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Salinity limits mosquitofish invasiveness by altering female activity during mate choice

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Biological invasions of freshwater habitats are of increasing biological and economical concern, and both, salinity and parasites are considered to be key contributors to invasion success. Salinity, for example, influences the distribution of invasive mosquitofish (*Gambusia holbrooki*) and native killifish (*Aphanius fasciatus*) in Europe, with the latter now predominantly confined to high-salinity habitats. Here, we examined how salinity might affect female activity and preference for large and non-parasitized males in multiple populations of mosquitofish and killifish in Sardinia, Italy. We predicted that (1) females of both species would associate preferentially with larger and uninfected males, and that (2) female behavior in both species would be significantly influenced by salinity. We used dichotomous choice tests, in which we presented focal females with video animations of photos of the same male but differing in body size and presence/absence of an ectoparasite (*Lernaea cyprinacea*). We calculated female preference based on association time and quantified female inactivity as time spent in the central neutral zone during trials. Contrary to prediction 1, females did not prefer the large or uninfected male stimuli over their counterparts in any of the populations. However, while salinity did not significantly affect female preferences, it did significantly affect their activity, with mosquitofish becoming more inactive at higher salinities and killifish exhibiting the opposite pattern, matching prediction 2. These results suggest that salinity limits mosquitofish invasiveness by reducing their activity and thus provides a refuge for the Mediterranean killifish.

KEYWORDS

video animations, female mate choice, sexual selection, parasites, *Gambusia holbrooki*, *Aphanius fasciatus*

1 Introduction

Mounting evidence documents the negative impacts of human activities on biodiversity and ecosystem functioning (1, 2). For example, biological invasions, which are often caused by human action, are considered one of the main threats to global biodiversity, ranking third behind habitat fragmentation and habitat loss (3, 4). The introduction of invasive species can be damaging for several reasons, including that they may affect native species through predation, competition for resources and transmission of parasites (5, 6). Freshwater ecosystems are deemed particularly susceptible to the establishment of invading species (3). Additionally, the invasive success of an introduced non-native species may depend on factors such as the level of parasitism in those species, and their personality and behavioral traits, including mate choice (7, 8).

Sexual selection is widely recognized as one of the main evolutionary forces driving the development and refinement of traits that influence mating success (9, 10), and sexual selection can drive differences in sexual traits within and between native and invasive populations of the same species (11, 12). Mate choice is a multi-phase process that can occur before, during and after mating (13, 14). It typically involves the detection of signals and cues, their evaluation, decisions on who to mate with and different fitness consequences arising from mating (13, 14). For instance, choosers' preferences are partially dependent on their ability to process multiple cues and signals, including, but not limited to, visual [e.g., body size and coloration, (15, 16)] and olfactory stimuli [e.g., as often observed in insects; e.g., (17)]. Moreover, there is evidence that other cues such as parasite load can also directly affect mate choice, with choosers often preferring to associate with non-parasitized mating partners (13, 18, 19).

Mating preferences can vary among individuals, species and populations (14, 20, 21), and the strength and direction of these preferences are often shaped by environmental factors (22–24). These, including factors influenced by human activities, can affect the production and expression of traits relevant to mate choice, the transmission of information between potential mates, and the reception and processing of received information (25); for example, in fishes, pollution with synthetic hormones and hormone analogs has been shown to reduce the motivation to make informed mate choice decisions (26), turbidity has been shown to interfere with mate choice (27) and higher salinity has been demonstrated to negatively affect the desire to mate, activity levels during mate choice, and mating preferences [(24); for a negative influence of salinity on other aspects of fish activity, such as maximum swimming speed, see (28), for example]. Thus, by altering mating preferences and activity levels, environmental factors may influence the population viability and survival of a species (20, 29). Moreover, freshwater habitats around the world are getting saltier, a process termed freshwater salinization syndrome [e.g., (30, 31)]. How might salinization (and other human induced environmental change) influence mate choice and activity levels during mate choice? Since invasive species have often been found to be bolder and show more exploratory behavior compared to non-invasive species [e.g., (32, 33)], behavioral inactivity could influence pathways of species introductions, the likelihood of successful establishment in novel habitats and the level of impact on native organisms and ecosystems (34, 35)? Knowledge of these patterns is crucial to effectively predict to what extent climate change will impact species invasiveness and implement correct management protocols.

As a result of their broad distribution and capacity to tolerate a wide range of environmental conditions, eastern mosquitofish (*Gambusia holbrooki*) provide excellent model organisms for investigating environmental effects on female mate choice. They are native to North America but are, and have been, intentionally introduced worldwide as mosquito biocontrol agents, making them one of the most widely introduced aquatic species globally (36). Several studies have documented *G. holbrooki* introductions being responsible of the displacement and decline of native biota [reviewed in (37)], and competition from mosquitofish has been proposed as one of the main causes for the displacement of many

Mediterranean fish species, like the Mediterranean banded killifish *Aphanius fasciatus* (38, 39). This cyprinodont fish is endemic to the central-eastern Mediterranean and is listed as a protected fauna species in the Annex II of the European Habitat Directive (92/43/CEE) and Annexes II and III of the Convention on the Conservation of European Wildlife and Natural Habitats. Like mosquitofish, killifish are capable of tolerating a wide range of temperatures and salinities [e.g., from freshwater to > 60‰; (39)]. Nonetheless, their distribution is now mostly confined to higher salinity waters due to the successful establishment of mosquitofish (39, 40). Therefore, it has been proposed that salinity may limit the invasive success of mosquitofish and that high salinity may act as a refuge for the native *A. fasciatus* (39, 40).

Both *A. fasciatus* and *G. holbrooki* display reverse sexual size dimorphism, with females being larger than males, but while males of *A. fasciatus* also differ from females in body and fin coloration (i.e., being generally more colorful), coloration of male and female *G. holbrooki* is very similar (41, 42). Mating behavior in *A. fasciatus* involves male courtship displays (43, 44) and the likelihood of successful mating has been proposed to be determined by both, aggressive interactions between males and female choice [e.g., through control on the latency to spawn; (45)]. Nonetheless, to our knowledge, no research has yet investigated female mate choice in this species. By contrast, mating in *G. holbrooki* is dominated by male coercion, where males approach females from behind and thrust their gonopodium (i.e., modified anal fin) into the females' genital pore (46–48). Although female cooperation is not necessary in this mating system, females can influence the likelihood that forced copulation attempts are successful by, for example, selecting a particular male and actively staying close to it, thus facilitating copulation and increasing the future reproductive success of the offspring (47, 49). Most studies on *G. holbrooki* have focused on evaluating the influence of male body size on female preference and females have often been reported to prefer larger males [e.g., (50, 51); but see (52, 53)]. However, while a recent study of Zhou et al. (24) has investigated the effects of salinity on female preference for larger males in a close relative, the western mosquitofish *Gambusia affinis*, no research has yet investigated the influence of parasites and salinity on female mating decisions in *Gambusia holbrooki*, despite these factors being documented as contributors to their invasiveness (39, 40, 54, 55). Thus, investigating how factors such as male body size and the presence of parasites influence female-mate choice in both species will substantially increase our understanding of their mating systems. Moreover, understanding how salinity influence mate-choice interactions in invasive mosquitofish and native killifish could provide more insight into mosquitofish invasiveness and establish whether or not salinity is a key determinant of successful mosquitofish invasions and their impact on killifish.

We examined how male body size and parasitism affect female mate preferences and activity patterns in *G. holbrooki* and the co-occurring native *A. fasciatus*, and how salinity influences the strength and direction of these behaviors. To do this, we sampled female *G. holbrooki* from four and female *A. fasciatus* from three distinct, allopatric populations in Sardinia, Italy. We investigated female mate preference using dichotomous choice tests, in which we presented focal females with computer animations of pictures

of the same male, but differing in body size (i.e., large vs. small) and presence of parasites (i.e., infected vs. uninfected). Thus, we aimed to assess: (a) variation in female preferences and activity within and between populations of each species and between the two species and (b) the potential influence of changing salinity on female preferences and activity. Based on previous research, which we outlined above (24, 50, 51), and given that both species are known to withstand a wide range of salinities (36, 39), we predicted that: (i) during baseline experiments (i.e., trials examining initial female preferences within the context of each population's natural salinity) females of both species would associate preferentially with larger and non-parasitized males, and (ii) female activity and mate choice in both species would be significantly influenced by salinity, with mosquitofish showing decreased activity in higher salinity and killifish exhibiting the opposite pattern.

2 Materials and methods

2.1 Field sampling and housing conditions

Fieldwork was performed in September and October of 2021 in the central-western part of Sardinia, Italy. Adult specimens of *G. holbrooki* and *A. fasciatus* were collected with dip nets (~1–2 mm mesh size) from four and three sites, respectively (hereafter G1–G4 for sampling sites of mosquitofish and K1–K3 for sampling sites of killifish; Table 1; Figure 1; due to logistical constraints, an even sampling scheme was not possible). We measured the following parameters three to four times across consecutive days *in situ* at each site: Water temperature (°C) was measured using a Handy Polaris Probe (OxyGuard[®], Denmark) and salinity (ppt) with a Handy Salinity Probe (OxyGuard[®], Denmark).

Immediately upon capture, fish were transported to the experimental facilities at CNR-IAS in Torregrande (Sardinia, Italy). In the laboratory, specimens of each species and population were sexed based on their respective sexual characteristics [modified anal fin in *Gambusia*, (48); bar patterning in *Aphanius*, (44)]. Specimens from each population and species were housed separately in mixed-sex groups (134.89 ± 57.86 total fish per tank) in large well-oxygenated housing tanks of ~148 L (44.4 × 37 × 90 cm) for a week. The tanks were provided with artificial vegetation to resemble their natural environments and ensure the animals' welfare. Furthermore, each tank was maintained at 25 ± 0.7°C and at a 9:15 h light:dark cycle photoperiod. Tanks were checked daily for the presence of ammonium, nitrate and nitrite, but values never reached thresholds of concern (i.e., ammonium was never above 0 ppm; nitrate was never >25 mg/L and nitrite was never > 0.2 mg/L). The salinity of each tank was kept as close as possible to the environmental values measured in the field. However, this was not possible for the housing tank with killifish from site K1 given that this site was characterized by extremely high salinity levels (>60 ppt). As artificial sea salt could not be obtained, these extremely high salinity levels were impossible to replicate in laboratory settings, and we, therefore, housed this populations in marine water (~36 ppt). All wild-caught specimens were daily fed *ad libitum* with commercial food flakes (Tetra) during the acclimation period. During this time, baseline mate-choice trials (i.e., trials performed at natural salinity levels for each population

prior to salinity manipulation) were performed on nine females of each population of each species (i.e., 36 female *G. holbrooki* and 27 female *A. fasciatus*) at the same salinity level as their housing tanks. These females were subsequently removed from the setup and were not reused in any other part of the study.

2.2 Salinity acclimation protocol

After the initial acclimation period described above, we investigated salinity-dependent mate choice in two populations of each species (i.e., mosquitofish: G1 and G2; killifish: K1 and K2); logistical constraints again prevented us from doing this for all populations. Before the experiments, female and male specimens from these populations underwent a salinity acclimatization protocol, which consisted of an initial gradual adjustment of salinity in the housing tanks until the two desired experimental salinities were reached (i.e., 15 and 30 ppt), followed by 2 weeks of acclimation at those salinities in circulating systems. This protocol was not applied to all populations simultaneously but was done in two blocks. Specifically, the first block consisted of mosquitofish population G1 and killifish population K1, and the second block of mosquitofish population G2 and killifish population K2 (see details below).

2.2.1 Populations G1 and K1

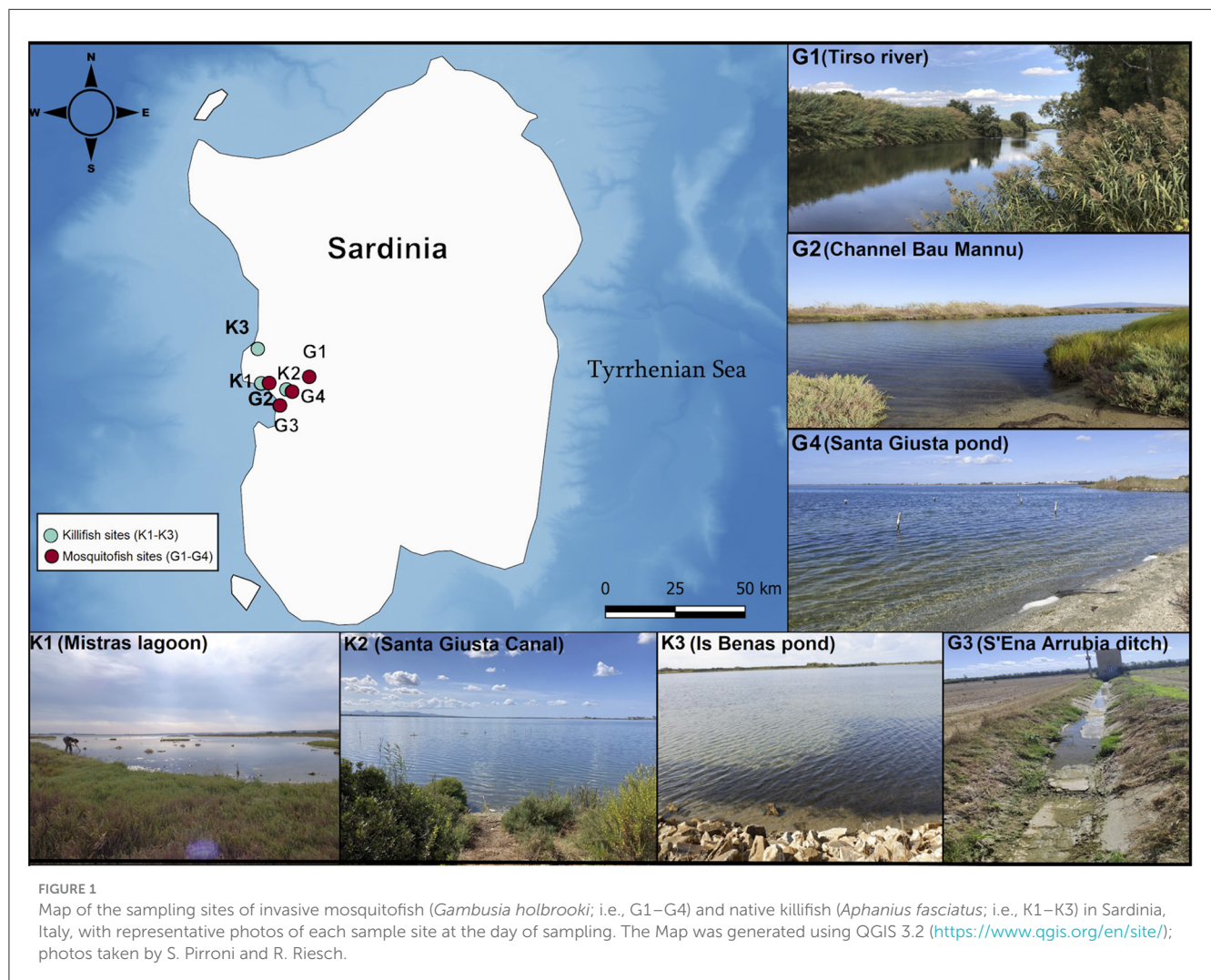
Because fish from G1 and K1 were captured at sites with divergent levels of salinity (Site G1 was a river with a mean salinity of 0.3 ppt, while site K1 was a lagoon with a mean salinity of 62.2 ppt), the protocol consisted of reducing or increasing the salinity of each housing tank by 15 ppt each day until the two desired experimental salinities (i.e., 15 and 30 ppt, ±0.7 ppt) were reached. These experimental conditions and adaptation protocols were chosen given that both mosquitofish and killifish have been reported to withstand significant spatial-temporal fluctuations in salinity (41, 56), and that similar salinity changes with these and similar species, had not revealed any potential problems in previous settings (R. Riesch, personal observation). Once experimental salinities were obtained, fish were transferred into four independent indoor recirculating systems. Specifically, three replicates per species and salinity (15 and 30 ppt) were set up, each tank holding 40 L (26 × 30.7 × 50.5 cm) and 24 fish each (12 females and 12 males). Fish of each species were kept in these tanks, and under these conditions, for 2 weeks prior to the start of behavioral assays. Water temperature in the systems was maintained at 25 ± 0.7°C given the temperatures observed during sampling. Water temperature and salinity were monitored daily using a Handy Salinity Probe (OxyGuard[®], Denmark). Furthermore, commercial food flakes were supplied *ad libitum* as a food source each day. Due to high mortality (90%) in *G. holbrooki* at 30 ppt within the first few minutes, female mosquitofish' mate choice preference and activity at 30 ppt could not be investigated.

2.2.2 Populations G2 and K2

Due to the high mortality of G1 mosquitofish at 30 ppt in block 1, the acclimation protocol for the fish for block 2 was

TABLE 1 List of *Gambusia holbrooki* and *Aphanius fasciatus* sampling sites, with site location (latitude and longitude), number of fish caught (F, females; M, males), number of tested females along with their standard length (SL), and mean values \pm SD of water temperature and salinity measured *in situ*.

Population	Location	Latitude	Longitude	Temperature (°C)	Salinity (ppt)	No. of fish caught (F/M)	No. tested females	SL (mm)
Gambusia holbrooki								
G1	Tirso river	39° 56' 2" N	8° 40' 49" E	27.63 \pm 1.42	0.30 \pm 0	232 (99/134)	18	22.8 \pm 4.6
G2	Channel Bau Mannu	39° 54' 42" N	8° 30' 43" E	26.40 \pm 3.68	23.30 \pm 7.26	339 (249/90)	27	21.0 \pm 6.9
G3	S'Ena Arrubia lagoon	39° 48' 56" N	8° 33' 35" E	24.17 \pm 1.50	8.50 \pm 11.95	58 (28/30)	9	25.7 \pm 5.5
G4	Santa Giusta pond	39° 52' 12" N	8° 36' 31" E	23.80 \pm 1.82	27.70 \pm 7.75	82 (63/19)	9	21.5 \pm 2.3
Aphanius fasciatus								
K1	Mistras lagoon	39° 54' 27" N	8° 28' 57" E	26.80 \pm 2.60	62.17 \pm 3.04	382 (257/125)	23	21.8 \pm 4.4
K2	Santa Giusta canal	39° 52' 54" N	8° 35' 11" E	27.25 \pm 2.79	33.15 \pm 4.14	194 (105/89)	27	22.4 \pm 6.5
K3	Is Benas pond	40° 2' 21" N	8° 27' 19" E	21.47 \pm 1.36	39.87 \pm 1.25	60 (30/30)	9	26.0 \pm 9.8



slightly modified. Similar to block 1, fish from these populations were found in habitats with different levels of salinity (Site G2 was a canal with a mean salinity of 23.3 ppt, while site

K2 was a lagoon with a mean salinity level of 33.15 ppt). Therefore, to avoid salinity stress (57), we adopted a protocol consisting of the gradual increasing or decreasing of salinity

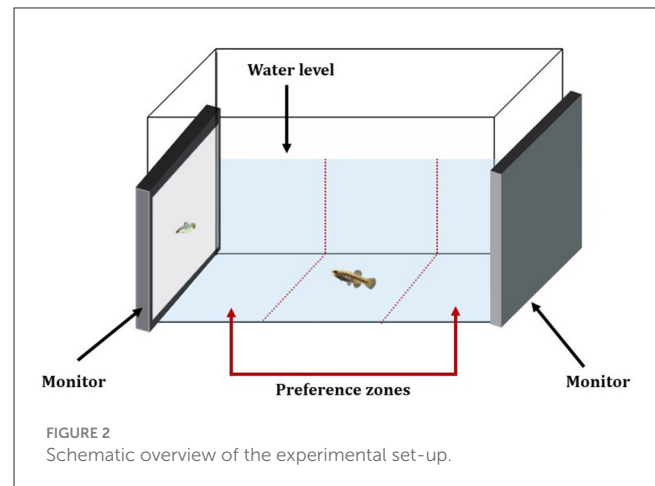
by 5 ppt every 2 days until the experimental conditions were reached. Fish of both species were then transferred into the 12 aquaria described above for block 1. Killifish mortality in the acclimatization tanks was $\sim 14\%$ whereas mosquitofish mortality was $< 5\%$.

2.3 Mate-choice experiments

2.3.1 Video animations

We investigated female preference for large and non-parasitized males in each sampled population of each species (G1-G4 and K1-K3) under different salinity settings. Specifically, we investigated female preference in nine females for each population within each testing condition. Female preference was assessed using 2D computer video animations as stimuli. The use of video animations is a validated technique to study female mate choice, and it has been previously applied in mate-choice studies on *Gambusia spp.* and other poecilids [e.g., (58–62)]. This technique allowed us to control and manipulate single male traits (i.e., male body size and parasitism) and test their effects on female preferences while keeping potential confounding factors (e.g., males' activity levels, boldness and length of gonopodium) constant. To generate animations, digital photographs were taken of individual males swimming in a narrow glass tank ($20 \times 20 \times 3$ cm) using a Nikon D70 digital camera. We took pictures of about three males from each population of mosquitofish. By contrast, because male killifish showed a high level of stress when transferred into the photo setup, we used only one picture of an original male taken in the lab and two pictures taken from the Web for this species. All fish photographed for video animations were not used in any of the experiments. Each resulting picture was then imported in Gimp [v2.10.24, (63)] to remove the background and only keep the image of the fish. Male size was manipulated at a later stage (see below), but to create parasitized males, the picture of an anchor worm (*Lernaea cyprinacea*) was pasted around the anal pore of a copy of each male's image. This freshwater parasite was chosen because it is a widely distributed common fish parasite (64, 65), it provides a clear visual cue and it was found on many collected specimens of *G. holbrooki* at our study sites (Supplementary Figure S1).

The resulting images were then animated using Adobe Animate 2021 (Adobe): The background of each picture was changed to white and the male size on the screen was digitally adjusted. Specifically, we created two pairs of animations: one pair showing two identical large males differing only in the presence or absence of *L. cyprinacea* and the other pair showing two identical males differing only in their size [i.e., one small (killifish SL: 18 ± 1.2 mm; mosquitofish SL: 14 ± 0.9 mm) and one large male (killifish SL: 27 ± 0.24 mm; mosquitofish SL: 19.6 ± 2.1 mm)]. The created animations were then converted into an mp4 file using Adobe Media Encoder 2021 (Adobe). Each animation was 130 s in duration and consisted of a male swimming vertically and horizontally, with invisible turns of 1 s. AVI video playbacks with infinite



loops of these animations were created with Windows Video Editor (Microsoft).

2.3.2 Experimental procedure

All mate-choice experiments were performed using the same experimental setup consisting of a 31L tank ($50 \times 30.4 \times 30.2$ cm) filled to a depth of 20.5 cm, covered with white opaque material on all sides to reduce external disturbance. Water temperature was again maintained at $25 \pm 0.7^\circ\text{C}$ while salinity matched salinity of the tank of origin for the tested fish (i.e., baseline salinity, 15 or 30 ppt). During each mate-choice trial, a randomly chosen female was placed at the center of the experimental arena and two CHUWI 10.1-inch tablets were placed on either side of the tank (Figure 2). After a 5-min acclimation period, during which the fish could swim freely and explore the test tank, the screens started playing the video animations of the stimulus males (e.g., one screen playing videos of a stimulus male “infected with *L. cyprinacea*” and the opposite screen playing videos of the same individual, but without *L. cyprinacea*).

Female mating behavior was recorded using a GoPro Hero9 (60 fps), mounted above the tank in a position not visible to the animals and the time spent by the focal female in the compartment near each of the monitors (i.e., preference zone) was measured for an observation period of 5 min. To detect side biases, the video playbacks were then switched off and after 1 min, they were switched on again but now with the location of each video being reversed. The behavior of the focal female was again recorded for 5 min. This procedure was repeated twice for each fish (i.e., one time with the screens displaying parasitized and non-parasitized males and another time with screens displaying animations of larger vs. smaller males). Mate-choice preference was calculated based on the association time in seconds near each of the screens (the bottom of the experimental arena was divided into three equal-sized zones using VSDC Video Editor: one central neutral zone and two peripheral preference zones near the screens; Figure 2).

In addition to estimating female preference, female behavioral inactivity was recorded as the time the focal female spent in the central neutral zone during trials. After behavioral assays, the standard length (SL in mm), total length (TL in mm) and body mass (g) of each experimental subject were measured

(Supplementary Table S1). Tested females were transferred into a new tank and were not re-used for any other procedure. To further reduce the risk of a tank effect, we used three replicates for each salinity treatment (i.e., three tanks at each level of salinity for each species) and tested a total of three females from each replicate (i.e., three females per tank) to investigate whether salinity influenced female mate choice preference. Furthermore, water in the experimental tank was replaced after every trial to avoid any potential confounding effects of chemical cues on fish behavior. After the completion of the experiments, all *A. fasciatus* were released back into the wild (depending on the opinion of the veterinarian) where they had been captured, while invasive *G. holbrooki* were sacrificed by percussive stunning in accordance with local guidelines (Art. 2, paragraph b of Legislative Decree No. 26 of 04/03/2014), and stored in 95% ethanol for potential future studies.

2.4 Statistical analyses

2.4.1 Do females exhibit a preference for large and non-parasitized males?

Following previous studies [e.g., (61, 66)] we excluded all trials from our analyses in which females exhibited a side bias; i.e., in which the focal females spent more than 85% of her total time during both 5-min trials in the same preference zone irrespective of which stimulus male was shown there. Trials in which females spent >50% of the total time in the preference zones were also discarded as females were considered not motivated to choose. For experiments with animations of large vs. small males, side biases occurred in 27 out of 122 total trials and only one trial was discarded due to low female response. For experiments with animations of parasitized vs. non-parasitized males, side biases occurred instead in 31 out of 122 trials and one trial was eliminated from the analyses due to low response. Furthermore, due to data loss from a faulty hard drive we could only analyse five out of nine females from K1 at 15 ppt.

First, we examined female preference separately for each population and testing condition (i.e., baseline, 15 and 30 ppt). This was done by comparing association times near both types of stimuli (i.e., large vs. small and parasitized vs. non-parasitized male) using paired *t*-tests.

Then, we investigated within- and between-species differences and the influence of salinity on the strength of preference (SOP). Each focal female's SOP for large vs. small and non-parasitized vs. parasitized male was calculated with the following equation:

$$\text{SOP} = \frac{\text{time spent near large (or non-parasitized) male} - \text{time spent near small (or parasitized) male}}{\text{time spent near both stimuli}}$$

SOP values ranged between from -1 (female spent all her time near small and parasitized males) to 1 (female spent all her time near large-bodied and non-parasitized males).

Analyses were conducted in two separate steps and once each for our two choice scenarios (i.e., once for large vs. small and once for parasitized vs. non-parasitized). First, because populations originated from habitats with drastically different salinities, we tested for differences in SOP between populations and species

within the baseline salinity treatments. This would help us identify if the baseline salinity already had a significant influence on fish behavior. We therefore used SOP as the dependent variable in a two-way ANCOVA, for which species (*A. fasciatus* vs. *G. holbrooki*) and “population-nested-within-species” [hereafter: population(species)] were included as independent variables along with the interaction term of “SL-by-species”, and log₁₀-transformed standard length (SL) was included as the covariate. If the interaction term had a *p*-value > 0.1, then it was subsequently removed from the final model. One ANCOVA model tested SOP within the context of a choice between a large and a small male, while a second ANCOVA model tested SOP within the context of a choice between a parasitized and a non-parasitized male of the same size.

The outcome of the above tests then determined how we planned to analyse mate choice quantified via SOP in the two experimental salinity treatments of 15 and 30 ppt. If we did not find any significant effects of “species” or “population(species)” in the baseline comparisons, then we constructed another ANCOVA with SOP as the dependent variable, SL as the covariate, and the independent variables were now “species”, “population(species)” and “salinity treatment” (15 vs. 30 ppt). We also initially added the interactions terms of “SL-by-species” and “salinity treatment-by-species”, but those were removed from the final model if *p* > 0.1. However, if we uncovered a significant effect in the initial (i.e., baseline) ANCOVA, we then implemented a mixed-effect ANCOVA on SOP with SL as the covariate, the independent variables now being “species” and “salinity treatment” (15 vs. 30 ppt), and the random effect being “baseline salinity”. We again initially added the interactions terms of “SL-by-species” and “salinity treatment-by-species”, but removed them from the final model if *p* > 0.1.

2.4.2 Does salinity influence female activity during mate choice?

To examine potential differences in female behavioral activity within and between species, and the potential influence of salinity, we applied the same statistical approach as outlined above for SOP (Section 2.4.1) also to inactivity time (i.e., time the focal female spent in the central neutral zone). Specifically, we again conducted these in two steps, because baseline salinities differed between species and populations. We therefore used “inactivity time” as the dependent variable in a two-way ANCOVA, for which “species” and “population(species)” were included as independent variables, and log₁₀-transformed standard length (SL) was included as the covariate. The interaction term of “SL-by-species” was removed from the models when it was associated with a *p*-value > 0.1 and final models were refitted with the remaining parameters. One ANCOVA model tested inactivity within the context of a choice between a large and a small male, while a second ANCOVA model tested inactivity within the context of a choice between a parasitized and a non-parasitized male of the same size.

The outcome of the above tests then determined how we planned to analyse inactivity in the two experimental salinity treatments of 15 and 30 ppt. If we did not find any significant effects in the baseline comparisons, we constructed another ANCOVA with SOP as the dependent variable, SL as the covariate, and the

independent variables were now “species”, “population(species)” and “salinity treatment” (15 vs. 30 ppt). We also initially added the interactions terms of “SL-by-species” and “salinity treatment-by-species”, but those were removed from the final model if $p > 0.1$. However, if we uncovered a significant effect of species in the initial ANCOVA, we then implemented a mixed-effect ANCOVA on inactivity, with SL as the covariate, the independent variables now being “species” and “salinity treatment” (15 vs. 30 ppt), and the random effect being “baseline salinity”. We again initially added the interactions terms of “SL-by-species” and “salinity treatment-by-species”, but those were removed from the final model if $p > 0.1$.

All *t*-tests were performed using the software R x60 3.5.1 (67), while all other analyses were performed using IBM SPSS Statistics VS 28.0.1.1 (IBM Corporation). Each model was applied after having checked model validation. Diagnostic plots were used to validate all models prior to consideration of estimated parameters.

3 Results

3.1 No female preference for large males and no influence of salinity

We did not find a significant difference in the amount of time females spent associating with the large vs. small male stimuli in any of the populations and treatment combinations for each species (all $P \geq 0.080$; Table 2). However, when not considering testing conditions (i.e., baseline, 15 and 30 ppt treatment) separately, there was substantial individual variation among females within populations, with some females spending considerably more time near the large male stimulus while others exhibited either no preference or an opposite pattern (>65% of the total time considered as an indicator of a preference; Figure 3). For instance, of the total number of female mosquitofish from population G3, 29% exhibited a preference for the small male whereas 14% showed a preference for the opposite male stimulus, and 57% did not exhibit a preference for any of the stimuli (Figure 3).

When only considering the baseline treatments, our nested ANCOVA testing for differences in SOP found no significant effects [e.g., SL: $P = 0.802$; population(species): $P = 0.599$], but indicated two non-significant trends, one for species ($P = 0.056$) and one for the interaction effect of “SL-by-species” ($P = 0.078$; Table 3A).

When testing females’ responses to 15 and 30 ppt, the interaction terms of “SL-by-species” ($P = 0.875$) and “salinity treatment-by-species” ($P = 0.262$) were removed in a stepwise fashion from the nested ANCOVA. In the resulting final model, no term had a significant effect on SOP (species: $P = 0.724$; salinity treatment: $P = 0.765$; SL: $P = 0.887$; Table 4A).

3.2 No female preference for non-parasitized males and no influence of salinity

There was no effect of non-parasitized or parasitized male stimuli on female preference within any of the populations or treatment combinations for each species (Table 5). Again, there was large variance between individuals in association time within

populations, with females exhibiting a preference for one of the stimuli on some occasions (Figure 3). For example, 33% of the female killifish from population K3 showed a preference for the non-parasitized male while 33% exhibited a preference for the parasitized male and 33% showed no preference (Figure 3).

When comparing the baseline treatments with each other, female SOP for non-parasitized males did not significantly differ between species ($P = 0.692$), between different populations of the same species [population(species): $P = 0.695$], and also not as a function of SL ($P = 0.307$; Table 3B). Furthermore, the interaction term “SL-by-species” had also not been significant ($P = 0.235$) and had been removed from the final model.

When testing females’ SOP in 15 and 30 ppt, both interaction terms (“SL-by-species”: $P = 0.887$; “salinity treatment-by-species”: $P = 0.762$) were removed in a stepwise fashion from the nested ANCOVA. Nonetheless, no term had a significant effect on SOP in the final model (species: $P = 0.078$; salinity treatment: $P = 0.762$; SL: $P = 0.145$; Table 4B).

3.3 Salinity significantly affects female activity levels during some aspects of mate choice

Our nested ANCOVA on inactivity during baseline trials and when given a choice between a large and a small male did not reveal any significant effects (species: $P = 0.340$; population(species): $P = 0.597$; SL: $P = 0.646$; Table 3C), and the interaction term of “SL-by-species” had also been removed from the initial model due to lack of effect ($P = 0.674$).

We therefore applied a nested ANCOVA also to the inactivity data from females tested in 15 and 30 ppt. Again, the two interactions “SL-by-species” ($P = 0.671$) and “salinity treatment-by-species” ($P = 0.219$) were removed from the final model. In that final model, no significant effects were discovered [species: $P = 0.271$; population(species): $P = 0.760$; SL: $P = 0.707$; Table 4C].

Our nested ANCOVA on inactivity during baseline trials and when given a choice between a parasitized and a non-parasitized male, however, did reveal significant effects of species ($P = 0.012$) and “population(species)” ($P = 0.019$), while SL was not significant ($P = 0.616$; Table 3D), and the interaction term of “SL-by-species” had been removed from the final model ($P = 0.835$). Species differences were due to mosquitofish spending a greater amount of time inactive (estimated marginal means \pm standard error: 73.89 \pm 8.38 s) than killifish (37.14 \pm 11.09 s). The effect highlighted by the nested term “population(species)” was driven by significant differences between some populations of mosquitofish, but not killifish. Specifically, G1 (estimated marginal means \pm standard error: 29.92 \pm 15.97 s) differed significantly in inactivity from G3 (113.37 \pm 17.06 s; *post-hoc* Bonferroni-corrected pairwise comparison: $P = 0.004$) and G4 (94.77 \pm 17.23 s; $P = 0.040$), while all other comparisons were not significant ($P \geq 0.108$ in all cases).

However, when we attempted to therefore implement a mixed-effect ANCOVA as outlined in our methods, the model failed to reach convergence. We therefore applied another nested ANCOVA also to this inactivity data (qualitatively, both models, the mixed-effect and the nested ANCOVA, provided the same results). This model revealed significant effects on inactivity of “salinity

TABLE 2 Parameter estimates of paired *t*-tests investigating differences in association times near large and small males.

Population	Treatment	<i>N</i>	Time (s) spent near the large male (mean ± SD)	Time (s) spent near the small male (mean ± SD)	<i>T</i> -value	<i>df</i>	<i>p</i> -value
<i>Gambusia holbrooki</i>							
G1	0 ppt (baseline)	5	292.40 ± 61.04	244.00 ± 56.19	0.963	4	0.390
	15 ppt	9	302.78 ± 55.64	232.33 ± 58.57	2.006	8	0.080
G2	15 ppt (baseline)	7	300.00 ± 144.74	249.57 ± 51.06	0.481	6	0.648
	15 ppt	8	205.75 ± 106.37	353.88 ± 112.89	−1.927	7	0.095
	30 ppt	7	236.00 ± 103.22	269.86 ± 129.51	−0.389	6	0.710
G3	0 ppt (baseline)	7	220.57 ± 110.17	319.29 ± 120.09	−1.139	6	0.298
G4	20 ppt (baseline)	9	250.11 ± 128.02	281.89 ± 100.51	−0.424	8	0.683
<i>Aphanius fasciatus</i>							
K1	35.5 ppt (baseline)	7	254.43 ± 127.81	277.86 ± 131.70	−0.241	6	0.817
	15 ppt	4	209.75 ± 67.11	268.25 ± 85.49	−0.942	3	0.416
	30 ppt	7	261.43 ± 98.40	254.71 ± 77.43	0.105	6	0.920
K2	30 ppt (baseline)	3	440.33 ± 129.28	134.33 ± 108.21	2.232	2	0.155
	15 ppt	9	320.00 ± 129.00	216.89 ± 125.02	1.232	8	0.253
	30 ppt	8	251.13 ± 110.71	244.50 ± 1124.93	0.082	7	0.937
K3	35.5 ppt (baseline)	4	224.50 ± 101.95	224.25 ± 103.01	0.998	3	0.392

“Treatment” indicates the salinity level, which was either held as close as possible to the natural salinity of the habitat of origin (indicated by “Baseline”) or was experimentally adjusted to 15 and 30 ppt (only populations G1, G2, K1, and K2). *N* refers to the number of fish tested. All *p*-values are two-tailed, and times are provided in seconds.

treatment-by-species” ($P = 0.004$), “SL-by-species” ($P = 0.002$), species ($P = 0.002$), salinity treatment ($P < 0.001$), and SL ($P = 0.016$), while “population(species)” was not significant ($P = 0.981$; Table 4D). Specifically, while there were general differences between *Gambusia* and *Aphanius*, with the former spending more time inactive than the latter (estimated marginal means ± standard error: 127.32 ± 12.99 s and 74.07 ± 9.26 s, respectively), and general differences in inactivity between salinity treatments (i.e., 71.29 ± 8.96 s at 15 ppt compared to 130.10 ± 12.59 s at 30 ppt), the way of how the salinity treatment affected inactivity, differed between the species with *Gambusia* showing a much greater increase in inactivity compared to *Aphanius* (Figure 4A). Similarly, while there was an overall decrease in inactivity with SL (Figure 4B), this pattern only held true for *Aphanius*, while in *Gambusia*, larger females increased their inactivity (Figure 4C).

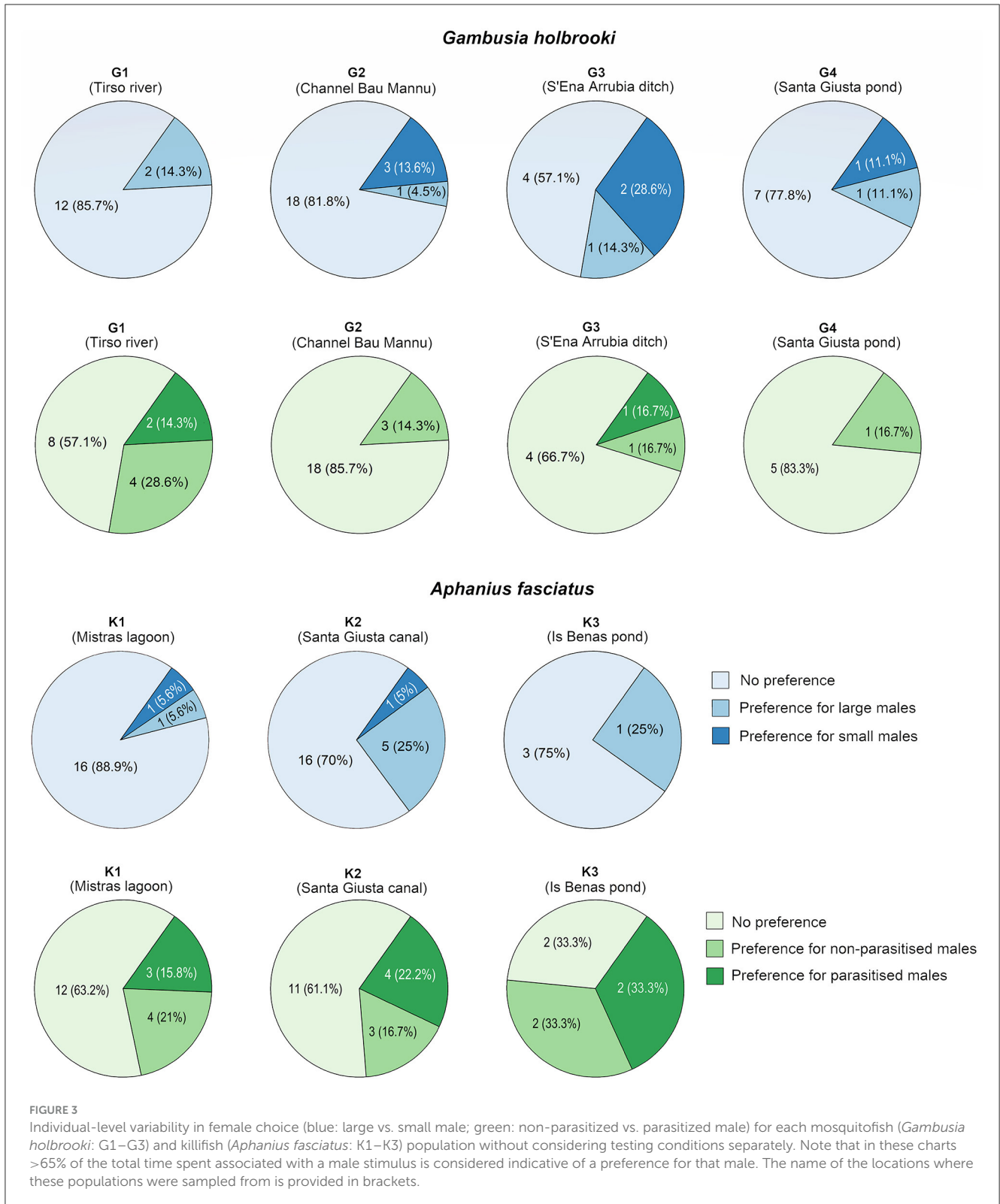
4 Discussion

We investigated how male body size and parasitism influence female behavioral activity and mating preferences in multiple populations of invasive eastern mosquitofish and native killifish. Furthermore, we examined how salinity influences the strength and direction of these behaviors. We did not find significant female preferences, significant effects of salinity on female preferences, or significant variation in preference between and within species. However, our analyses did reveal that salinity affected female activity during mate choice trials, with mosquitofish becoming less active at high salinities and killifish exhibiting the opposite pattern.

4.1 Female mosquitofish and killifish do not prefer to associate with larger males

Male body size is often considered an indirect signal of male dominance and a critical component of male fitness that can have direct benefits (e.g., increased fecundity and reduced predation risk) to the choosing female and/or confers indirect benefits to its offspring fitness and viability (13). Our first prediction stated that females of both species would exhibit a preference for large males. However, our analyses did not reveal a significant preference by females for larger males in either species. To our knowledge, this is the first study to investigate female mating decisions in *Aphanius fasciatus*. However, across mosquitofish more broadly, our findings contrast with those reported by several previous studies on mosquitofish [e.g., (51, 68)] and other poeciliids [e.g., in *Heterandria formosa*: (69); in *Xiphophorus nigrensis*: (70)], but are congruent with a previous study of Bisazza and Marin (52) on another population of invasive *G. holbrooki* from Italy.

Our results, thus, suggest indifference of female mosquitofish and killifish toward male stimuli differing in body size but also that male body size might play only a small role in sexual selection for these species. In many poeciliids, large males court females while small males rely on sneaking to copulate (46). Such sexual harassment by small males can be highly costly to females [e.g., reduce their foraging efficiency; (71)], hence, resulting in females preferring to associate with large males to avoid the costs of harassment (46). In *G. holbrooki*, however, as males do not court females, but males of all sizes try to force copulation, the cost of associating with a large male could be equal to the cost of associating with a smaller male. This may result in



females not exhibiting any preference, but rather associating with males apparently at random (i.e., potentially based on individual circumstances that might change from day to day). Furthermore, this could also explain why we found strong individual variability in mating preference within mosquitofish populations in our study.

Such variation among females in their choosiness has also been documented before [e.g., (72–74)], indicating that it is important to distinguish between population- and individual-level preferences when interpreting the mating behavior of a species. With respect to *A. fasciatus*, a large male body size alone may not be a strong

TABLE 3 Parameter estimates of nested ANCOVAs on baseline (i.e., natural salinity) treatments investigating the effects of species (*Aphanius fasciatus* vs. *Gambusia holbrooki*), "population nested within species" [population(species)], standard length (SL) and the interaction SL-by-species on female strength of preference (SOP) for (A) larger males and (B) non-parasitized males; and the effects of species, population(species), SL and the interaction SL-by-species on female inactivity during mate choice trials investigating preferences for (C) larger males and (D) non-parasitized males.

Variable	df	Sum of squares	F-value	P-value
(A) Female SOP during trials "large vs. small"				
SL	1, 33	0.010	0.064	0.802
Species	1, 33	0.593	3.641	0.065
Population(species)	5, 33	0.603	0.740	0.599
SL × species	1, 33	0.538	3.304	0.078
(B) Female SOP during trials "non-parasitized vs. parasitized"				
SL	1, 34	0.307	2.075	0.307
Species	1, 34	0.046	0.160	0.692
Population(species)	5, 34	0.869	0.608	0.694
SL × species	1, 33	0.412	1.461	0.235
(C) Female inactivity during trials "large vs. small"				
SL	1, 34	311.482	0.215	0.646
Species	1, 34	1,357.893	0.937	0.340
Population(species)	5, 34	5,380.893	0.743	0.597
SL × species	1,33	267.342	0.180	0.674
(D) Female inactivity during trials "non-parasitized vs. parasitized"				
SL	1, 34	447.536	0.256	0.161
Species	1, 34	12,263.582	7.026	0.012
Population(species)	5, 34	27,443.619	3.145	0.019
SL × species	1,33	78.760	0.044	0.835

Interaction terms in gray were removed from the final model to increase statistical power if $P > 0.1$ and significant P -values are in bold.

indicator of male dominance and benefits for the female but other traits such as number and span of the bars along the body flank could have a stronger influence in male mating success as suggested by Malavasi et al. (45) and observed in swordtails (75).

Furthermore, we cannot exclude that multiple-interacting factors drove the observed patterns. Personality traits also play an important role in female mating decisions and often affect male body size effects on female preferences (68, 74). For instance, Chen et al. (68) found an increasing female preference for larger males with increasing male boldness and activity levels in western mosquitofish, and other studies found female mating decisions to be influenced by social context (62, 76). Thus, we cannot exclude that our results may have been due to our specific setup (i.e., no additional conspecifics or cues on male personality).

We used computer animations to examine female mate-choice, therefore, it is possible that the lack of female preference for larger males in both species was the result of females not being able to discriminate animated male stimuli. However, we think this to be unlikely for two reasons. First, the use of animations for investigating mate-choice has been validated in multiple systems,

including Poeciliidae [e.g., swordtails: (59, 77); guppies: (78); mollies: (72, 79); western mosquitofish: (24, 68)]. Second, we conducted several trial runs of this experimental setup using our laboratory stocks of *G. holbrooki* (an invasive population from southern Italy) at Royal Holloway, University of London, in the summer of 2021. These trial runs resulted in strong trends for preferences for (a) large males [$t_{(8)} = 1.095, p = 0.093$] and (b) non-parasitized males [$t_{(8)} = 2.055, p = 0.070$]. Third, while computer animations have not yet been applied to test mating decisions in *A. fasciatus*, the finding of high visual acuity in the congener species, *A. sirhani* (80), suggest that *A. fasciatus* should be able to discriminate between animated potential partners. Nonetheless, we acknowledge that a computer image may not allow size- or parasitization-perception as clearly as a real conspecific would be and so this is a potential limitation of the employed methods. Additionally, we used testing protocols with 2×5 min observation periods and video animations that are well-established for poeciliid fishes [e.g., (81–83); including previous studies used in our lab, such as (72, 84)], but we cannot completely rule out that this is not long enough, or that the rather uniform movement of the animated fish is not stimulating enough, to elicit a response for *A. fasciatus*.

TABLE 4 Parameter estimates of nested ANCOVAs on experimental salinity (i.e., 15 and 30 ppt) treatments investigating the effects of species (*Aphanius fasciatus* vs. *Gambusia holbrooki*), "population nested within species" [population(species)], salinity treatment, standard length (SL) and the interactions SL-by-species and salinity treatment-by-species on female strength of preference (SOP) for (A) larger males and (B) non-parasitized males; and the effects of species, population(species), salinity treatment, SL and the interactions SL-by-species and salinity treatment-by-species on female inactivity during mate choice trials investigating preferences for (C) larger males and (D) non-parasitized males.

Variable	df	Sum of squares	F-value	P-value
(A) Female SOP during trials "large vs. small"				
SL	1, 46	0.003	0.020	0.887
Species	1, 46	0.018	0.126	0.724
Population(species)	2, 46	0.613	2.196	0.123
Salinity treatment	1, 46	0.013	0.091	0.765
SL × species	1,44	0.004	0.025	0.875
Salinity treatment × species	1,45	0.179	1.290	0.262
(B) Female SOP during trials "non-parasitized vs. parasitized"				
SL	1, 42	0.409	2.206	0.145
Species	1, 42	0.606	3.270	0.078
Population(species)	2, 42	0.062	0.168	0.846
Salinity treatment	1,42	0.017	0.093	0.762
SL × species	1,40	0.004	0.020	0.887
Salinity treatment × species	1,41	0.018	0.093	0.762
(C) Female inactivity during trials "large vs. small"				
SL	1, 46	384.363	0.143	0.707
Species	1, 46	3,333.004	1.239	0.271
Population(species)	2, 46	1,488.461	0.277	0.760
Salinity treatment	1,46	7,211.325	2.680	0.108
SL × species	1,44	494.777	0.183	0.975
Salinity treatment × species	1,45	4,139.493	1.557	0.219
(D) Female inactivity during trials "non-parasitized vs. parasitized"				
SL	1, 40	12,975.464	6.299	0.016
Species	1, 40	21,587.625	10.480	0.002
Population(species)	2, 40	77.122	0.019	0.981
Salinity treatment	1,40	31,939.083	15.505	<0.001
SL × species	1,40	23,888.434	11.597	0.002
Salinity treatment × species	1,40	19,348.800	9.393	0.004

Interaction terms in gray were removed from the final model to increase statistical power if $P > 0.1$ and significant P -values are in bold.

4.2 Female mosquitofish and killifish do not prefer to associate with non-parasitized males

In addition to mating with large males, mating with non-parasitized males is also thought to have fitness benefits for choosing females (79). Thus, we predicted females of both species to exhibit a preference for non-parasitized males. However, contrary to our expectation, we did not find any population-level preference for non-parasitized vs. parasitized male stimuli. While we know of no other studies that have examined the influence of parasites on female mating preferences in both *Aphanius* and *Gambusia* [but

see (18) for male mate choice in *G. affinis*], our results contrast with several previous studies in other Poeciliids (79, 85), where females preferred to mate with males that had no (or few) parasites over (more heavily) parasitized males.

Here, we digitally "infected" males with the ectoparasitic copepod (also called anchor worm), *Lernaea cyprinacea*. This parasite has a direct life cycle consisting of adult females releasing eggs onto the sediment, which hatch into non-parasitic nauplii that molt into parasitic copepods, attach to several parts of a fish host and undergo further metamorphosis. While attached to the host, this parasite penetrates fish skin and causes inflammation and lesions that might become necrotic or lead to secondary

TABLE 5 Parameter estimates of paired *t*-tests investigating differences in female association times near parasitized and non-parasitized males.

Population	Treatment	<i>N</i>	Time (s) spent near the non-parasitized male (mean ± SD)	Time (s) spent near the parasitized male (mean ± SD)	<i>T</i> -value	<i>df</i>	<i>p</i> -value
<i>Gambusia holbrooki</i>							
G1	0 ppt (baseline)	7	188.33 ± 168.80	384.33 ± 165.85	-1.438	5	0.210
	15 ppt	7	266.14 ± 98.49	249.71 ± 129.00	0.193	6	0.853
G2	15 ppt (baseline)	6	301.33 ± 79.40	245.83 ± 75.98	0.966	5	0.378
	15 ppt	8	305.50 ± 101.16	243.88 ± 86.26	0.898	7	0.399
	30 ppt	7	254.57 ± 121.79	205.71 ± 24.07	0.727	6	0.495
G3	0 ppt (baseline)	6	268.22 ± 120.56	244.33 ± 151.16	0.27	8	0.794
G4	20 ppt (baseline)	6	277.22 ± 110.37	247 ± 121.35	0.399	8	0.700
<i>Aphanius fasciatus</i>							
K1	35.5 ppt (baseline)	8	259.50 ± 213.11	305.38 ± 211.99	-0.306	7	0.769
	15 ppt	5	159.40 ± 122.19	357.20 ± 121.91	-1.853	4	0.137
	30 ppt	6	260.33 ± 134.59	268 ± 130.29	-0.071	5	0.946
K2	30 ppt (baseline)	3	267.33 ± 160.13	297.33 ± 186.83	-0.15	2	0.895
	15 ppt	7	278.66 ± 95.78	269.43 ± 86.71	0.138	6	0.895
	30 ppt	8	204.70 ± 175.34	326.00 ± 158.08	-1.168	9	0.273
K3	35.5 ppt (baseline)	6	272.00 ± 177.38	285.00 ± 155.37	-0.096	5	0.927

"Treatment" indicates the salinity level, which was either held as close as possible to the natural salinity of the habitat of origin (indicated by "baseline") or was experimentally adjusted to 15 and 30 ppt (only populations G1, G2, K1, and K2). *N* refers to the number of fish tested. All *p*-values are two-tailed, and times are provided in seconds.

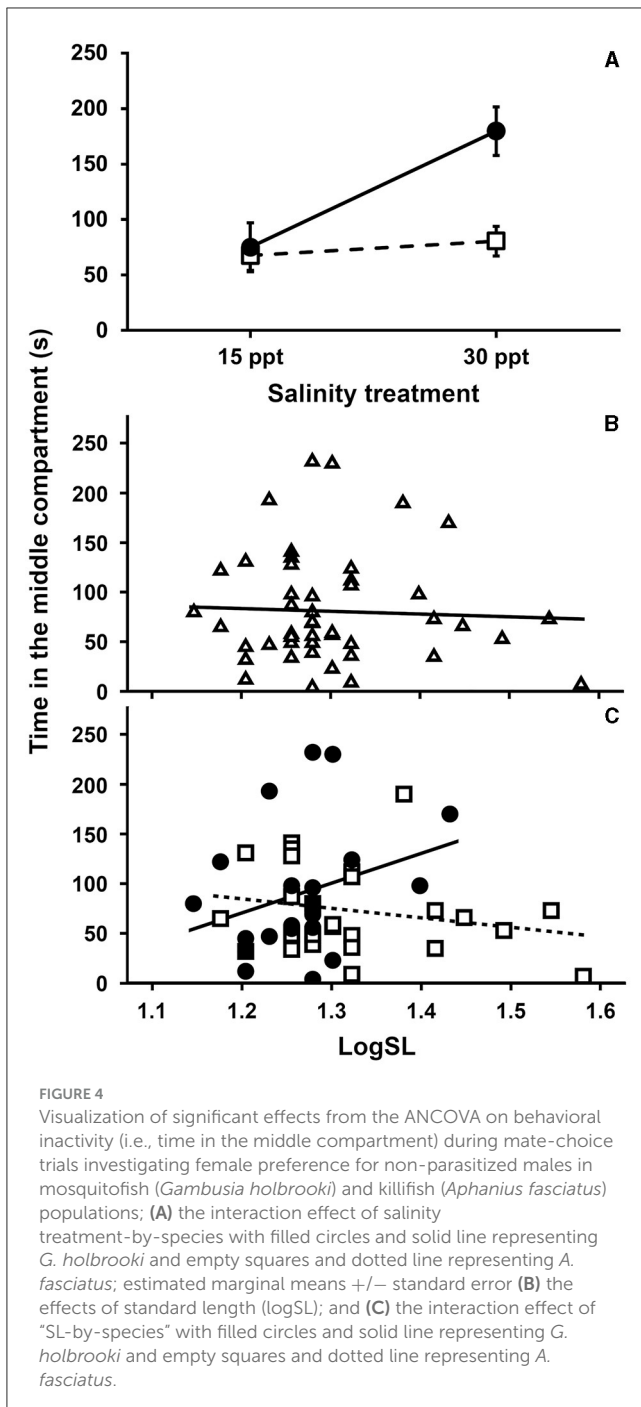
infections (65). Moreover, infection by this parasite often leads to a reduction in fish growth, fecundity and swimming abilities (64, 86). Fish in the wild are often able to reject these parasites even after penetration has occurred (87), so the observed lack of female responsiveness toward male stimuli differing in the parasites could be due to the fact females did not perceive this parasite to affect male reproductive state. Alternatively, we cannot rule out that females showed no preference because they did not observe any secondary infections (e.g., fungal infection) or other characteristics such as reduced swimming performance on the infected males in our video animations.

Furthermore, salinity has been documented to affect how well this parasite reproduces, with direct infection being significantly reduced at high salinities (65). This could explain why we did not find this parasite in any of the female specimens of *A. fasciatus* captured for this study (killifish were found in habitats often characterized by salinities >30 ppt) and a female preference for non-parasitized males in this species. However, we found anchor worms in almost all mosquitofish populations and a preference for uninfected males was not found even in the mosquitofish populations sampled in freshwater habitats (i.e., G1 and G3).

4.3 Salinity does not influence female preferences but activity levels during choice

In partial contrast with our prediction 2 (i.e., salinity effects on both female activity and mate-choice), our analyses did not reveal a significant effect of salinity on the strength and direction

of female preferences in both species. However, congruent with this prediction, salinity significantly affected female activity levels, with mosquitofish being more active at lower salinities and killifish showing the opposite pattern. To our knowledge, this is the first study to investigate salinity effects on female preference and activity levels during mate choice in these species. Our results align with those of a recent study by Zhou et al. (24), who found female western mosquitofish (*G. affinis*) reduced their activity levels with increasing salinity. However, that study also found female *G. affinis* to prefer larger males only under freshwater and low-salinity conditions. Such context-dependent reduction in female activity during mate-choice has also been documented in invasive guppies, *Poecilia reticulata* (88). Specifically, the authors uncovered a reduction in female sexual activity and preference for male stimuli under predation threat. Together with our results, this suggests that the levels of female sexual activity in a species can be highly dependent on local habitat characteristics and environmental factors. In invasive species, changes in sexual activity may potentially be a way to adapt to novel environments. In this context, it is important to note that we only found a significant effect of salinity on inactivity in one of our two choice scenarios (i.e., when given the choice between a parasitized and a non-parasitized male) but that the underlying patterns of inactivity were the same in both settings (i.e., when choosing between a large and a small male, estimated marginal mean inactivity ± standard error for *G. holbrooki* at 15 ppt: 52.12 ± 12.66 s; at 30 ppt: 105.22 ± 23.54 s; *A. fasciatus*, at 15 ppt: 84.31 ± 14.94 s; 30 ppt: 95.83 ± 13.49 s). Nonetheless, we only measured inactivity within the context of mate choice and therefore call on future studies to investigate if this pattern holds also across other behavioral contexts.



Moreover, in our comparison of inactivity during baseline trials when females were given a choice between a parasitized and a non-parasitized male, we had found *G. holbrooki* to spend more time inactive than *A. fasciatus*, and that the *G. holbrooki* from G1 (habitat/baseline salinity of 0 ppt) spent less time being inactive than *G. holbrooki* from G3 (0 ppt) and G4 (20 ppt). Since baseline salinities between *G. holbrooki* and *A. fasciatus* were consistently greater in *A. fasciatus*, we cannot tease apart whether the species difference is based on taxon-specific differences or rather based on the salinity differences between the sampled habitats. However, the second effect on population differences in *G. holbrooki* partially

supports the interpretation that salinity affects patterns of inactivity at least in *G. holbrooki*. However, since we also found significant differences between two habitats of the same salinity (i.e., 0 ppt), this also highlights the importance of testing multiple population replicates for certain environmental conditions, when trying to identify the impact of that environmental factor on certain organismal traits.

Observed salinity effects on female behavioral activity in our study species alone do not help explain mosquitofish invasiveness in Europe. However, taking these results together with those of another study we performed on the same populations in Sardinia (all authors, unpublished data) and a study of Alcaraz et al. (40), where mosquitofish food consumption and aggressiveness were significantly reduced at high salinities while killifish showed opposite patterns, this maps onto the current distribution patterns of invasive mosquitofish and native killifish in the Mediterranean. Hence, this suggests that salinity may limit the negative effects of invasive mosquitofish and that high-salinity habitats may act as a refuge for native killifish.

5 Conclusion

In conclusion, the results of this study suggest that male body size and parasitization with *Lernea* may play little role in the sexual decisions of invasive mosquitofish and native killifish females in Sardinia. In contrast, salinity appears to profoundly alter female sexual activity in both species and these effects also help explain their distribution patterns in other parts of Europe. Specifically, our findings suggest that while increasing salinization of freshwater habitats poses a serious global threat to ecosystem health and biodiversity (31), it may decrease the potential for freshwater invasive species such as *G. holbrooki* to spread in aquatic systems. Hence, salinization may reduce their impacts on native biota such as *A. fasciatus* (24).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The datasets generated and analyzed for this study can be found on Figshare (doi: 10.17637/rh.26067916).

Ethics statement

The animal study was approved by the Animal Welfare Ethical Review Board at Royal Holloway, University of London (RHUL-NRR-0038-2020). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SP: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. FL: Investigation, Writing – review & editing.

JC: Investigation, Writing – review & editing. PD: Methodology, Resources, Writing – review & editing. MB: Methodology, Supervision, Writing – review & editing. SM: Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. RR: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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