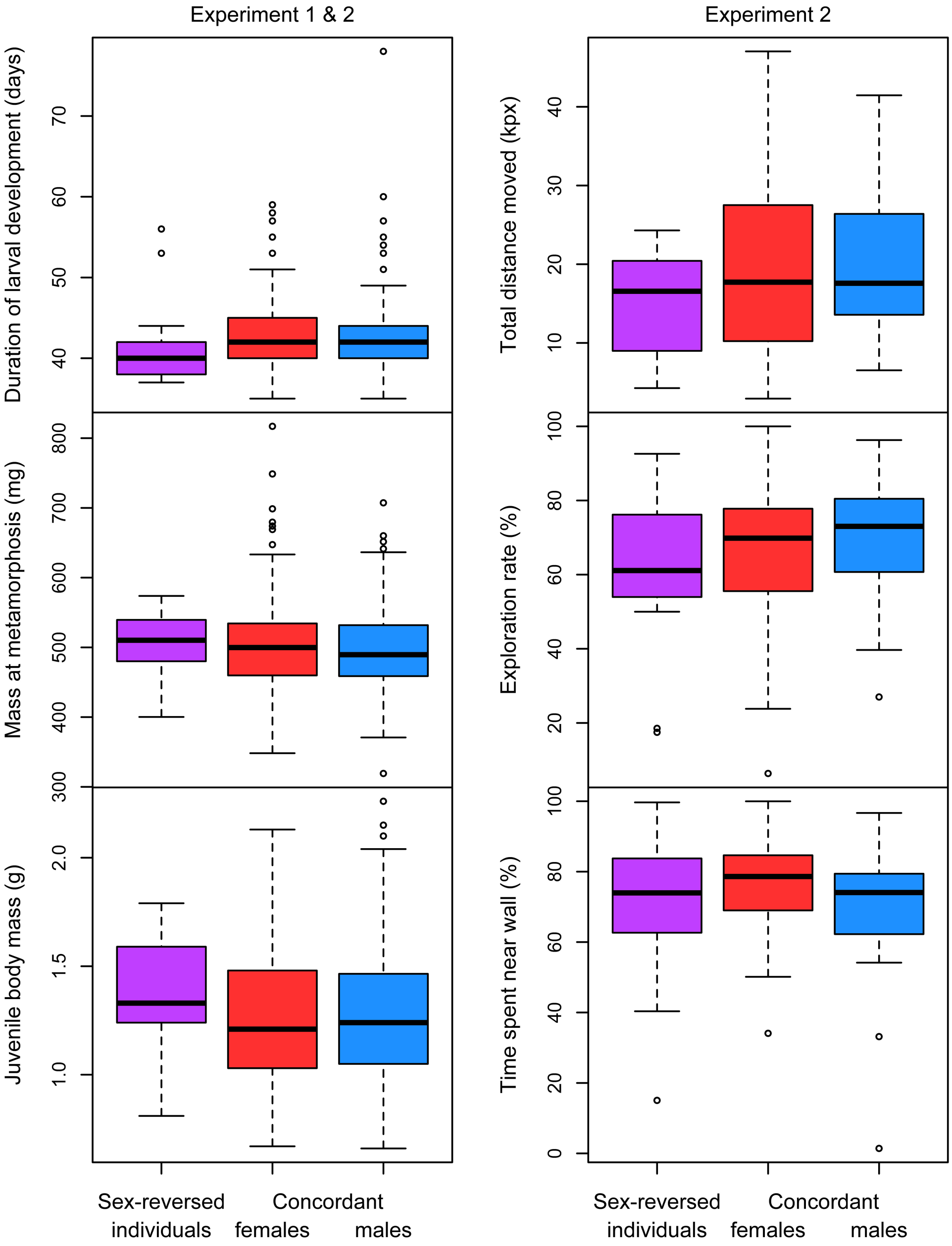

FIGURE 2 | Development, growth, and behavior before the addition of predator cues in sex-reversed individuals (XX males), concordant (XX) females and concordant (XY) males. In each box plot, the thick middle line and the box represent the median and interquartile range, respectively; whiskers extend to the most extreme data points within 1.5 × interquartile range from the box.

happen when only one marker is used (Toli et al., 2016). Because the animals were raised in the laboratory at benign temperatures with *ad libitum* food and no predators, and their sex development was not altered by the chemical treatments, it seems likely that these instances of sex reversal occurred independently of any obvious environmental stressor. Furthermore, the sex-reversed individuals in this study were similar to their sex-concordant siblings in almost all morphological, life-history and behavioral traits that we examined. Taken together, these results may be explained by two, not mutually exclusive ideas. First, the theory of random sex determination (Perrin, 2016) postulates that, in the lack of strong genetic and environmental effects on sex, developmental noise (i.e., random fluctuations in the expression of sex-determining genes) decides the sexual fate of individuals. Second, the threshold model of sex determination (Quinn et al., 2011; Nemesházi et al., 2021) assumes that phenotypic sex depends on whether the amount of "male signal" (i.e., expression of male-producing developmental signals, which can be influenced by both genotype and environment) exceeds the individual's threshold for male development, a trait encoded by

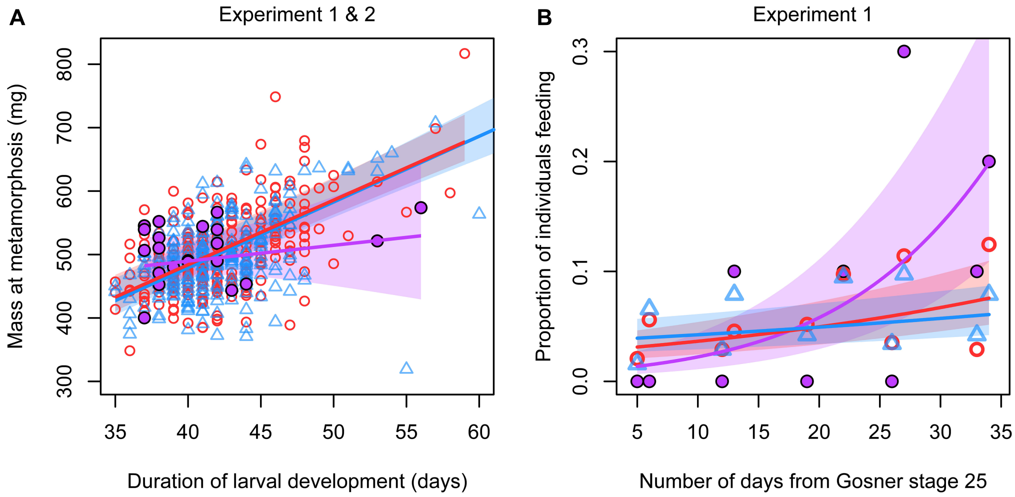

FIGURE 3 | Relationships between mass at metamorphosis and duration of larval development **(A)** and between feeding rate and tadpole age **(B)** in sex-reversed individuals (XX males; purple, filled circles), concordant females (XX females; red, open circles), and concordant males (XY males; blue, triangles). The lines and surrounding polygons, respectively, represent the slopes and their confidence intervals given in **Table 3**. To facilitate graph readability, panel A was cropped to exclude an outlier point (a concordant male with 373.4 mg mass at metamorphosis and 78 days of larval development; note that this individual was included in all analyses and also in the estimation of the curve fitted here).

TABLE 3 | Trade-off between larval development and growth, change of feeding rate with age, and effects of predator cues on behavior in the three sex categories, shown as non-standardized effect sizes with 95% confidence intervals (CI).

Effect	Sex-reversed individuals (XX males)	Concordant (XX) females	Concordant (XY) males
Slope of relationship between duration of larval development and mass at metamorphosis (mg)	2.49 (−4.06, 9.04)	**10.19 (8.74, 11.64)**	**10.36 (8.57, 12.16)**
Slope of relationship between feeding rate and age (odds ratio)	1.63 (0.71, 3.74)	1.31 (0.84, 2.04)	0.81 (0.52, 1.25)
Effect of predator cues on intensity of startle response (cumulative odds ratio)	0.47 (0.03, 6.53)	**3.24 (1.09, 9.68)**	1.26 (0.25, 6.37)
Effect of predator cues on escape duration (hazard ratio)	**0.31 (0.10, 0.93)**	1.51 (0.74, 3.08)	1.03 (0.37, 2.83)
Effect of predator cues on freezing time (hazard ratio)	2.22 (0.77, 6.39)	**4.07 (1.78, 9.29)**	1.46 (0.58, 3.62)

Significant effects (i.e., CI excluding 0 or, in case of odds ratios and hazard ratios, 1) are marked with bold text.

genetic elements. Thus, for individuals who happen to have genes encoding high "male signal" levels and/or low threshold levels, even a small elevation of environmentally induced "male signal" expression may result in female-to-male sex reversal. This theory is supported in the present study by the non-random distribution of sex reversals among agile frog families, and by the high rate of sex reversal in those families where we detected only genetic females and no genetic males at all. The latter fits the threshold theory because the agile frog has an XX/XY sex determination system, so families containing 100% female offspring suggest that the sire in those families had been a female-to-male sex-reversed individual (i.e., an XX male, mating with a concordant XX female) who may have passed on his alleles encoding high propensity for sex reversal to his offspring. Such a combination of genetic variation and random environmental noise might explain at least some occurrences of sex reversal in natural populations, especially where sex-reversal rate is not correlated with environmental factors such as the level of urbanization (Lambert et al., 2019), climate (Castelli et al., 2021), or elevation (Phillips et al., 2020).

The fact that the sex-reversed individuals in this study did not differ from concordant individuals in growth and development stands in stark contrast with our findings from another experiment on agile frogs (Mikó et al., 2021b), where sex reversal was induced by a six-days heat treatment during larval development. Heat treatment resulted in high rates of female-to-male sex reversal, but also reduced survival, development, growth, and fat reserves (Mikó et al., 2021b). Thus, in that experiment, sex reversal was strongly associated with signs of developmental stress and poor fitness prospects, similarly to what has been reported about various fishes (Senior et al., 2012; Baroiller and D'Cotta, 2016). Combining those findings with our current results, we speculate that the fitness of sex-reversed individuals may depend on the etiology of sex reversal. When it arises by stochastic variation in the biochemical processes of sex determination or in individual sensitivity to environmental effects on sex, it might not be systematically accompanied by changes in fitness-related traits. In contrast, when sex reversal is triggered by strong environmental effects and/or high physiological stress, it might be associated with

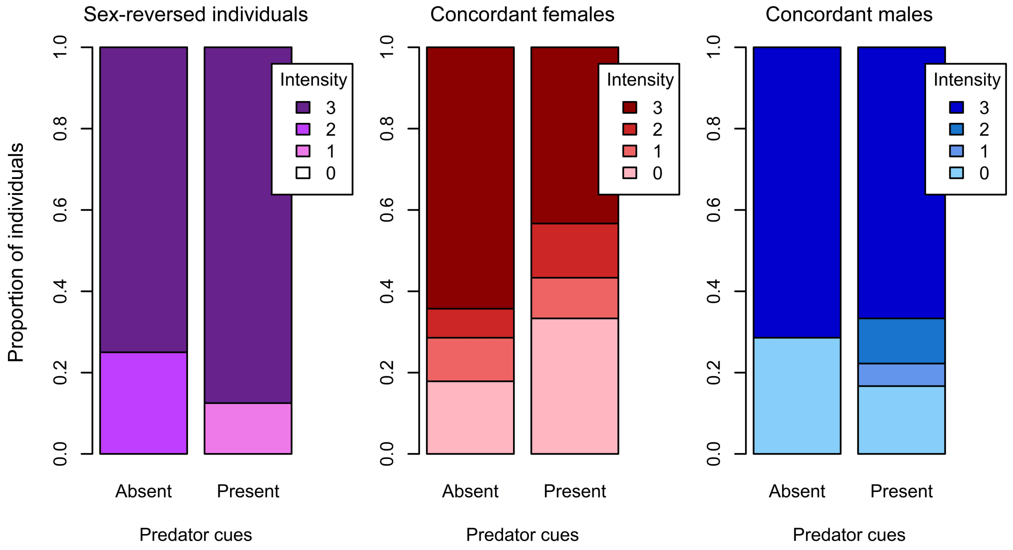

FIGURE 4 | Intensity of response to the startling stimulus with and without predator cues in sex-reversed individuals (XX males), concordant (XX) females, and concordant (XY) males in experiment 2 (0: no movement, 1: slight movement, 2: swimming away apparently calmly, 3: swimming around fervently).

poor health or reduced performance in life-history traits. This association may arise by the same stressor affecting both sex and fitness-related traits, perhaps mediated by stress-induced glucocorticoid hormone effects (Geffroy and Douhard, 2019) or cellular calcium–redox regulation (Castelli et al., 2020). For example, a meta-analysis concluded that the poor fitness of fish that underwent chemically induced sex reversal was not due to sex reversal *per se*, but was the result of the chemical treatments themselves (Senior et al., 2012). Additionally, the association between sex reversal and fitness might be exacerbated by sex reversal itself directly affecting some fitness-related traits (Mikó et al., 2021b) or by making the offspring of sex-reversed individuals more sensitive to environmentally induced sex reversal (Piferrer and Anastasiadi, 2021). If one sex can do better than the other under stressful conditions, environment-induced sex reversal may serve as an adaptive sex-allocation strategy (Geffroy and Douhard, 2019). However, in agile frogs that spawn in early spring and develop in cool waters, high temperatures during larval development might not have been frequent enough in their evolutionary past for such an adaptive strategy to evolve. Nevertheless, recent findings indicate that sex-reversed agile frogs occur more frequently in anthropogenic habitats (Nemesházi et al., 2020), and phenotypic sex ratios have become more male-biased in some amphibian species since the start of contemporary climate change (Bókony et al., 2017), suggesting that sex reversals might be shifting from mostly spontaneous or stochastic to increasingly stress-induced incidences. These speculations would deserve further empirical testing.

In the few instances where we found significant differences between sex-reversed and sex-concordant individuals in the present study, the former stood out by having lower locomotor activity and responding to disturbance with shorter escape duration when predator cues were present. Furthermore, the natural trade-off between larval development speed and growth rate seemed to be lacking in sex-reversed individuals. These findings support neither higher nor lower performance in terms of overall fitness for sex-reversed animals. First, while low activity may constrain foraging success, sex-reversed tadpoles were feeding at least as often as concordant individuals. Second, while short escape duration may lower the probability of being noticed by predators, it may be disadvantageous for escaping predators if they are already in pursuit. Third, although both early metamorphosis and large metamorphic mass are considered beneficial for amphibians in general (Smith, 1987; Berven, 1990; Altwegg and Reyer, 2003), sex-reversed individuals in our study tended to perform either well or poorly in both traits. Those sex-reversed individuals that did well in these traits tend to conform to the theory of heritable, random variation in the propensity for sex reversal, because most of them originated from two families with only XX genotypes (suggesting a sex-reversed sire; see above). Almost all sex-reversed individuals that did poorly in both development and growth appeared unsuccessful also in executing sex reversal completely, as they had oogonia in their testes. This supports the above idea that sex-reversed individuals may represent a heterogeneous group whose life history and health might depend on the etiology of sex reversal.

When comparing males and females (either genetically or phenotypically), we found no difference in development and growth, and only a few differences in behavior. Concordant females were the only group that reacted to predator cues by less intense startle response and longer freezing. This may indicate lower risk taking by females, which may agree with the behavior of adult agile frogs observed in nature, where females were reported to forage less in open areas than males (Cicort-Lucaciu et al., 2011). Phenotypic males, including sex-reversed individuals and concordant males, did not show the same responses to predator cues as females did, suggesting that

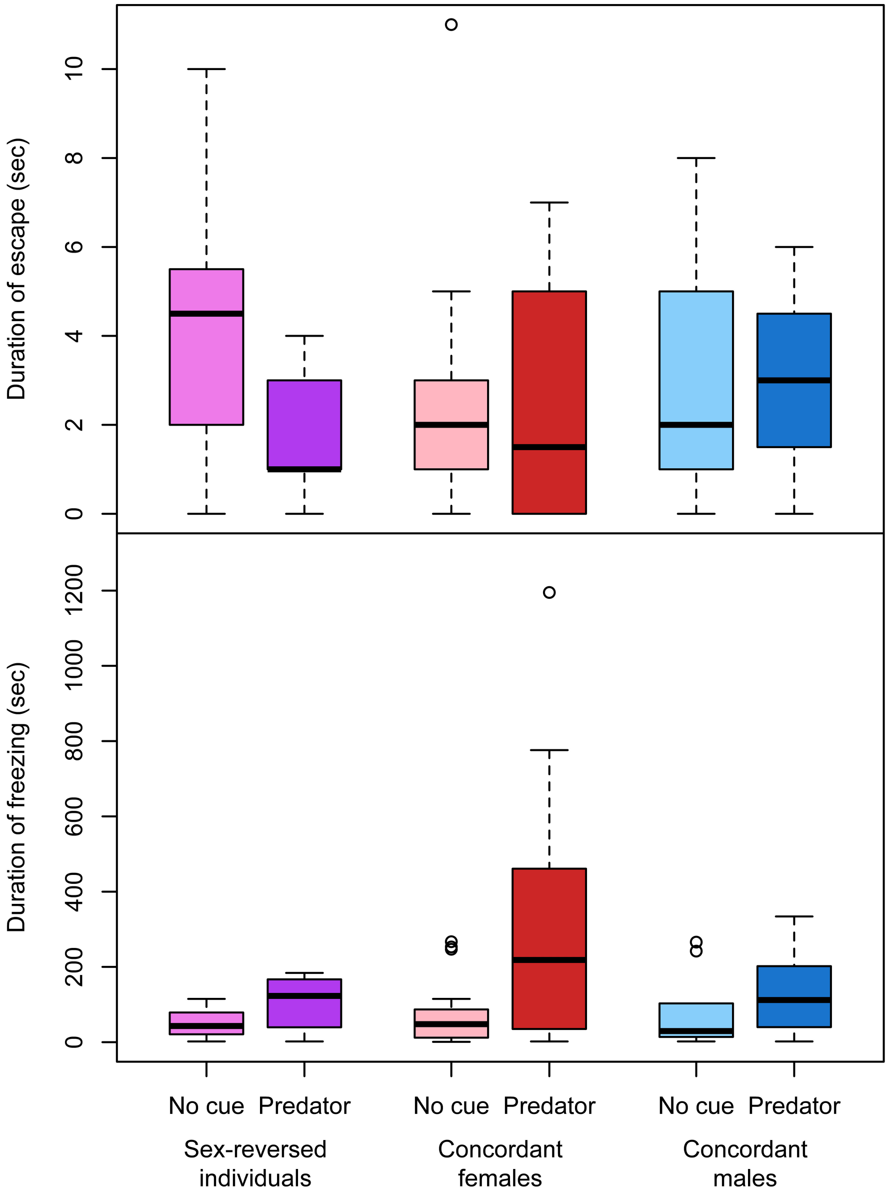

FIGURE 5 | Duration of escape and freezing after the startle stimulus with and without predator cues in sex-reversed individuals (XX males), concordant (XX) females, and concordant (XY) males in experiment 2, excluding those individuals that did not respond to the stimulus by moving. In each box plot, the thick middle line and the box represent the median and interquartile range, respectively; whiskers extend to the most extreme data points within 1.5× interquartile range from the box.

sex differences in these aspects of risk-taking behavior may not be genetically determined, but rather may develop after sex determination, e.g., by sex hormones. However, because none of the male-female differences in our study were statistically significant despite the relatively large sample for both sexes, we conclude that most of the divergent life histories and behaviors making up sex roles in agile frogs do not seem to arise in their larval life. In this species, males search and compete actively for females at high densities (Lodé et al., 2004), whereas at lower densities males maintain territories and females appear to choose males by their call characteristics (Lesbarrères et al., 2008), but both parents abandon the eggs after spawning. It would be very interesting to perform similar studies with species where either the male or the female parent takes the risky job of providing care to the offspring, as the developmental determinants of sex roles and therefore the effects of sex reversal may vary greatly between traditional and sex-role reversed systems. Amphibians and fish offer excellent model systems for such studies given their great diversity in mating and parental-care systems (Mank et al., 2005; Vági et al., 2019), but notably, sex reversal can also be experimentally induced in birds and mammals to study the development of sex roles (Adkins-Regan and Wade, 2001; Renfree et al., 2014).

Since sex reversal occurs relatively rarely under natural circumstances, most of our existing knowledge about ecologically relevant sex reversal comes from studies that include relatively small numbers of sex-reversed individuals in each population, year or treatment group (e.g., Li et al., 2016; Lambert et al., 2019; Jones et al., 2020; Nemesházi et al., 2020). The present study is no exception to this constraint. However, the fact that sex-reversed individuals do not make up a large proportion of current populations does not mean that they are merely a curiosity: they may be powerful catalyzers of evolutionary change (Holleley et al., 2016; Nemesházi et al., 2021). Therefore, we call out for many more empirical studies to solidify our understanding of the evolutionary-ecological significance of sex reversal, and to extend it from a few species to a broad spectrum of ectothermic vertebrates faced with the challenges of rapid human-induced environmental change.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study, along with an annotated R script, can be found in the Figshare repository (https://doi.org/10.6084/m9.figshare.14974050).

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Commission of the Plant Protection Institute, Centre for Agricultural Research, Hungary.

AUTHOR CONTRIBUTIONS

VB conceived the idea, conducted the statistical analyses, wrote the first draft, and supervised the project. VB, EN, NU, ZM, and AH collected the data. RE analyzed the video recordings. EN and NV isolated DNA and performed PCRs. ZG and OH performed HRM analyses, which were then evaluated by EN. VB, OH, and AH acquired funding. EN supervised all molecular work. All authors contributed to writing the article and approved the submitted version.

FUNDING

This study was funded by the National Research, Development and Innovation Office of Hungary (NKFIH, grants 115402 and 135016 to VB, 124375 to AH, and 124708 to OH). The authors were supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (to VB, AH, and OH), the ÚNKP-20-5 New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund ("Bolyai + Scholarship" to VB and AH), the Ministry of Human Capacities (National Program for Talent of Hungary, NTP-NFTÖ-17-B-0317 to EN), and by the Young Researcher program of the Hungarian Academy of Sciences (to NU). None of the funding sources had any influence on the study design, collection, analysis, and interpretation of data, writing of the manuscript, or decision to submit it for publication.

ACKNOWLEDGMENTS

We thank all members of the Lendület Evolutionary Ecology Research Group for insightful discussions, Márk Szederkényi, Viktória Verebélyi, Patrik Katona, László Sipõcz, and Mátyás Szin for help with animal handling and data archiving, Gergely Zachar for the fixative solution, and Gergely Tholt and the NÖVI Department of Zoology for allowing us to use their stereomicroscope and camera and for providing helpful advice. We are grateful to Renáta Pop and the Department of Pathology at the University of Veterinary Medicine Budapest for preparing the histological sections, and Kinga Lefler and Beata Rozenblut-Kościsty for help with interpreting histological images.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.745752/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 17 November 2021
doi: 10.3389/fevo.2021.768263

Sex-Specific Stress Tolerance in Embryos of Lake Char (*Salvelinus umbla*)

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *31 August 2021*
Accepted: *25 October 2021*
Published: *17 November 2021*

Citation:
Nusbaumer D, Garaud L, Ançay L and Wedekind C (2021) Sex-Specific Stress Tolerance in Embryos of Lake Char (Salvelinus umbla). Front. Ecol. Evol. 9:768263. doi: 10.3389/fevo.2021.768263

Salmonid fish have become important models in evolution and ecology, but possible effects of embryo or larval sex are mostly ignored, probably because morphological gonad formation starts only months after hatching and sexual maturation years later. However, recent gene expression studies and first observations in domestic strains suggest that sex-specific life histories could already start at an embryonic stage. Here we test this hypothesis in embryos and larvae of lake char (*Salvelinus umbla*). We sampled wild char and used their gametes to produce embryos of 40 different families. Embryos were raised singly in a stress or a non-stress environment until a late larval stage (stress was induced by allowing remainders of ovarian fluids to support microbial growth). Genetic markers were then used to sex the fish and reconstruct paternity ($N = 1{,}463$, including dead embryos). Primary sex ratio did not differ among families and was about 1:1. Female embryos hatched on average later and showed lower stress tolerance than male embryos. There were significant parental effects on offspring growth and mortality, but the sex differences in embryo performance were not family specific. We conclude that the sexes differ in their life history and susceptibilities to environmental stress already at embryonic stages. Environmental stress during incubation can therefore affect population sex ratio and hence population growth and genetics.

Keywords: fish, genetic sexing, embryo, hatching, yolk-sac larvae, environmental stress, ovarian fluid, salmonid

INTRODUCTION

Salmonid fish usually reach sexual maturity after their second year of life or later. At this stage, male and female differ significantly in behavior, life history, and morphology. There can also be sex differences in juveniles of migratory salmonids, with females being typically more inclined to become migratory than males (Klemetsen et al., 2003; Pearse et al., 2019). Apart from that, little is known about sex differences at embryo and juvenile stages, especially also of non-migratory salmonids. Population models typically assume that such differences do not exist or are of little relevance for demography and population growth (e.g., Nislow and Armstrong, 2012; Tsuboi et al., 2013; Louison and Stelzer, 2016). However, Morán et al. (2016) found variation in family sex ratios in crosses of three domestic brown trout (*Salmo trutta*) populations that they speculated could be due to sex-specific mortalities during embryogenesis. In natural populations of the same species, juvenile males were found to be more aggressive and having a lower perception of predation risks than juvenile females (Johnsson et al., 2001) and they sometimes also showed sex-specific habitat preferences (Giller and Greenberg, 2015). Bylemans et al. (in preparation) found that sex ratios in about half-year-old brown trout caught from a streamlet depended on whether the fish were hatchery or wild born. Any such early sex differences in behavior and life history can affect tolerance to various types of environmental stressors, including new ones that are linked to, for example, different types of pollution or climate change (Geffroy and Douhard, 2019). They therefore have the potential to affect adult sex ratio and hence population growth, genetics, and evolutionary potential.

Salmonid populations are often monitored over decades because of their ecological, cultural, and economic relevance. Some of these monitoring programs revealed changes in sex ratios due to, for example, sex-specific mortality during migration (Thompson et al., 2016). Others revealed significant changes in sex ratios that could statistically be linked to environmental factors such as changes in water temperatures (Geffroy and Wedekind, 2020). In the late 1980s, for example, mean temperatures changed abruptly in Europe and remained elevated since then (Reid et al., 2016). This sudden temperature change seemed to be responsible for a significant male bias in a wild and protected population of European grayling (*Thymallus thymallus*) (Wedekind et al., 2013). The distorted sex ratio has remained largely unchanged until today (Wedekind, unpublished observations). Interestingly, the distortion of the adult sex ratio was recorded among mostly 5-year old fish and started only 5 years after the change of the mean water temperatures (Wedekind et al., 2013). This observation, combined with the fact that grayling sex determination is genetic (Yano et al., 2013) and stable under various temperature conditions (Pompini et al., 2013) suggested that the changed environment affects sex-specific survival mainly during the first year of life, i.e., long before sex differences in behavior or life history are obvious in this species. A closer look at the timing of gonad development then revealed that the two sexes differ significantly during their first summer: sex differentiation starts early in genetic females and it is much delayed in genetic males who instead grow faster during that time (Maitre et al., 2017). Gene expression studies revealed that the sexes differ already at an embryonic stage (Maitre et al., 2017), long before morphological gonad development. Selmoni et al. (2019) therefore tested whether sex-specific gene expression during such early stages could be affected by a typical anthropogenic change of the environment, namely pollution by a synthetic estrogen at ecologically relevant concentration. They found that this form of pollution can indeed affect sex-specific gene expression at ontogenetic stages as early as the embryo and yolk-sac stages. Their findings suggest that the genetic sexes can differ in their response to environmental challenges from the earliest stages on, and that environmental conditions that affect embryo development can therefore have long-lasting effects on population sex ratios that may then potentially affect, for example, spawning behavior, population genetics, and population growth. Sex differences and their link to environmental conditions therefore need to be studied already at early developmental stages.

Here we focus on embryos and yolk-sac larvae of another salmonid, the lake char (*Salvelinus umbla*) from Lake Geneva (Switzerland). This population has been exposed to the typical challenges of lakes of the Swiss Plateau that include eutrophication (Feulner and Seehausen, 2019), pollution by toxic metals or organic matter deposition (Diez et al., 2017), and a significant increase in winter temperatures (Kelly et al., 2020) that not only affects embryo development rates but also sediment load and its effects on embryos (Mari et al., 2016). We test whether the genetic sexes differ in mortality, embryo development, timing of hatching, and larval growth in a stress and a non-stress environment. Previous experiments on other salmonids established that exposure of embryos to organic nutrients supports growth of symbiotic microbial communities (Wedekind et al., 2010) and affects their compositions and functional pathways (Wilkins et al., 2016). Such nutrient-induced changes then leads to net increases in total virulence (Jacob et al., 2010; Wedekind et al., 2010; Wilkins et al., 2016, 2017), either because of additive effects linked to increased microbial density or changed microbial compositions, or because of density-dependent transitions of microbial life histories (Diggle et al., 2007). Because ovarian fluids are rich of carbohydrates, amino acids, and other organic and inorganic components that support for the growth of microbial communities (Lahnsteiner et al., 1995), we induced such environmental stress by varying the level of remainders of ovarian fluids on freshly fertilized eggs.

MATERIALS AND METHODS

Sampling of Breeders and Production Families

Ten adult males and 4 females were caught during their spawning season in Lake Geneva. Their gametes were stripped and used in a study on male and female reproductive strategies (Nusbaumer et al., 2021b). Briefly, the eggs and ovarian fluids of a female were stripped into a container from which ovarian fluids (as much as possible) were extracted with a syringe before washing the eggs twice with Ovafish® (IMV Technologies, l'Aigle, France; the content of the container was each topped up to 200 mL before discarding the Ovafish). Twenty batches of 24 eggs per female (80 batches in total) were then separated in wells of 6-well plates (Falcon, BD Biosciences, Allschwil, Switzerland). The ovarian fluids of each female were diluted with standardized water to a ratio of 1:2 (ovarian fluid to water). The milt of each male was diluted in the inactivating medium Storfish (IMV Technologies, France) to 50 million active sperm per mL. The males were assigned to 5 haphazardly chosen pairs of competitors, and the milt of each pair of competitors was mixed equally. Each batch of 24 eggs was then exposed to 1 mL of such a mix of milt that had been then activated in either 4 mL of standardized water or in 4 mL of diluted ovarian fluids (of the female the eggs were from). After 2 min (when fertilization can be expected to have happened), 16.8 mL of standardized water was added to each well and the eggs were left undisturbed for 2 h to allow for hardening before immediate transport to the laboratory.

This experimental design of these sperm competition trials was full-factorial and fully balanced and finally led to 40 different full-sib families (each female crossed with each male) after paternity had been determined with genetic markers (see below). However, 10 of the 80 batches (all of the same female, prepared to be exposed to sperm activated in water only) were lost due to a handling accident, leaving 1,680 eggs that could be monitored.

Monitoring of Embryos and Larvae

After fertilization, the freshly fertilized eggs were transported to the laboratory to be incubated for later parental analyses (Nusbaumer et al., 2021b). Briefly, eggs were rinsed for 30 s under cold running tap water (4 L/min) in a sterilized tea strainer before being distributed singly in 24 well plates (Falcon, BD Biosciences, Allschwil, Switzerland) filled with 1.8 mL of autoclaved standardized water (OECD, 1992), and incubated at 4.5°C in a 12 h:12 h light-dark cycle (to simplify monitoring and minimize variation of light conditions) until 14 days post-hatching as in von Siebenthal et al. (2009). Under these environmental conditions, embryo mortality until hatching is typically $< 3\%$ in similar-sized eggs of brown trout (Nusbaumer et al., 2021c). We considered a short rinsing of the eggs necessary because we had previously observed that not rinsing eggs before incubation in 24-well plates can lead to very high embryo mortalities (Wedekind, unpublished observations).

Two weeks after fertilization, fertilization success was estimated by the presence of absence of a neurula in the egg, and embryo mortality was monitored from then on. At the day of hatching, yolk-sac larvae were transferred to 12-well plates filled with 5 mL autoclaved standardized water and larval mortality and malformations (**Supplementary Figure 1**) were recorded until 14 days after hatching. Dead embryos and dead larvae were transferred to 2 mL microtubes filled with 1 mL 96% ethanol, and stored at −20°C for further analysis.

On the day of hatching and 14 days later, larvae were photographed in a custom-made photo box with a digital camera from below the 12-well plate (Canon 70D, 50 mm, f/3.2, 1/400 s, ISO 200, WB 6000 K, RAW format 24 Mpx). After the second photo was taken, larvae were euthanized with a spike of 100 μL of Koi Med® Sleep 4.85% (Koi & Bonsai Zimmermann, Germany), transferred to 2 mL microtubes filled with 1 mL 96% ethanol, and stored at −20°C for further analysis. Photos were analyzed in ImageJ (Schneider et al., 2012). At each stage (hatching day, 14 days post-hatching), the standard body length was measured (from snout to the base of the caudal fin), and the minor and major axis of the yolk sac were measured to infer yolk sac volume as in Jensen et al. (2008).

Paternity Analyses and Genetic Sexing

In total 1,475 embryos and larvae of known maternal origin were genotyped in one or two multiplexes that each contained 3 polymorphic microsatellite markers (Savary et al., 2017). The second multiplex was only used if the first one was not sufficient yet for identifying the paternal origin. The detailed protocol is described in Nusbaumer et al. (2021b). Briefly, DNA was extracted from unhatched embryos, larvae and parents. Genotypes were read using Genemapper v4.0 (Applied Biosystems, United States), and paternity was assigned using CERVUS v3.0 (Kalinowski et al., 2007). Peak heights in adult males ($n = 10$) that were genotyped twice ranged from 1,513 to 3,743 in replicated PCRs and showed high repeatability ($r^2 = 0.95$).

The assignment to fathers was used in Nusbaumer et al. (2021b) to evaluate the competitiveness of sperm. In the present study, the family assignments are used to test for the maternal and paternal influence on the performance of male and female offspring. An *sdY* sex marker (226 bp; primers: 5′-CCCAGCACTGTTTTCTTGTCTCA-3′ and 5′-CTTAAAACCACTCCACCCTCCAT-3′) (Yano et al., 2013) was therefore added to the first multiplex to identify the genetic sex of each embryo or larva. Successful amplification of the *sdY* marker as observed in Genemapper was interpreted as male, while no amplification of the *sdY* marker was interpreted as female if the three other loci of the multiplex were successfully amplified. In total 1,463 offspring could be assigned to both parents and genetically sexed (amplification of the first multiplex that included the *sdY* marker failed for one offspring that could nevertheless be assigned to a father with the second multiplex).

Statistical Analyses

All analyses were done in RStudio (R Development Core Team, 2015) and JMP 15.2.1. General (GLMM) and linear (LMM) mixed-effects models were run in the package *lme4* (Bates et al., 2015). Categorical traits such as sex, mortality until hatching, and malformation were analyzed in GLMM fitted with a binomial error distribution. Continuous variables were analyzed in LMM fitted with maximum likelihood. For all models, the model assumptions were visually checked in Q-Q plots and residuals plots. Sex and experimental treatment (presence or absence of ovarian fluids during fertilization) were entered as fixed effects, while sire and dam identities were random effects. In models on embryo growth, length at hatching was added as fixed effect to account for variability in initial length. The significance of model terms was evaluated in likelihood ratio tests. Terms of interest were dropped or added and the new model was compared to the reference model using likelihood ratio tests. Adding terms of interests allowed testing for possible effects of two-way interactions, while higher level interactions were not tested. Larval mortality was not further analyzed because only 13 larvae died during the observational period ($<1\%$).

RESULTS

Sex Ratios

The 1,463 embryos that could be successfully assigned to the 40 experimental families were represented by 36.6 ± 15.8 (± SD, range 6–71) offspring per full-sib family. The overall sex ratio was 49.96% males, did not differ among paternal or maternal sib groups and was not affected by the presence or absence of ovarian fluids during fertilization (**Table 1** and **Figure 1**). There were also no significant effects of any possible interactions between parents and treatment on sex ratio (**Table 1**).

Sex Differences in Embryo Performance

Offspring sex played a role in embryo development in the two experimental groups. There was a non-significant tendency for males to reach higher hatching rates than females (i.e., males tend to have lower embryo mortality; **Table 2A**). In total 38 embryos

TABLE 1 | Likelihood ratio tests on generalized mixed-effects models testing for parental and treatment effects (fertilization based on sperm competition with or without ovarian fluids) on primary sex ratios.

Model terms	Effect tested	AIC	df	χ^2	*P*
of + d + s		2034.6	4		
d + s	of	2032.6	3	< 0.1	0.92
of + s	d	2034.1	3	1.5	0.22
of + d	s	2032.6	3	0	1
of + d + s + of × d	of × d	2040.5	7	0.2	0.98
of + d + s + of × s	of × s	2040.3	7	0.3	0.96
of + d + s + d × s	d × s	2036.6	5	0	1

The table gives the degrees of freedom (df) and the Akaike information criterion (AIC) that provide estimates of the quality of models relative to each other. The significance of model terms was tested by comparing reduced or extended models to the respective reference model (bold).
Fixed effect: of, ovarian fluid. Random effects: d, dam; s, sire.

were classified as malformed (2.6%) of which 34 hatched. These malformed fish hatched on average earlier than non-malformed ones ($t = -2.0$, $p = 0.046$). Females suffered from higher malformation rates but hatched on average later than males (**Tables 2B,C** and **Figures 2B,C**). Despite the later hatching, freshly hatched females did not differ from males in body length, yolk sac volume or growth during than first 14 days after hatching (**Tables 2D–F** and **Figures 2D–F**).

The experimental treatment during gamete fusion produced significant effects on offspring performance: Embryos that resulted from fertilization in diluted ovarian fluids suffered from lower hatching rates, higher malformation rates, hatched at smaller size, and then grew slower than larvae that resulted from fertilization in water only (**Table 2** and **Figure 2**). The two sexes reacted differently to this treatment: While hatching rates appeared unaffected in males, they significantly declined in females that resulted from gamete fusion in the ovarian fluids treatment (sex × treatment interaction in **Table 2A** and **Figure 2A**). However, the sexes' reactions to the treatment did not produce any significant effects on embryo growth and development (**Tables 2B–F** and **Figure 2**).

Parental Effects on Embryo Performance

There were significant parental effects on all measures of embryo performance (see significant main effects of sire and dam in **Table 2**, and the significant sire × dam interaction in **Table 2A**). Sires differed in embryo survival, the timing of hatching, larval size at hatching, and larval growth after hatching (**Table 2**), while dams differed significantly in offspring malformation rates, timing of hatching, larval size and yolk sac volume at hatching, and larval growth after hatching (**Table 2**). Embryo mortality was also dependent on the combination of sire and dam (see dam × sire interaction in **Table 2A**).

There were significant parental effects on the strength of the treatment effects, i.e., significant dam × treatment interactions on embryo mortality, malformation rates, and larval growth after hatching (**Tables 2A,B,F**), and a significant sire × treatment

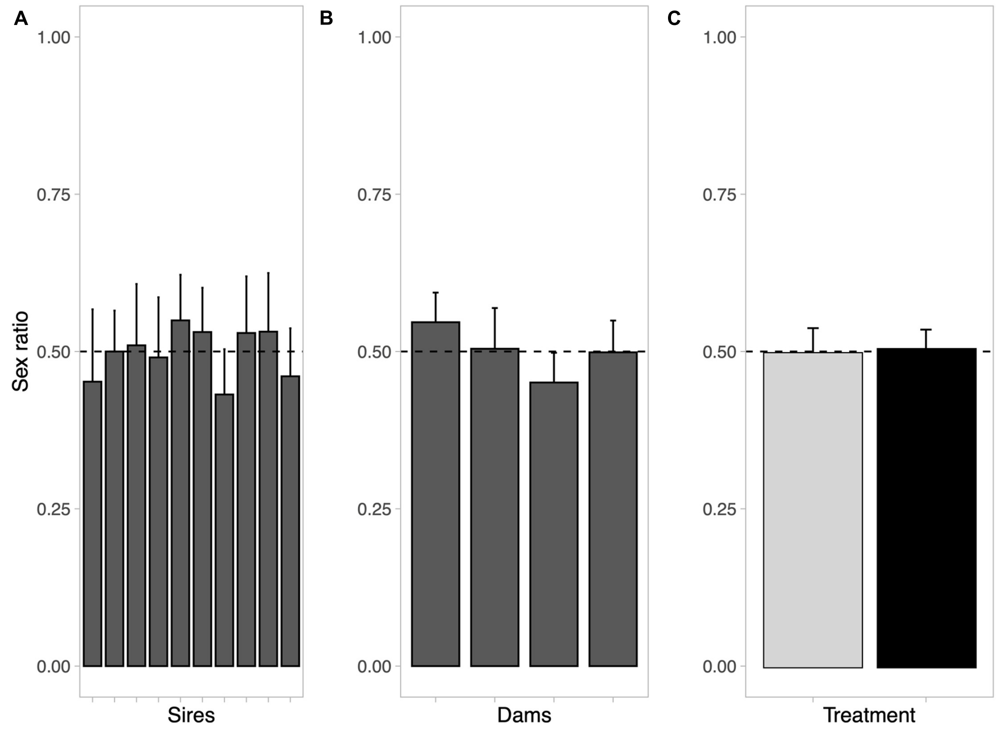

FIGURE 1 | Average sex ratios of **(A)** all paternal sib groups, **(B)** all maternal sib groups, and **(C)** of embryos fertilized with water only (gray bar) or with diluted ovarian fluid (black bar). Bars indicate means per full-sib family and treatment, with 95% confidence intervals. Sex ratio range from all females (0) to all males (1). The horizontal dashed lines indicate even sex ratio.

TABLE 2 | Likelihood ratio tests on generalized mixed-effects models on (A) embryo hatching rate and (B) malformation rate, and likelihood ratio tests on linear mixed-effects models on (C) hatching time, (D) larval length at hatching, (E) yolk sac volume at hatching, and (F) increase in larval length during the first 14 days after hatching.

Model terms	**Effect tested**	**AIC**	**df**	χ^2	***P***
(A) Hatching rate					
Sex + d + s + of		734	5		
d + s + of	Sex	735.6	4	3.6	0.06
Sex + s + of	d	735.5	4	3.4	0.06
Sex + d + of	s	758.4	4	26.4	**<0.001**
Sex + d + s	of	748.7	4	16.7	**<0.001**
Sex + d + s + of + sex × d	Sex × d	733.8	7	4.2	0.12
Sex + d + s + of + sex × s	Sex × s	737.7	7	0.3	0.85
Sex + d + s + of + sex × of	Sex × of	719.5	6	16.5	**<0.001**
Sex + d + s + of + d × s	d × s	718.7	6	17.3	**<0.001**
Sex + d + s + of + d × of	d × of	729.2	7	8.9	**0.01**
Sex + d + s + of + s × of	s × of	734.6	7	3.4	0.18
(B) Malformation rate					
Sex + d + s + of		326.1	5		
d + s + of	Sex	329.9	4	5.8	**0.02**
Sex + s + of	d	334	4	12.9	**<0.001**
Sex + d + of	s	324.1	4	0.1	0.81
Sex + d + s	of	341.2	4	17.1	**<0.001**
Sex + d + s + of + sex × d	Sex × d	328.9	7	1.2	0.55
Sex + d + s + of + sex × s	Sex × s	325	7	5	0.08
Sex + d + s + of + sex × of	Sex × of	327.9	6	0.1	0.73
Sex + d + s + of + d × s	d × s	328.1	6	0	1
Sex + d + s + of + d × of	d × of	322.1	7	8	**0.02**
Sex + d + s + of + s × of	s × of	329.8	7	0.3	0.88
(C) Hatching time					
Sex + d + s + of		4983.6	6		
d + s + of	Sex	4987.1	5	5.5	**0.02**
Sex + s + of	d	5208.2	5	226.6	**<0.001**
Sex + d + of	s	5080	5	98.4	**<0.001**
Sex + d + s	of	4981.6	5	< 0.1	0.94
Sex + d + s + of + sex × d	Sex × d	4987.6	8	< 0.1	0.99
Sex + d + s + of + sex × s	Sex × s	4987.6	8	< 0.1	0.98
Sex + d + s + of + sex × of	Sex × of	4985.1	7	0.54	0.46
Sex + d + s + of + d × s	d × s	4985.3	7	0.3	0.58
Sex + d + s + of + d × of	d × of	4987.5	8	0.1	0.96
Sex + d + s + of + s × of	s × of	4963.4	8	24.2	**<0.001**
(D) Length at hatching					
Sex + d + s + of		1430.3	6		
d + s + of	Sex	1428.3	5	0.1	0.82
Sex + s + of	d	1650.8	5	222.5	**<0.001**
Sex + d + of	s	1450.5	5	22.3	**<0.001**
Sex + d + s	of	1448	5	19.7	**<0.001**
Sex + d + s + of + sex × d	Sex × d	1433.2	8	1	0.6
Sex + d + s + of + sex × s	Sex × s	1433	8	1.3	0.53
Sex + d + s + of + sex × of	Sex × of	1430.8	7	1.4	0.23
Sex + d + s + of + d × s	d × s	1430.2	7	2.1	0.15
Sex + d + s + of + d × of	d × of	1433.4	8	0.5	0.78
Sex + d + s + of + s × of	s × of	1430.8	8	3.5	0.18
(E) Yolk sac volume					
Sex + d + s + of		8373.5	6		
d + s + of	Sex	8371.5	5	< 0.1	0.92

(Continued)

TABLE 2 | (Continued)

Model terms	Effect tested	AIC	df	χ^2	*P*
Sex + s + of	d	8678.1	5	306.6	**<0.001**
Sex + d + of	s	8371.5	5	0	1
Sex + d + s	of	8371.5	5	< 0.1	0.84
Sex + d + s + of + sex × d	Sex × d	8377	8	0.4	0.8
Sex + d + s + of + sex × s	Sex × s	8377.5	8	0	1
Sex + d + s + of + sex × of	Sex × of	8375.3	7	0.2	0.66
Sex + d + s + of + d × s	d × s	8375.5	7	0	1
Sex + d + s + of + d × of	d × of	8376.1	8	1.3	0.51
Sex + d + s + of + s × of	s × of	8377.5	8	0	1
(F) Growth					
Sex + d + s + of + l		1230.3	7		
d + s + of + l	Sex	1230.7	6	2.4	0.12
Sex + s + of + l	d	1402.6	6	174.3	**<0.001**
Sex + d + of + l	s	1240.8	6	12.5	**<0.001**
Sex + d + s + l	of	1239.3	6	11	**<0.001**
Sex + d + s + of	l	1403.5	6	175.1	**<0.001**
Sex + d + s + of + l + sex × d	Sex × d	1234.8	9	< 0.1	1
Sex + d + s + of + l + sex × s	Sex × s	1234.3	9	< 0.1	1
Sex + d + s + of + l + sex × of	Sex × of	1232.2	8	< 0.1	0.77
Sex + d + s + of + l + d × s	d × s	1232.3	8	0	1
Sex + d + s + of + l + d × of	d × of	1224.4	9	9.9	**0.007**
Sex + d + s + of + l + s × of	s × of	1233.2	9	1.1	0.59

The AIC gives the Akaike information criterion. Models were compared to the respective reference model by removing or adding the term of interest. Significant p-values are highlighted in bold.

Fixed effects: of, ovarian fluid; sex, sex; l, length at hatching. Random effects: d, dam; s, sire.

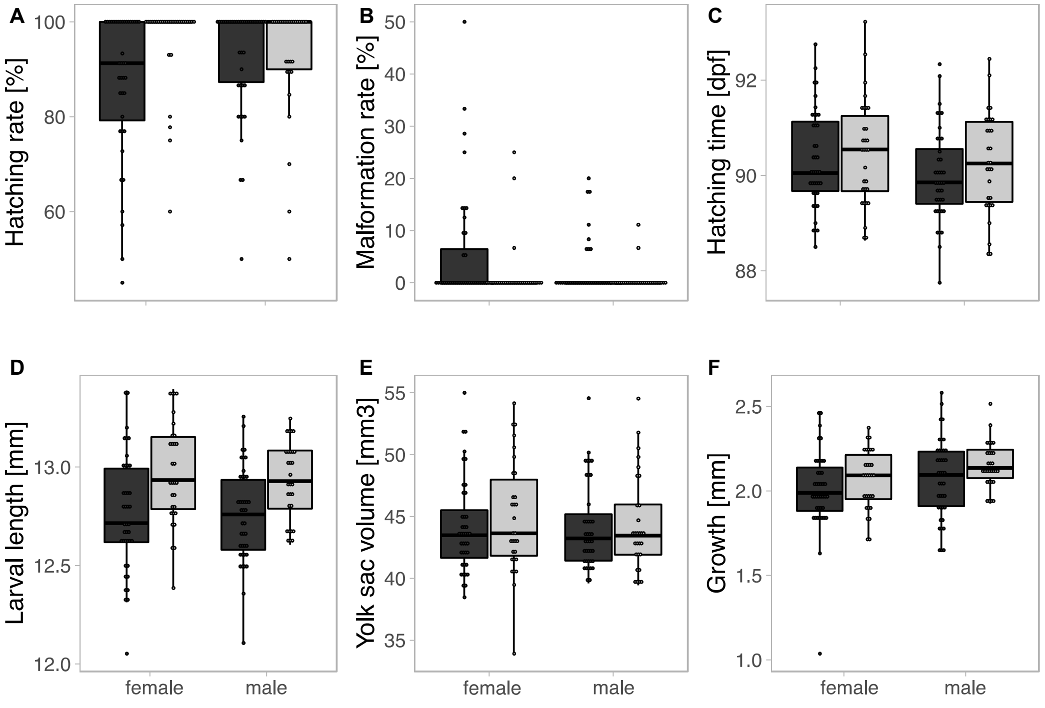

FIGURE 2 | Effects of genetic sex and environment on **(A)** hatching rate (as measure of embryo survival), **(B)** rate of malformations among hatchlings, **(C)** the timing of hatching (dpf = days post-fertilization), **(D)** larval length at hatching, **(E)** yolk sac volume at hatching, and **(F)** increase of larval length during the first 14 days after hatching. Tukey outlier boxplots with quartiles, whiskers, and jittered family means for female and male embryos. Black and gray symbols represent stressful and non-stressful environments, respectively. See **Table 2** for statistics.

interaction on the timing of hatching (**Table C**). However, effects of offspring sex were never significantly influenced by parental origin (see non-significant sex × dam and sex × sire interactions in **Table 2**).

DISCUSSION

The primary sex ratio of fish with genetic sex determination can be expected to be around 1:1, i.e., 50% males in all families (Fisher, 1930). This is what we observed in our study population when sexing all offspring, including those that had died before hatching. With on average 36.6 sexed offspring per family and 40 full-sib families in total, we had much statistical power to detect potential variation in family sex ratios. We therefore conclude that the primary sex ratio is not different to 1:1 in this population of lake char. There were, however, increased rates of malformations in female larvae. We also found that the sexes differed in their susceptibility to environmental stress, i.e., that sex-specific embryo mortality depended on the rearing environment. Therefore, sex-specific embryo mortality needs to be controlled for when testing for variation in primary family sex ratios.

We found that female larvae hatched on average later than male larvae (even if they had higher rates of malformed fish that hatched earlier than non-malformed fish). Despite this difference in hatching date, both sexes hatched at similar body sizes and similar yolk sac volumes. We therefore conclude that male embryos developed faster than female embryos. After hatching, male larvae also tended to grow faster than females, but this difference was not significant.

The genetic differences between the sexes are small in salmonids and seem to be restricted to the *sdY* master sex determining gene that Guiguen et al. (2019b) described as "*. . . the first example of a totally de novo evolved gene functionally capable of hijacking the leadership of the gonadal gene regulatory network*" (p. 47). This novelty may at least partly explain why, in salmonids, the Y chromosomes that bear the *sdY* gene have not significantly degenerated as would generally be expected from Y chromosomes (Bachtrog, 2013). In rainbow trout (*Oncorhynchus mykiss*), the *sdY* gene has been found to be expressed already around hatching, i.e., long before the initiation of histological sex differentiation (Guiguen et al., 2019a). Our results suggest that sex-specific gene expression happens even earlier in lake char, as it does in grayling (Maitre et al., 2017).

Females vary in the amount and viscosity of ovarian fluids (Lahnsteiner et al., 1995; Turner and Montgomerie, 2002), and during natural spawning, eggs are swirled around to various degrees depending, for example, on male and female movements during the spawning. Gamete fusion is therefore expected to happen in various concentrations of ovarian fluids. This is what we simulated in our experimental treatment that revealed significant effects of ovarian fluids on sperm velocity and the outcome of sperm competition (Nusbaumer et al., 2021b). Here we concentrate on what happens after gamete fusion when eggs may be contaminated to various degrees by viscous and somewhat sticky residues of ovarian fluids.

Ovarian fluids of char and other salmonids contain high levels of carbohydrates including glucose and fructose, proteins and free amino acids, lactate, phospholipids, cholesterol, and various inorganic components (Lahnsteiner et al., 1995). Residues of ovarian fluids are therefore expected to support the growth of symbiotic microbial communities on freshly fertilized eggs, even after much of the ovarian fluids had been washed off the eggs before incubation (in order to avoid the very high embryo mortalities that could be expected when not rinsing eggs before incubation; Wedekind, unpublished observations). We found that embryos that resulted from fertilization trials with ovarian fluids suffered indeed from increased mortalities and malformation rates and showed reduced growth at the embryo and larval stages than eggs of which ovarian fluids had been washed of before fertilization. Analogous detrimental effects have previously been observed when nutrient broth was experimentally added to eggs of the whitefish *Coregonus suitieri* (Wedekind et al., 2010) or to eggs of brown trout (Jacob et al., 2010; Wilkins et al., 2016, 2017). Organic pollution, therefore, creates a stressful environment to salmonid embryos, and contamination by residues of ovarian fluids is expected to be a stressor under hatchery and laboratory conditions. For this reason, salmonids eggs are often separated from ovarian fluids in aquaculture production. It remains to be tested whether contamination by residues of ovarian fluids can also be a stressor of ecological relevance at the natural spawning ground.

We found significant parental effects on all offspring characteristics that we determined. We also found that parental effects affected the tolerance to the environmental stress that has been inflicted by the experimental treatment. Many of these effects were very strong, as expected from estimates of parental effects in other salmonids (see below). However, despite all these effects of parental origin on embryo performance, and despite the high number of replicates that we used here and that provided much statistical power, there seemed to be no parental effect on sex-specific embryo performance. The families did not differ in sex ratios and no family effects on sex-specific growth and life history were detected.

Parental effects on embryo and larval performance have been repeatedly analyzed in other salmonids, usually based on full-factorial breeding experiments with sufficient replication on the maternal and the paternal sides and large numbers of embryos that were often singly reared as in the present study (recent examples include Clark et al., 2016; Forest et al., 2016; Marques da Cunha et al., 2019; Nusbaumer et al., 2021c). Such experiments allow separating and quantifying the variance components and hence testing, for example, for the evolutionary potential of natural populations to adapt to changing environments (Lynch and Walsh, 1998). Sire effects on offspring traits then directly reveal additive genetic effects, while dam effects are a mixture of maternal environmental and genetic effects, and dam × sire interaction effects provide a good estimate of non-additive genetic effects. Our experimental treatment led to 40 different full-sib families, i.e., all families that are possible with gametes from 10 males and 4 females, and we also raised large numbers of embryos singly in 24-well plates. However, our breeding design is different from the full-factorial breeding that was used in previous experiments on salmonid fish, because the families resulted from sperm competition experiments. Sperm competitiveness can vary within the milt of a male (Sutter and Immler, 2020) and has been shown to affect offspring traits (Immler et al., 2014). We can therefore not exclude that sperm competitiveness is linked to genetic quality. If so, our experiment would not provide reliable estimates of the variance components that would result from non-competitive breeding experiments. Instead, it could provide a first estimate of variance components affected by sperm competition. In future studies, such estimates could be compared to estimates that result from non-competitive breeding to discuss the effects of sperm competition on population genetics.

CONCLUSION

In conclusion, we found that male and female lake char embryos differ significantly in the timing of hatching and in their tolerance to an ecologically relevant stress. These sex-specific differences happen long before histological sex differentiation starts, and years before the sexes assume their different role at the spawning place. Sex-specific susceptibilities to environmental challenges can affect growth and survival and hence adult population sex ratios. They can therefore be a reason for sex-ratio biases that may not only affect population growth (e.g., if population growth is limited by the number of females) but also population genetics by reducing the genetically effective population size and hence increasing average inbreeding coefficients. Our findings demonstrate that sex-specific susceptibilities to environmental factors can already be relevant as early as during egg incubation on the spawning ground.

DATA AVAILABILITY STATEMENT

Data can be downloaded from the Dryad depository (Nusbaumer et al., 2021a).

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because data were taken from embryos and larvae before the onset of exogenous feeding.

AUTHOR CONTRIBUTIONS

DN and CW designed the study. LG and DN organized and conducted fieldwork. DN performed the *in vitro* fertilization, reared, and monitored embryos and larvae, did the genotyping, and analyzed the data. LG and LA measured the larvae. CW wrote a first draft of the manuscript that was revised by all authors.

FUNDING

Funding was provided by the Swiss National Science Foundation (31003A_159579 and 31003A_182265).

ACKNOWLEDGMENTS

We thank G. Levray from the Pistolet fishery for catching the fish, L. Adhia Eya, A. Atherton, C. Berney, C. de Guttry, F. Dolivo, L. Espinat, S. Kreuter, E. Longange, L. Marques da Cunha, N. Sironi and for assistance in the field or in the lab, F. Schütz for discussion on statistics, and E. Lasne for discussion and access to the INRA facilities.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.768263/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 07 January 2022
doi: 10.3389/fevo.2021.799996

Genetic and Social Transmission of Parental Sex Roles in Zebra Finch Families

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Parental care plays a central, reinforcing role in the evolution of sex roles and its development is often reported to be driven by genetic, rather than environmental effects. Based on these studies, however, genetic inheritance does not account fully for the often-significant phenotypic variability observed within species, a variation that we hypothesized may be explained by social effects from parents. Following a full cross-fostering design, here we aimed at disentangling genetic and social parental effects in the ontogeny of parental behaviours. Clutches of eggs were swapped, and we monitored parental behaviours in two consecutive generations of a captive population of the socially monogamous, biparental zebra finch (*Taeniopygia guttata*). Using nest box cameras, parental behaviour was recorded for 3 h in two reproductive stages: on day 8 of incubation and day 10 post-hatching. These fostered birds, after becoming fully matured, received a pair randomly and we observed parental care of this second generation too, following the same protocol. We then compared various parental behaviours (such as time spent incubating, or number of nest attendances during offspring provisioning) in the second generation to those of their genetic and social parents. Based on the results of our experiment, both genetic and social effects can contribute to intergenerational transmission of specific parental behaviours, with various weights. However, the strongest and most consistent effect that we found is that of the current mate; a social effect that can manifest both in negative and positive directions, depending on the behavioural trait. Our study suggests context-specific and sexually different genetic, social and non-social environmental effects in the ontogeny of parental sex roles and outline the importance of parental negotiation in explaining individual variation of parental behaviour in biparental species.

Keywords: sex differences, parental care, heritability, social environment, parental coordination, negotiation, *Taeniopygia guttata*

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Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *22 October 2021*
Accepted: *13 December 2021*
Published: *07 January 2022*

Citation:
Morvai B, Fazekas EA, Miklósi Á and Pogány Á (2022) Genetic and Social Transmission of Parental Sex Roles in Zebra Finch Families. Front. Ecol. Evol. 9:799996. doi: 10.3389/fevo.2021.799996

INTRODUCTION

Males and females often differ in various aspects of their reproductive behaviours, for instance, in their competitiveness and choosiness during mating and their parental behaviour, so that they exhibit distinctive sex roles (Kokko et al., 2006; Fairbairn, 2013). Although the traditional definition of sex roles focuses on the competition aspect only, the frequent association with biased parental care and the reinforcing, positive interaction between them justifies a wider definition including parental sex roles (Kokko and Jennions, 2008). Parental sex role differences in terms of workload may manifest in one parent providing full care and the other providing no care at all (such as in uniparental systems), or it may manifest in unequal relative amount of care provided by the two sexes in biparental systems. Explaining the origin and consequences of parental sex role differences are central questions in evolutionary biology and behavioural ecology, due to their direct and significant impact on individual fitness and ultimately, their contribution to breeding system evolution (Clutton-Brock, 1991; Kokko and Jennions, 2008; Royle et al., 2012; Kahn et al., 2013; McNamara and Wolf, 2015; Fromhage et al., 2016; Fromhage and Jennions, 2016; Henshaw et al., 2019).

Sex-roles involve social behaviours, and as such, understanding how they change, develop, and evolve in non-human animals by genetic evolution, social learning and the interaction between them is challenging. Accumulating evidence suggests that social learning [defined as 'learning that is influenced by observation of, or interaction with, another animal (typically a conspecific) or its products'; (Heyes, 1994; Hoppitt and Laland, 2013)] may play a more important role across a broad range of animal taxa than previously anticipated (Galef, 2012; Kendal, 2015; Whiten, 2017). In general, large adaptive value, and consequently, the prevalence of social learning are expected in situations when genetically determined behavioural variability is unproductive, non-social learning is costly or individuals are faced with uncertain, frequently changing environment (Laland, 2004; Rendell et al., 2011; Heyes and Pearce, 2015; Kendal et al., 2018); see also Rieucau and Giraldeau (2011) for a detailed review on the costs and benefits of social learning). Theory suggests that besides influencing whether social learning of a given trait is adaptive and thus expected, the speed of environmental change also influences the mode of social transmission (Laland and Kendal, 2003). Specifically, vertical (from parents to offspring) or oblique transmission (from non-parent adults to offspring) is expected if environmental changes are not significant between subsequent generations, and horizontal transmission (between peers, either immatures or adults) is expected when environment changes rapidly, e.g., from generation to generation (Cavalli-Sforza and Feldman, 1981; Laland and Kendal, 2003).

Parental care, especially when intense or extended such as in great apes and in most bird species, provides ample opportunity for vertical transmission to take place (Whiten, 2017). The predominant avian breeding system is social monogamy with biparental care that has been described in 90% of bird species (Cockburn, 2006). The high prevalence, great diversity and often substantial within-species variation of parental behaviours make biparental passerines an ideal group for investigating the origin of phenotypic variation in terms of genetic and social environmental effects. Since heritability estimates are often difficult to acquire in avian species due to dispersal and low survival, repeatability (the proportion of phenotypic variation in parental effort that is explained by genetic or consistent environmental variation among individuals) was first used as a proxy and upper limit of heritability (Falconer, 1981; Lessells and Boag, 1987). Although the validity of such interpretation of repeatability has later been challenged [repeatability does not necessarily set an upper limit to heritability in various scenarios, e.g., when genotype-environment interactions, or maternal effects are strong; (Dohm, 2002)], numerous studies reported individually consistent parental efforts in different bird species and for various parental behaviours. Remarkably, most of these studies reported sex differences in the repeatability estimates. In the house sparrow (*Passer domesticus*), a study reported highly repeatable male but unrepeatable female provisioning rates (Schwagmeyer and Mock, 2003), and another study corroborated this finding in addition to showing similar sex differences in repeatabilities for incubation time in this species (Nakagawa et al., 2007). Consistency of care decisions (care or desert the clutch and mate) at subsequent nests in the variable breeding system of the sequentially polygamous Eurasian penduline tit (*Remiz pendulinus*) showed a contrasting pattern with consistent female, but individually variable male care decisions (Pogány et al., 2008). Similarly, parental work load (measured as daily energy expenditure) was repeatable in female, but not in male pied flycatchers (*Ficedula hypoleuca*) (Potti et al., 1999). In Manx shearwaters (*Puffinus puffinus*), however, neither male nor female provisioning rates were repeatable (Gray et al., 2005).

The empirical studies that aimed at directly estimating heritability reported high and moderate heritabilities for parental efforts in birds. The study of Dor and Lotem (2010) stands out by applying an experimental approach. After cross-fostering nestlings between broods in four generations of house sparrows, their analysis revealed that approximately 50% of variation in feeding rates of sons can be explained by the previous generation, whereas genetic heritability was not significant in daughters. The majority of heritability studies, however, applied a quantitative genetic approach, and these early studies investigated heritability as a joint effect of genetic and social environmental effects, hindering discrimination between them. In line with the results of repeatability studies, these investigations also revealed sex differences in heritabilities. For instance, while more than 80% of the phenotypic variation in male feeding rates could be attributed to that of the previous generation in Savannah sparrows (*Passerculus sandwichiensis*), female feeding rates were not heritable (Freeman-Gallant and Rothstein, 1999). A long-term field study in long-tailed tits (*Aegithalos caudatus*), besides linking offspring survival to feeding rates, also found significant heritability for parental effort with ca. 43% of variation in male feeding rates explained by the parental generation (MacColl and Hatchwell, 2003).

With the advances of quantitative genetics in the last decades, environmental effects could be further partitioned, considering the indirect genetic effects by the social partners that may have significant influences on the interacting phenotypes (Moore et al., 1997; Wolf et al., 1998). With regards to parental care, a handful of investigations applied such a more-in-depth quantitative genetic framework by further partitioning environmental effects in variation of parental care into indirect genetic effects (IGEs) and indirect environmental effects (IEEs). Brommer and Rattiste (2008), for instance, reported 14.5% of variance in laying date of female common gulls (*Larus canus*) being explained by direct genetic effects, and an additional 4.8% of variance explained by indirect genetic effects via the males. Furthermore, their analysis revealed that the direct (female) and indirect (male) genetic effects were negatively correlated. Adams et al. (2015) also focused on social environmental effects in explaining variation in the feeding rates of parents and helpers in long-tailed tits, although their analysis did not reveal significant IGEs. Schroeder et al. (2019), however, found large social effects via IGEs in nest visit frequencies of both sexes in house sparrows. In addition to the conventionally calculated heritability estimates (13% in both sexes), IGEs accounted for an additional 11–12% variation, resulting in a total heritable variation of 24 and 25% in females and males, respectively.

The above studies demonstrate that parental care can often have large heritable variation. However, in monogamous species with no or insignificant extra-pair copulations, the transmission of genetic and social effects from a given parent toward the young are inseparable non-experimentally. Here we aim at filling a gap by experimentally investigating the potentially determining social effects of parenting. Previous studies revealed social interactions to have prominent influence on sex role-related behaviours in the socially monogamous, biparental zebra finch (*Taeniopygia guttata*), such as on mating preferences, nest building and parental care (Immelmann, 1972; Jones et al., 1996; Swaddle et al., 2005; Guillette et al., 2016; Katsis et al., 2018; Pogany et al., 2019). Based on these studies, we chose this small passerine as our model species, and applied a full cross-fostering design to disentangle experimentally genetic and social effects deriving from the parents. We hypothesized that vertical social learning is a crucial process in the ontogeny of parental behaviour. If so, we expected that a larger proportion of the phenotypic variation in nest attendance, incubation, brooding and nestling provisioning will be explained in the cross-fostered second generation by variation of the same traits of their same-sex social than that of their genetic parent.

MATERIALS AND METHODS

Study Population and Housing Conditions

This experiment was carried out using a population of captive zebra finches in the Animal House of Eötvös Loránd University, Hungary, between October 2015 and June 2019. All subjects were born in the Animal House and were approximately the fourth-generation descendants of our stock population [our stock population was established in 2013 from a domesticated population of Bielefeld University (Germany) (Forstmeier et al., 2007)]. Adult birds were kept in same-sex indoor aviaries until the beginning of the experiment. We established 35 breeding pairs (first generation, henceforth; mean ± SD brood size on day 10, post-hatching = 2.61 ± 1.34) avoiding genetic relatedness, but otherwise randomly assigning the pair members. None of the birds had any successful breeding attempts before the experiment. The breeding of the first-generation pairs allowed us to establish 43 pairs when birds from the offspring generation became sexually mature (second generation, henceforth). In the statistical analysis of the incubation period, we excluded four pairs (one pair each due to failed video recording, the female being clearly afraid of the camera, because the male was aggressive and hurt its mate, and because the pair had missing parental incubation data), while in the analysis of the post-hatching period we excluded five pairs (in one pair, the female, in two other pairs, the offspring died before the video recording, whereas two more pairs had to be excluded due to the male and the female hurting their mates, respectively, during feeding). Pairs were kept in separate cages (100 × 30 × 35 cm) with a wooden nest box attached to each (12 × 12 × 12 cm), and they were provided with fresh nest material (coconut fibres) every second day. Numbered aluminium rings (one ring per bird; Principle Kft., Újlengyel, Hungary) were used for individual identification of the subjects. Optimized light conditions were maintained in the experimental room; a 14:10 h light:dark cycle was adjusted (using full-spectrum tube lights, NASLI, Prague, Czechia). The air conditioning system of the animal house were set to provide moderate conditioning so that temperature and humidity varied somewhat over the 4-year course of the experiment (mean ± SD temperature: 23.7 ± 2.0°C, humidity: 47.7 ± 12.0), which we considered in our analyses. Food and water were provided *ad libitum* to the birds. Nourishment consisted of a seed mixture, supplemental egg-food (Egg food tropical finches, Orlux, Versele-Laga, Belgium) and home-made germinated seeds [for more details on the diet, see Morvai et al. (2016)].

Experimental Design

Nest boxes of the pairs (first generation) were monitored every second day to determine the start of incubation. On day 6 of incubation, we swapped clutches with fertile eggs (presence of embryos were checked by lamping the eggs) between nests in similar stages, thus all pairs took care of an unrelated clutch from that point onward (full cross-fostered design). On day 8 of incubation, between 10:00 and 13:00 [time window for observations were chosen based on Morvai et al. (2016)], parental behaviour was recorded through a hole on the top of the nest boxes. Small digital cameras (Mobius Action Cam, JooVuu Store, United Kingdom) were used to monitor the birds, equipped with wide-angle lenses (116° field of view). The cameras were attached onto the nest boxes a day before timed recording started, and dummy cameras (black wooden blocks) covered the hole on the nest boxes every other day when there was no video recording. After the recording, clutch mass was measured (Radwag WTB 2000, Poland). Nest checks were continued every second day after the recording to determine hatching date (day 13–14 of incubation). On day 10 post-hatching (counted from hatching of the first nestling), between 10:00 and 13:00, parental behaviour was again recorded, and brood mass was measured. Nestlings fledged at around day 18–20 and were removed from their parents well after becoming independent, on day 35 post-hatching. They were kept together with other birds of similar age until their sex could be determined unambiguously. Male tutors were provided in a neighbouring cage to ensure normal development of acoustic communication. Juveniles were then placed into same-sex aviaries until they became sexually mature [approximately on day 90 post-hatching (Zann, 1996)]. Sexually mature second-generation birds were randomly assigned as pairs, and we repeated the same protocol that we applied for the first generation (including full cross-fostering of similar-stage clutches) so that data on their parental behaviour was collected.

Behavioural Coding and Statistical Analyses

Behavioural coding from the video recordings was carried out using Solomon Coder software (Péter, 2015). From the recordings, male and female parental behaviour was coded, simultaneously. We coded the frequency of, and time spent on the following parental behaviours: nest attendance (defined as whenever the bird is seen on the recording of the nestbox camera, i.e., irrespective of what he/she is doing), incubation (sitting on eggs) and brooding (sitting on nestlings). We also coded actual feedings (provisioning begging offspring with regurgitated seeds). For each of the above frequency variables, we calculated the hourly rates, whereas for time variables, the proportion of observation time spent with the given behaviour was calculated.

R statistical environment [v. 4.0.3; (R Core Team, 2020)] was used to analyse genetic and social inheritance in parental behaviour. Since we had multiple variables that were likely highly correlated (e.g., rate and time of any given behaviour, or incubation and nest attendance), to avoid redundancy in our analysis, first we filtered our response variables based on a Spearman's rank correlation matrix ($r > 0.8$ between two variables were considered as conveying the same information, so that only one of the variables were included in further analysis). This resulted in the following rate response variables in our analyses (incubation and post-hatching period taken together): hourly incubation or brooding rate, feeding nestlings, and nest attendance. For time (duration) variables, we selected nest attendance time (i.e., the proportion of time the bird was seen on recordings; this variable highly correlated with incubation time (Spearman $r = 0.88$, df = 76, $p < 0.001$) and brooding time ($r = 0.94$, df = 74, $p < 0.001$), respectively, in the two reproductive stages). Our explanatory variables in focus were the same behaviours as the response variable from the same- and opposite-sex genetic and social parents (e.g., the analysis of nest attendance time (response variable) included nest attendance time by the genetic and social fathers and mothers as explanatory variables). In addition to these variables, in each model we tested for other, potentially confounding effects, such as clutch or brood size, temperature, humidity and the behaviour of the mate.

Response variables were analysed in separate Linear Mixed-effects Models [LMM; R package 'nlme' (Pinheiro et al., 2019)]. In addition to the above independent variables, all models included nest ID (the number of the nest where the subject grew up) as a random term, to account for non-independent sampling due to genetic and social relatedness between siblings. We carried out stepwise model selection based on likelihood ratio tests (LRT); we provide χ^2 and the corresponding p-values of LRTs of models with and without the explanatory variables in question. We also report parameter estimates ($\beta \pm$ SE) and odds ratios [exp(β), with 95% confidence intervals for log-transformed responses in LMMs] for significant explanatory variables.

We applied two different approaches in our analyses. In our first approach, we analysed the same reproductive stages of the two generations (e.g., a given parental behaviour during incubation (or post-hatching) period of the second generation explained by the same behaviour during incubation (or post-hatching) of the first, parental generation). In our second approach, we analysed whether a given behaviour during incubation period in the second generation is explained by a similar behaviour during post-hatching period in the first generation. In the latter analysis, our aim was to compare the first reproductive stage of the second generation (incubation) with the reproductive stage of their parents which allowed them to socially interact and learn from them (i.e., provisioning during the post-hatching period). Hourly feeding rate and time spent feeding was excluded from the latter analysis, as these could not be matched with any behaviour in the incubation period.

ETHICAL NOTE

The study was carried out following the Hungarian Laws for the experimentation with animals and with permission of the ethical board of our university (ELTE MÁB #02/2014). All experimental birds and their offspring remained for their entire life at the Animal House, Eötvös Loránd University, Budapest. Birds were visually monitored for health status daily.

RESULTS

Our analysis revealed significant variation in all investigated behavioural variables, allowing us to detect any potential genetic or social effects (see **Supplementary Table 1**). For a summary of statistical models with significant effects, see **Supplementary Table 2**).

Incubation Period

Neither male, nor female incubation rate was explained by the same behaviour of their genetic (LMM, LRT: all $p > 0.074$) or social parents (all $p > 0.738$). However, incubation rate was strongly correlated between pair members (to avoid redundancy, for pair effects we report results with the behaviour of one sex as response and that of the other sex as explanatory variable only henceforth – the reverse analyses provided very similar results in each case; LMM of (log) hourly male incubation rate, effect of pair's incubation rate: LRT: $\chi^2_1 = 30.78$, $p < 0.001$; exp(β) = 1.15 [1.10; 1.19], $t_{15} = 6.67$, $p < 0.001$; **Figure 1**.

Similar to incubation, male and female nest attendance frequencies were not explained by the genetic (all $p > 0.218$) or social parents' behaviour (all $p > 0.065$), although we found positive correlation between the behaviour of pair members [LMM of (log) hourly male nest attendance rate, effect of pair's attendance rate: LRT: $\chi^2_1 = 6.48$, $p = 0.011$; exp(β) = 1.13 [1.03; 1.25], $t_{15} = 2.59$, $p = 0.021$; **Figure 2A**]. Time spent attending the nest in males was not influenced by the same behaviour of their genetic or social parents (all $p > 0.350$), whereas humidity had a negative effect (LMM of proportion of time spent attending the

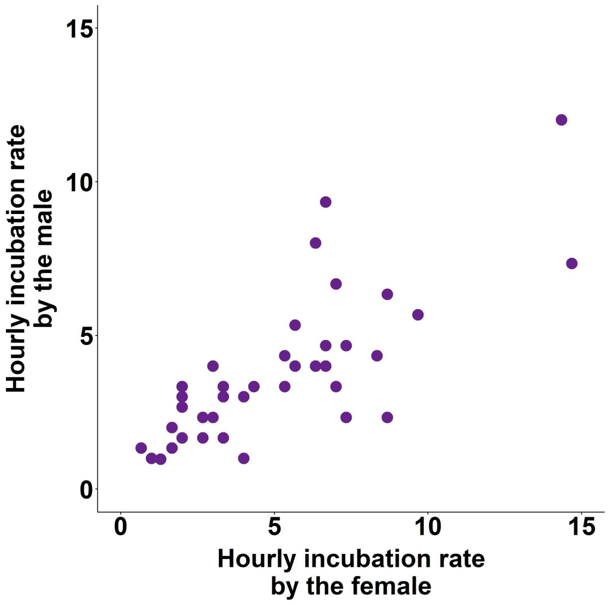

FIGURE 1 | Hourly incubation rate in zebra finch pairs.

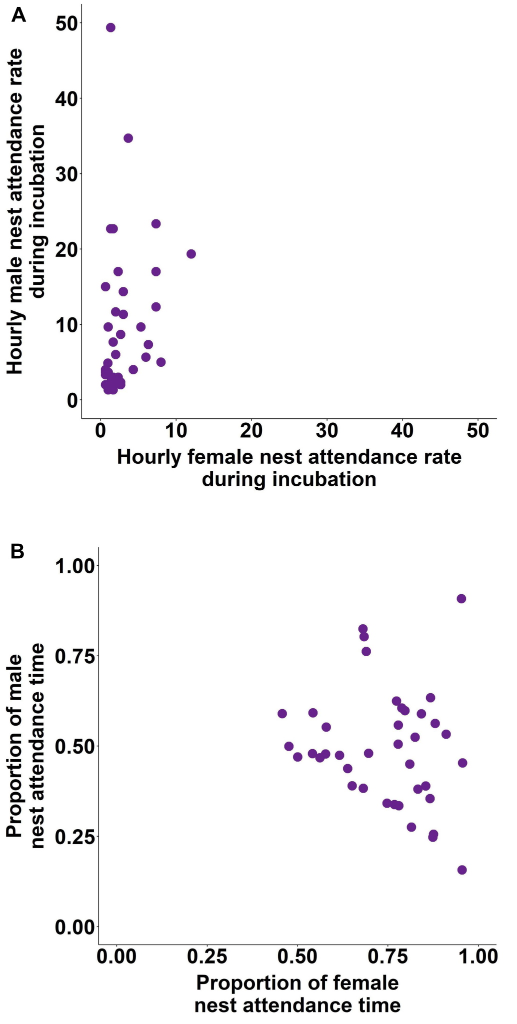

FIGURE 2 | Hourly nest attendance rate **(A)** and proportion of 3 h observation time spent attending the nest **(B)** in incubating zebra finch pairs.

nest by males, effect of humidity: LRT: $\chi^{2}_{1} = 3.74$, $p = 0.053$; $\beta \pm \text{SE} = -0.004 \pm 0.002$, $t_{12} = -1.90$, $p = 0.081$). In contrast, female nest attendance time was explained by the behaviour of their genetic mother [LMM of (quadratic) proportion of time spent attending the nest by females, effect of genetic mother's nest attendance time: LRT: $\chi^{2}_{1} = 4.45$, $p = 0.035$; $\beta \pm \text{SE} = 0.58 \pm 0.27$, $t_{24} = 2.13$, $p = 0.043$], but not those of their social parents ($p > 0.285$). Furthermore, nest attendance time was influenced negatively by the pair's behaviour (LRT: $\chi^{2}_{1} = 4.12$, $p = 0.042$; $\beta \pm \text{SE} = -0.37 \pm 0.18$, $t_{12} = -2.02$, $p = 0.066$; **Figure 2B**).

Post-hatching Period

Male brooding rate (sqrt-transformed) was influenced by the behaviour of the genetic father (LMM of hourly male brooding rate, effect of genetic father's brooding rate, LRT: $\chi^{2}_{1} = 4.06$, $p = 0.044$; $\beta \pm \text{SE} = 0.06 \pm 0.03$, $t_{19} = 1.95$, $p = 0.066$; **Figure 3A**), but not that of the social parents ($p > 0.150$). Besides brood size (LRT: $\chi^{2}_{1} = 4.12$, $p = 0.042$; $\beta \pm \text{SE} = -0.17 \pm 0.08$, $t_{10} = -1.96$, $p = 0.078$), temperature also had a negative effect on male brooding rate (LMM, LRT: $\chi^{2}_{1} = 5.68$, $p = 0.017$; $\beta \pm \text{SE} = -0.14 \pm 0.06$, $t_{10} = -2.33$, $p = 0.042$). In contrast, female brooding rate was not explained by the genetic (all $p > 0.093$) or social parents' behaviour [all $p > 0.570$), whereas brood size had a negative effect (LRT: $\chi^{2}_{1} = 5.98$, $p = 0.015$; $\exp(\beta) = 0.79$ [0.66; 0.95], $t_{11} = -2.48$, $p = 0.031$].

In contrast to brooding, neither male, nor female nest attendance frequencies were explained by the genetic (all $p > 0.191$) or social parents' behaviour (all $p > 0.100$). However, nest attendance rate was positively influenced by the pair's behaviour [LMM of (log) hourly male nest attendance rate, effect of pair's nest attendance rate: LRT: $\chi^{2}_{1} = 12.21$, $p < 0.001$; $\exp(\beta) = 1.11$ [1.05; 1.17], $t_{14} = 3.69$, $p = 0.002$]. Male and female nest attendance times were also not explained by either their genetic (all $p > 0.100$), or their social parents' behaviour (all $p > 0.503$). Brood size, however, had negative effect on nest attendance time in both sexes [LMM of (sqrt-transformed) nest attendance time in males: LRT: $\chi^{2}_{1} = 5.94$, $p = 0.015$, $\beta \pm \text{SE} = -0.05 \pm 0.02$, $t_{14} = -2.47$, $p = 0.027$; LMM of (logit) nest attendance time in females: LRT: $\chi^{2}_{1} = 9.65$, $p = 0.002$, $\exp(\beta) = 0.64$ [0.50; 0.83], $t_{11} = -3.25$, $p = 0.008$].

Offspring feeding rate was not influenced by the feeding rate of either the genetic (all $p > 0.116$) or social parents (all $p > 0.157$), or by any other investigated variables.

Incubation Period of the Second Generation Compared to Post-hatching Period of the Parental Generation

Incubation rate in males was influenced by their social father's brooding rate (LMM of (log) hourly male incubation rate, effect

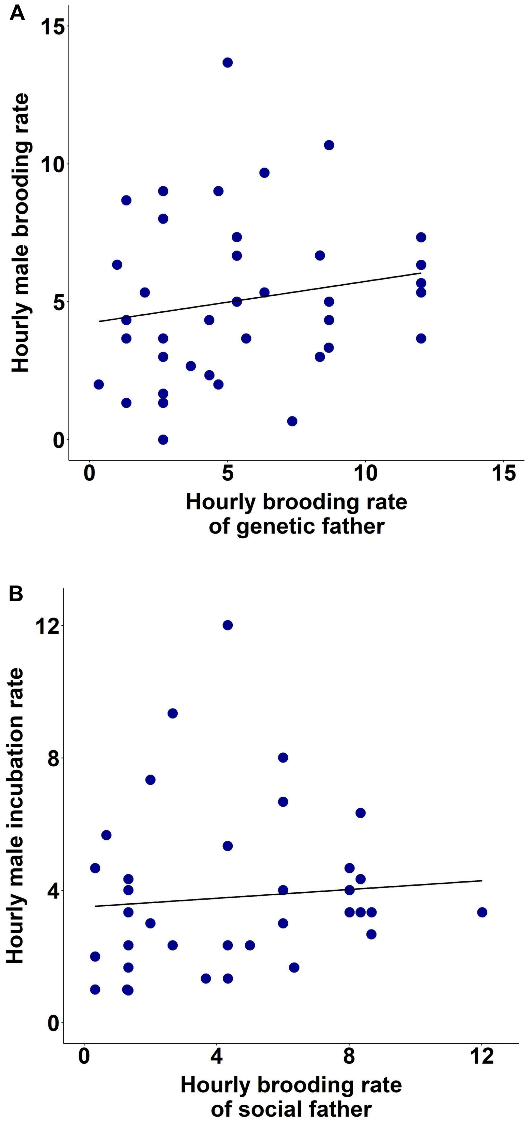

FIGURE 3 | Hourly brooding rate **(A)** and incubation rate **(B)** of male zebra finches based on hourly brooding rate of their genetic and social father, respectively.

of brooding rate of social father; LRT: $\chi^2_1 = 8.59$, $p = 0.003$; exp(β) = 1.06 [1.02; 1.10], $t_{14} = 2.98$, $p = 0.010$; **Figure 3B**), besides the effect of their pairs' behaviour (see also above (Results, Incubation period); LRT: $\chi^2_1 = 37.50$, $p < 0.001$; exp(β) = 1.15 [1.11; 1.20], $t_{14} = 7.63$, $p < 0.001$). On the other hand, female incubation rate was not influenced by brooding rate of their social ($p > 0.780$) or genetic parents ($p > 0.071$).

Neither male, nor female nest attendance rate were explained by the corresponding behaviour of their genetic (all $p > 0.146$) or social parents (all $p > 0.442$) during the post-hatching period.

For the effects of the pair's behaviour and humidity on parental care during incubation of the second generation, see the analysis of both generations' incubation period above.

DISCUSSION

Applying a full cross-fostering experimental design, our study disentangled genetic and social parental effects in transmission of parental sex roles to the next generation. We revealed further evidence for sex-specific genetic and social inheritance, as well as non-social environmental effects, but in contrast with our expectations, social effects from parents overall proved to explain very little variation in different parental behaviours. Another component of the within-family social environment, nevertheless, turned out to be determining; we found strong interdependence between the behaviour of the focal parent and that of its current mate in various parental behaviours. Hence, our results are in line with current behavioural studies underlying the importance of negotiation between parents.

We found significant genetic transmission in two of the investigated parental behaviours: female nest attendance time and male brooding rate. In addition, social effects were revealed in incubation rate, whereby males, but not females, adjusted their incubation rate based on the brooding rate they had previously experienced from their social father. This similarity could be the result of social learning from parents (i.e., vertical social learning), as well as other mechanisms, including physiological and asocial environmental effects (such as the direct effects of low quality food from parents, instead of observing and learning parental behaviour *per se*). However, while previous studies reported heritable variation in various parental traits (Freeman-Gallant and Rothstein, 1999; Potti et al., 1999; MacColl and Hatchwell, 2003; Schwagmeyer and Mock, 2003; Nakagawa et al., 2007; Brommer and Rattiste, 2008; Pogány et al., 2008; Dor and Lotem, 2010; Adams et al., 2015; Schroeder et al., 2019), to our knowledge, our study stands out by presenting a detailed analysis of multiple parental behaviours while experimentally separating heritability via genes and social experiences from parents. Our findings on genetic and social heritability, in combination, suggest sex- and context-specific transgenerational transmission of parental behaviours. Corresponding with previous studies reporting sex differences in repeatability and heritability of parenting (Freeman-Gallant and Rothstein, 1999; Potti et al., 1999; Schwagmeyer and Mock, 2003; Pogány et al., 2008; Dor and Lotem, 2010), males and females were different in the relative strength of genetic and social effects that shape any particular parental behaviour. It is also apparent from our results that neither genetic, nor social inheritance should be considered as a universal process behind variation in parental behaviours, in general. In contrast to our separately caged breeding pairs, individuals in natural populations experience further social and asocial environmental influences, including competition over resources, interference by conspecifics, potential extra-pair activities. The lack of an overall strong social effect from parents under controlled laboratory conditions (where all the above mentioned potentially influential effects are excluded) suggest that social effects from parents cannot be significant in natural conditions, either.

Focusing on the sex differences in the three behavioural traits revealing heritability, female nest attendance time appears to be under stronger genetic influence. In contrast, male nest attendance time was not related to the behaviour of the genetic father, however, it was influenced negatively by humidity, suggesting a more flexible response and compensation to changes in the non-social environment by males than by females during incubation. Our results are in line with previous studies reporting male and female parents to react differently to environmental changes in various bird species (Magrath et al., 2005; Charmantier et al., 2008; Kosztolányi et al., 2009; Vincze et al., 2017; Rohwer and Purcell, 2019; Sharpe et al., 2021; Ton et al., 2021). In zebra finches, a recent study also corroborates our finding suggesting sexually different flexibility and reactions to changing environmental conditions, however, with a sexually contrasting effect of ambient temperature (Ton et al., 2021). In this study females, rather than males, decreased their nest attendance time in response to high ambient temperatures. Time spent attending the nest during incubation is one of the most expressed parental sex role differences in zebra finches, with a bias toward females in captive populations [ca. 39% vs 72% male vs female nest attendance time during incubation in our population, see also Gorman and Nager (2003), Morvai et al. (2016), and Ton et al. (2021)]. This bias, taken together with our results on male response to humidity changes and those of Ton et al. (2021) on female response to temperature changes suggest sexually different capacities to respond to specific environmental conditions, involving a possible ceiling-effect in females. Males might respond more to environmental changes (such as lower humidity) at lower temperatures because females perhaps already spend as much time with nest attendance as their metabolism and energy demands and overall condition allow under these conditions (reflected in a decreased effort when higher temperatures allow this). In addition, only female zebra finches have a brood patch, so that their more effective heat transfer may contribute to sexually different optimization strategies of incubation effort (Zann and Rossetto, 1991; Hill et al., 2014). Similar to female nest attendance, the analysis of male brooding rate also revealed genetic transmission, in addition to a negative effect of brood size (a similar effect was also found in female brooding rate analysis) and temperature.

Our cross-reproductive stage analysis (comparing post-hatching behaviour of the parental generation with parental behaviour of the second generation during incubation) provided the only evidence for parental social effects in our experiment. Variation of male incubation rate was explained by the brooding rate experienced from social fathers. The behavioural pattern experienced from parents, however, appeared to have only a temporal effect, because our analysis of the next reproductive stage, post-hatching period, revealed brooding rate to be influenced by the genetic, rather than the social father's behaviour. Our previous experiment, in which we applied a split-family experimental design to create uniparentally raised young, resulted in very similar transitional social effects (Pogany et al., 2019). Both female-only and male-only cared zebra finches showed different parental sex role division when incubating during their first breeding based on their social experiences with each of their parents. This effect, however, could no longer be observed during the post-hatching period of their first breeding, or their second breeding attempt, suggesting that their own breeding experiences with their mate overrode any behavioural patterns they might have taken over from their parents socially. The results of our present study corroborate this scenario; with two strikingly different experimental approaches, both studies point toward the importance of current (due to the pair), rather than past (parental), social environmental effects in shaping parental behaviours, while suggesting a temporal social effect form the parents.

The most important and consistent social effect we found in our study is that of the current mate. This reflected in the number of parental behaviours and that we found significant pair effects in both reproductive stages (incubation rate, nest attendance rate during incubation and post-hatching period), but also in the effect sizes in relation to the above genetic and social effects due to parents. Pair members provided parental care in both reproductive stages in close coordination with each other. Considering the flexibility of parental coordination and the substantial differences between laboratory and natural conditions including the presence-absence of environmental constraints, our results are likely underestimating the importance and intensity of parental coordination in nature. Even so, during incubation, time spent with nest attendance correlated negatively, and visit rate correlated positively between pair members, suggesting turn-taking (cf. Johnstone and Savage, 2019), whereby males and females invest only after each contribution by their partner. During post-hatching, nest attendance rate was also positively correlated, suggesting coordinated, synchronized feeding of young (cf. Mariette and Griffith, 2012). Synchronized, rather than independent nest visits may reduce sibling competition through more equal food distribution between offspring or decrease the chance of depredation as a result of lower number of overall nest visits (Shen et al., 2010; Mariette and Griffith, 2012; Leniowski and Wegrzyn, 2018; Wojczulanis-Jakubas et al., 2018; Ihle et al., 2019). In addition, synchronized provisioning behaviour might be the result of other, non-parental activities, for instance foraging patterns (Masello et al., 2006). Incubation is a prolonged and continuous behaviour compared to offspring provisioning bouts. Therefore, the two behaviours are inherently different in the opportunity for pair members to replace each other. Turn-takings during incubation and synchronized offspring provisioning are in line with theoretical models of biparental care suggesting pair members should respond to each other's parental effort by real-time negotiation (McNamara et al., 1999, 2003; Johnstone and Hinde, 2006; Lessells and McNamara, 2012; Johnstone and Savage, 2019; Barbasch et al., 2020). Accumulating empirical evidence support this view (Hinde, 2006; Hinde and Kilner, 2007; Kosztolányi et al., 2009; Meade et al., 2011; Lendvai et al., 2018); a recent study, besides reporting coordinated turn-taking during incubation, identified acoustic communication between zebra finch pair members as a key factor facilitating coordination (Boucaud et al., 2016, 2017). Since variability in female nest attendance time had a significant genetic component in our analysis, it is possible that female nest attendance behaviour is less flexible, whereas males more likely adjust their parental investment to maintain the level of total care needed for successfully raising the offspring (cf. Adams et al., 2015).

Our results further corroborate that parental behaviour of zebra finch pairs are in strong interdependence, as partners respond to each other's behaviour in both reproductive stages, providing ample opportunities for IGEs to shape parental behaviour in this species. Although our study did not aim at investigating IGEs, our results suggest they likely play a prominent role in the evolution of parental care in this species, in line with recent theoretical and empirical studies (Brommer and Rattiste, 2008; Royle et al., 2014; Bailey et al., 2018; Schroeder et al., 2019).

Our findings also contribute to the growing body of empirical research reporting parental response to offspring needs (Gilby et al., 2011; Rehling et al., 2012; Royle et al., 2014; Trillmich et al., 2016; Lendvai et al., 2018; Westneat and Mutzel, 2019). Besides more food demands of a larger brood, thermoregulatory needs of non-feathered nestlings can differ depending on the number of nestlings in the nest, as more nestlings can keep each other warmer, allowing parents to leave a bigger brood alone for longer periods. The degree of recognizing offspring needs can be an important factor that can also influence parental negotiation. Lendvai et al. (2018), for instance, experimentally increased begging calls of tree swallow nestlings in the presence of their mother only. In response, females fed their offspring more, which elicited similar behaviour from their partner, providing an example for real-time response in a situation where males presumably relied on the female's information about the offspring needs.

CONCLUSION

Our results provided further evidence for sex- and context-specific genetic and social heritability of parental behaviours. Instead of a universal genetic inheritance or vertical social learning, the relative importance of parental effects and the mode of transmission appears to vary between the sexes and various aspects of parenting. Changes in the non-social environment elicited sexually different parental care responses. In addition, we found strong interdependence between parental behaviour of the pair members, suggesting that current social effects due to the mate are more important than past social experiences or genetic transmission from parents in explaining variation in parental behaviours.

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 Animal Welfare Committee, Eötvös Loránd University, Budapest, Hungary (ELTE-MÁB).

AUTHOR CONTRIBUTIONS

ÁP conceived the study with input from ÁM. ÁP, BM, and EF contributed to experimental design and managing the experiments. BM and EF carried out the experiments and collected data. BM coded video recordings and managed the database. ÁP and BM analysed the data. BM and ÁP wrote the manuscript with input from ÁM and EF. All authors contributed to the revision and final manuscript.

FUNDING

This study was supported by the Hungarian Scientific Research Fund (OTKA K109337 and FK131966) and was completed as part of the ELTE Thematic Excellence Programme 2020 supported by the National Research, Development and Innovation Office (TKP2020-IKA-05). ÁP was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences, the ÚNKP-20-5 New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund and the Hungarian Ethology Foundation.

ACKNOWLEDGMENTS

We thank László Zsolt Garamszegi for statistical advice. We also thank Buddhamas Kriengwatana and Roi Dor for their valuable comments on previous versions of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.799996/full#supplementary-material

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