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THE EVOLUTION OF MATE CHOICE IN THE GENUS *LIMIA*

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BY THE COMMITTEE CONSISTING OF

Dr. Ingo Schlupp, Chair
Dr. Piers Hale
Dr. Mariëlle Hoefnagels
Dr. Michael Patten
Dr. Cameron Siler

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ABSTRACT

Mate choice is a discipline of sexual selection research describing the process of an individual biasing resource use to reproduce with preferred mates. Classical theory describes mate choice as only occurring in females or extreme cases, such as sex-role reversal. However, mounting evidence of male mate choice in various animal systems has since debunked this theory. Although male mate choice occurs in many taxa, empirical studies examining the factors that shape male choice are lacking. Theory suggests that male preference can persist if the benefits of mating with high-quality females outweigh the costs of mate choice.

Given that many males have evolved elaborate secondary sexual traits (SST), such as ornaments, armaments, and courtship displays, we sought to understand how male SST influence mate choice in polygynous mating systems (i.e. populations were males and females mate multiple times). We predicted more elaborate SST should select for more exaggerated preferences for high quality mates. To test my prediction, we examined how interspecific and intraspecific variations in SST impacted mate choice in the genus *Limia*. *Limia* is an understudied group of livebearing fishes endemic to the Caribbean, with many mating strategies. In both my intraspecific and interspecific mate choice experiments, we found a lack of preference in both males and females regardless of male SST, except for the species with minimal male SST. These results were unusual because both male and female choice, which has been widely described in livebearing fishes, were absent in multiple species. We began to suspect that *Limia*'s unique preferences were a product of their unique geographic distribution. *Limia* are native to four islands in the Greater Antilles: Cuba, Hispaniola, Jamaica, and Grand Cayman. Biogeography theory predicts island landmass is positively correlated with species diversity, suggesting Cuba should host the greatest diversity of *Limia* species. However, of the 22 species of *Limia,* 19 are

on Hispaniola, the second largest island, while only one species is on each of the other islands. To begin understanding if this unusual pattern influenced mate preference, we constructed a phylogeny (i.e., a computational hypothesis of the evolution of a group of organisms based on genetic similarities) of *Limia*. We found evidence of two relatively recent radiation events on Hispaniola; however, further work is necessary to determine if these have impacted mate choice in this group.

CHAPTER 1: Males can't afford to be choosy: Male reproductive investment does not influence preference for female size in *Limia* (Poeciliidae)

Authors: Montrai Spikes *, Ingo Schlupp

Department of Biology, University of Oklahoma, United States

Abstract

Reproductive investment was initially thought to be the key selective force behind male mate preference for female characters, like size or ornamentation (i.e., the preference by males for certain females). Yet, evidence of polygynous species, where male reproductive investments are often inexpensive compared to those of females, have also been described to possess male preference. Our study aims to understand how reproductive investment influences the selection of choosy males in polygynous systems using two species of livebearing fishes varying in reproductive investment: *Limia perugiae*, in which males invest heavily into reproduction, and *L. zonata* in which males invest minimally into reproduction. We hypothesized that male reproductive investment when combined with fecundity selection will favour the evolution of male mate preference and thereby lead to males that invest heavily into reproduction being choosier than males that invest minimally. When male *Limia* were exposed to two females simultaneously, one from the small size class and one from the large size class, *L. zonata* chose large females over small ones, whereas *L. perugiae* allocated the same amount of time regardless of female size class. Although we fail to find support for our original hypotheses, our study highlights the need for a more thorough examination of non-model species like *Limia*. We suggest future studies analyse reproductive investment as it interacts with cryptic choice, multiple sensory cues as well as expand comparisons to multiple *Limia* species, especially those endemic to Cuba, Grand Cayman, and Jamaica.

1. Introduction

Reproductive investment was initially thought to be the key selective force behind male mate preference (i.e. how much an individual is attracted to a given character or potential mate) and male mate choice (i.e. which potential mate an individual decides to focus their reproductive efforts on) (Cummings, 2012; Bisazza et al., 2001; Godin and Briggs, 1996). Because females make a larger initial reproductive investment (e.g. oocyte production and gestation) than males, female choice evolves more readily than male choice. Only when male reproductive investment rivals or exceeds female reproductive investment, such as in monogamous or polyandrous systems, was it thought that males can evolve preference (Bateman, 1948). Yet, evidence of male mate preference has been found in polygynous species, where male reproductive investments were thought to be inexpensive compared to females, such as in fruit flies (*Drosophila*) (Byrne and Rice, 2006), guppies (*Poecilia reticulata*) (Herdman et al., 2004), and marine snails (*Littorina saxatilis*) (Johannesson et al., 2008) and in livebearing fishes in general (Schlupp, 2018). In separate reviews, Bonduriansky (2001) and Edward and Chapman (2011) argued that in addition to male investment, in populations where males are unable to mate with all available females, females vary in quality and the operational sex-ratio (OSR) is male biased are more likely to evolve male mate preference and choice. Theory suggests selection against male mate preference in polygynous systems is strong but can be overcome by male preference for female fecundity (Fitzpatrick and Servedio, 2017). The growing evidence of male preference in polygynous systems has reformed our current understanding of mate choice and sexual selection. Because males are dependent upon females for reproduction and the OSR is usually male biased

in polygynous systems, males that decline matings suffer a high opportunity cost. This opportunity cost directly selects against choosy males and can be exacerbated or relaxed depending on if mates are presented sequentially or simultaneously, respectively (Barry and Kokko, 2010; Head et al., 2015). Opportunity costs are similarly increased or reduced if males can copulate with a few or every available female, respectively. If males are unable to mate with all available females, which has been shown in livebearing fishes (Schlupp and Plath, 2005; Aspbury and Gabor, 2004), opportunity costs can also quality offspring (Riesch et al., 2008; Nakahashi, 2008). Empirical evidence in a diverse array of taxonomic groups has supported theoretical models finding males often evolve a preference for female traits that indicate fecundity such as female size (Jones et al., 2001; Rosenqvist, 1990; Sargent et al., 1986; Verrell, 1985). Although male preference for more fecund females can be selected for in polygynous systems, the role reproductive investment plays in the evolution of male mate preference remains unclear (Arriaga and Schlupp, 2013; Schlupp, 2018). Evidence does suggest pre-copulatory investments, such as courtship, ornamentation, and nuptial gifts, often involve a variety of tradeoffs, and can be quite costly. As predicted by life history theory, for instance, male secondary sexual characters present tradeoffs between predation and foraging, growth rate and mortality, growth and immune function, and growth and physiological stressors (Godin and McDonough, 2003; Devigili et al., 2015; Weinstein et al., 2019). Mate preferences are most beneficial when fecundity selection is sufficient to compensate for the trade-offs associated with pre-copulatory reproductive investment. Therefore, we hypothesize that a combination of fecundity selection and pre-copulatory reproductive investment would increase the probability of male preferences evolving in a population. Furthermore, males that invest more heavily into pre-copulatory secondary sexual traits risk extremely reduced fitness if they do not maximize the

number of offspring through preference for highly fecund females (Fig. 1). To understand how reproductive investment influences the selection of choosy males in polygynous systems, we used two species of *Limia, L. perugiae* and *L. zonata. Limia* is a genus of livebearing fishes (Poeciliidae) native to the Caribbean. *Limia* are closely related to and share many life history characteristics with other livebearing fishes that possess male mate preferences for female body size such as guppies (*Poecilia reticulata*), mosquitofish (*Gambusia holbrooki*), and sailfin mollies (*Poecilia latipinna*) (Auld and Godin, 2015; Auld et al., 2015, 2016, 2017; Jeswiet et al., 2011, 2012; Hoysak and Godin, 2007; Ptacek and Travis, 1997). Thus, it seems likely that male preference for fecundity is present in the group. Additionally, *Limia* possess over 20 described species with a diversity of male sexual traits and behaviours varying in reproductive investment (Farr, 1984). This study compares two species of *Limia* which have similar life histories but differing investments into ornaments and courtship (Farr, 1984; Goldberg et al., 2019). *Limia perugiae* males are heavily ornamented and perform courtship displays to solicit copulations in contrast to *L. zonata* which have minimal ornamentation and rely on coercion to copulate with females (Fig. 2, Farr, 1984; Goldberg et al., 2019). We infer that *Limia perugiae's* high pre-copulatory reproductive investment relative to *L. zonata* males likely are at the expense of reduced longevity, immune function, foraging, and increased predation risk. Together, these qualities make *Limia,* in particular *L. perugiae* and *L. zonata,* ideal for our comparative study. To compare male mate preference between *Limia* species, we ran dichotomous choice tests and absolute preference function assays on the two *Limia* species. Dichotomous choice tests are commonly used to determine directionality and presence of mate choice (Dougherty and Shuker, 2015) while absolute preference function assays measure mate preference in the form of preference functions which are used to determine the shape and degree of preference (Wagner,

1998). We define preference functions as the correlation between the individual's allocation of reproductive resources (time) and the phenotypic trait (female size) of potential mates (Lande, 1981; Edward, 2015, Fig. 1). Additionally, dichotomous choice tests are more sensitive than absolute preference function assays to assess? "mate choice" because the chooser is forced to select between mates (Wagner, 1998). This choice provides an idea of which potential mate's phenotype is relatively more attractive but introduces social information which can influence mating decisions (Auld and Godin, 2015; Auld et al., 2015, 2017). In contrast, absolute preference function assay measures the chooser's preference by showing multiple potential mates varying in the selected phenotype in sequence rather than simultaneously, thus omitting the social context and choice, thereby measuring what choosers find absolutely attractive (i.e. mate preference) (Wagner, 1998). These absolute measurements allow for more nuanced comparisons, of preference between and among populations, than results of dichotomous choice tests. However, sequentially introducing potential mates introduces an opportunity cost to choosers, which can influence results (Barry and Kokko, 2010). Both methodologies are critical to the understanding of mate choice but differ in what they measure (Dougherty and Shuker, 2015; Head et al., 2015). Our study aims to compare both methodologies while contrasting the interpretations that can be extrapolated from the results. Although meta-analyses comparing dichotomous choice test and sequential or no-choice tests have been conducted, they have seldom been compared empirically (Dougherty and Shuker, 2015; Head et al., 2015). Dichotomous choice tests will yield what males choose when offered two females of varying sizes, and absolute preference functions will show what males determine to be an ideal mate. We also compared preference functions of males of both *Limia* species using female size as a proxy for fecundity and association time as a proxy for preference, which is well established (Helfman

et al., 2009; Bischoff et al., 1985; Berglund, 1995; Kodric-Brown, 1993; Witte, 2006). We predicted that males from both *Limia* species will choose larger mates over smaller mates, although *L. perugiae*'s high reproductive investment will lead to a stronger degree of preference than *L. zonata*. Using both dichotomous choice tests and absolute preference functions we examine the underlying assumption in sexual selection, that investment into costly traits favours mate choice and preference. In addition, we compare the strength and weakness of both widely used methodologies employed in mate choice research, thus calling attention to the need for more thoughtful use of terminology and experimental practices.

2. Methods

2.1. Collection and size classification Two species of the genus *Limia* were used for this study, *L. perugiae* and *L. zonata*. *Limia perugiae* were collected from a small ditch near the south shore of Lake Enriquillo in the Dominican Republic (18∘24′4.61″ N, 71∘34′16.61″W) in 2014. *L. zonata* were collected in 2012 in shallow water of the Río Yuna near Bonao in the Dominican Republic (18∘57′33.5″ N, 70∘24′32.1″ W). After field collection, fishes were transported to a greenhouse at the University of Oklahoma's Aquatic Research Facility, where they are kept under common garden conditions. Fishes used in this study were approximately four generations removed from the wild-caught populations. Behavioural trials were conducted during September and October of 2016 at the University of Oklahoma Norman Campus. Forty individuals (20 male and 20 female) of each species were haphazardly obtained from the Aquatic Research Facility using a small seine, then transported to an indoor climate-controlled fish-room held at 26 ∘C ($\pm$3 ∘C) on a 12 -h day-night cycle. Each species was separated by sex and placed into 37 L holding tanks. The fishes were given 14 days to acclimate to the laboratory environment before any subsequent handling. While in the fish-room, fishes were fed *ad libitum* a mixture of blood

worms, *Daphnia*, brine shrimp, and TetraMin flakes twice daily. After the fishes acclimated to the laboratory, we measured both male and female body size as standard length (tip of the snout to the end of the vertebral column) using a laminated millimetre grid to the nearest tenth of a millimetre. During measurements, individuals were photographed (Nikon D52000 camera with a Nikon AF-S DX NIKKOR 18 200 mm f/ 3.5–5.6 G ED VR II Standard Zoom Lens). To keep fishes immobile while being photographed, fishes were anesthetized with 100 mg of tricaine methane sulfonate (MS222) in a 1 L tank. Fishes were immediately placed in a 5 L recovery tank after being photographed and monitored for 20 min to assure recovery. Fishes were then returned to the fish-room for two days before subsequent handling. Female body size was compiled for each species into body size distributions. Using these distributions, we determine discrete female size classifications for each species (small, medium, and large). Using median female body size as anchor, we split each species' females size distribution into four equal quadrants. The two most extreme quadrants were deemed small and large classes, while the two interior quadrants were combined into the medium class. Size cut-offs varied slightly between species and are reported in Fig. 3. The classification of females into discreet size categories is independent of the classification of male investment. We used male mating strategies to classify the two species into varying degrees of pre-copulatory reproductive investment relative to one another. *Limia perugiae* males' use of courtship and ornamentation classified them as high precopulatory reproductive investment (Goldberg et al., 2019; Farr, 1984). *L. zonata* served as the low pre-copulatory reproductive investment species, due to their minimal ornamentation and lack of courtship (Goldberg et al., 2019; Farr, 1984).

2.2. Experimental setup Dichotomous choice tests and absolute preference functions assays were conducted in a 76 L tank without gravel, divided into three equal sections lengthwise, denoted by

two vertical lines drawn on the front pane of the tank. Within each section was an 8.5 cm × 8.5 cm × 46 cm hollow clear unperforated Plexiglas rectangular cylinder. Cylinders were used to restrict movement, mechanosensory signals, and chemical cues, while still allowing visual communication of fish (Fig. 2). We elected to use only visual stimuli, because visual cues are key in mate assessment in livebearing fishes (Houde, 1997), and the use of one variable simplifies statistical analyses. Moreover, previous studies have demonstrated visual cues are sufficient to elicit preference in Cyprinodotiform fish. (Méndez-Janovitz and Macías Garcia, 2017; Fisher et al., 2009). The observer sat 3 m away directly in front of the test tank with two stopwatches. The stopwatches were used by the observer to record the amount of time a male spent within the outer sections of the tank.

2.3. Dichotomous choice tests Dichotomous choice tests began by placing focal males in the cylinder within the neutral zone of the testing tank. Then a small and a large class conspecific female were placed on either side of the focal male, in a randomized outer section of the test tank. Fish were then undisturbed for 5 min to acclimate to the test tank. After acclimation, the focal male was released from the neutral zone to swim freely throughout the test tank for 5 min. The observer then recorded the amount of time the focal male spent within the section with either the small female or the large female (association time). Males were deemed to be in a section if the entire head passed the vertical line on the front pane of the test tank. Afterward, the focal male was returned to the cylinder in the neutral zone and the female stimuli were switched to the opposite outer section, to control for male side bias. The focal male was then allowed to reacclimate, then he was released and observed for an additional 5 min. After tests, a partial water change was conducted and fish were placed in recovery tanks. Females were kept in recovery tanks for at least 2 days before being used in additional tests.

2.4. Absolute preference functions assays During assays, the focal male was placed in the cylinder in the centre of the middle section, hereafter called the neutral zone. Then one conspecific female, from a randomly selected size class, would be placed in a cylinder, in a randomly chosen outer section, hereafter referred to as the preference zone. The other outer section would then be deemed the non-preference zone. Both fish were then left to acclimate to the test tank for 5 min. Once the 5 min elapsed, the focal male would be gently released from the cylinder and allowed to swim freely throughout the test tank. The observer would record the time the focal male spent in the two preference zones. After the allotted time elapsed, the focal male was returned to the cylinder in the neutral zone. The female would then be replaced with a new female, of a differing size class. The new female was also placed in a randomly chosen outer section of the tank. The steps were then repeated until the focal male was exposed to all three female size classes. Once an assay was complete, the focal male would be removed from the test tank and all fishes placed in a recovery tank. The females remained in the recovery tank for a minimum of two days before being used in another assay.

2.5. Statistical analysis All analyses were conducted using R (R Core Team, 2017) in RStudio (RStudio team, 2016). Prior to inferential analysis, equal variances, population normality and power were checked using Levene's test, a Shapiro-Wilk's, and Cohen's D test, respectively. Data collected using absolute preference function assays were analysed using a linear mixed model. The model compared male association time with the various female classes among and between both *Limia* species, including male size as a covariate and male identity as a random factor. The two species (*L. perugiae* and *L. zonata*) were analysed independently to identify the presence of mate preference within either species, then combined into a single linear mixed model, after species preferences were determined. A paired *t*-test assuming equal variances

compared data collected in the dichotomous choice assays. The time males associated with small

and large females when presented simultaneously was also compared for both species. In both

the absolute preference function assay and the dichotomous choice test, non-independence was

accounted for by loading male ID as a random factor or pairing the data, respectively. Models

were constructed using Bayes Factor analysis with the BayesFactor R package (Morey and

Rouder, 2018). Bayes factor analyses run multiple mixed models, then select for the model with

the most support based upon Bayesian inference. The benefit of using a Bayes Factor analysis

over more classical hypothesis testing methodologies is that Bayes factors allow for the

quantification of the support of the hypothesis, as well as the null hypothesis. Our hypothesis is

based on the assumption that males should mate with large females because they produce more

offspring, thereby increasing that male's fitness. To ensure that female fecundity was positively

correlated with female size we ran a liner regression model using the R base package on *L.*

perugiae and *L. zonata* female size and fecundity provided to us by Cohen et al. (2015).

3. Results

3.1. Dichotomous choice tests When male *Limia* were exposed to two females simultaneously,

one from the small size class and one from the large size class, the species with reduced

reproductive investment displayed biased association times based on size class. Specifically,

overall there was no difference in association time between large and small females in male *L.*

perugiae (df = 19, t = 1.18, p = 0.25), whereas male *L. zonata* associated more time with larger

females over smaller females (df = 19, t = 2.73, p = 0.01). *Post-hoc* Bayes factor analyses

revealed strong evidence for no interaction between male ID and association time in

dichotomous choice tests (BF = 6.14e□ 4, Fig. 3). Considerable variance among individuals'

association time with females, irrespective of size, was detected across species.

3.2. Absolute preference function assay When presented with the three female size classes sequentially, overall male *Limia* of both species displayed no differences in association time as a result of changes in female size (BF = 0.01, Fig. 3). Specifically, the Bayes factor analysis for male *L. perugiae* showed moderate support for association time not being affected by female size (BF = 0.17), and (?) *L. zonata* showed strong support for female size not influencing association time (BF = 0.09). When male ID and size were included as a covariate and random factor, respectively in the analysis, male association time for female size remained unaffected (BF = 0.01). Individual males' association times with the various size classes varied considerably within all species. A summary of descriptive and inferential statistics is provided in Supplemental Table 1.

3.3. Female fecundity analysis In both species, larger females are more fecund than smaller females. The Pearson correlation coefficient for *L. perugiae* and *L. zonata* are 0.85 and 0.78, respectively. The linear regression model produced the following R2 and p values for *L. perugiae* and *L. zonata*: $R^2 = 0.71$, p = 1.91e-12 and $R^2 = 0.60$ p = 7.16e-07, respectively.

4. Discussion Dichotomous choice tests indicated male *L. zonata* choose larger females over smaller females whereas *L. perugiae* do not show pre-copulatory choice. This result was unexpected, as we predicted both species would choose larger (more fecund) females over smaller (less fecund) females. Our prediction was based on previous studies of closely related species indicating males tend to choose more fecund mates (Fitzpatrick and Servedio, 2018; Jones et al., 2001; Rosenqvist, 1990; Sargent et al., 1986; Verrell, 1985). These findings are especially surprising because *L. perugiae* (with the higher male reproductive investment than *L. zonata*) showed no preference and *L. zonata* did, suggesting minimal reproductive investments favour mate choice. Interestingly, no notable differences were found between *L. perugiae* and *L.*

zonata preference functions, suggesting both species lack pre-copulatory preference for female size, implying reproductive investment is not a strong selective force in shaping male mate preferences in polygynous systems. This result conflicts with both our hypothesis that increased reproductive investments would reinforce preferences for large females and our finding from the dichotomous choice tests revealing mate choice in *L. zonata.* Taken together, our results suggest, in regard to female size, that male *L. perugiae* lack both pre- copulatory mate choice and mate preference and *L. zonata* do possess pre-copulatory mate choice but lack preference in the absence of social information. One possible interpretation of our results is that increased reproductive investment reduces the likelihood of the evolution of male choice and mate preference. Therefore, reproductive investment is negligible to the evolution of male preference, which would explain the prevalence of male preference in polygynous systems despite minimal male investment. However, we find this interpretation unsatisfactory, chiefly because the lack of preference found in both species makes meaningful interpretation and comparison of reproductive investment's role in the two species' preferences difficult. In addition, other studies provide evidence that livebearing males are capable of and demonstrate preference for female size (M´endez-Janovitz and Macías Garcia, 2017). Our results pose critical questions about what factors have precluded *L. perugiae* and *L. zonata* from evolving male mate preference but allowed for male choice in *L. zonata*. Our study also provides support for the meta-analyses by Dougherty and Shuker (2015) suggesting that dichotomous choice tests are more sensitive measurements of mate choice than absolute preference function assays, which raises the question of how absolute preference functions can be optimized for mate preference studies. Therefore, we offer the following potential hypotheses for the results found in our present study. (1) Male reproductive investment and fecundity selection are not strong enough selective forces to favour

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the evolution of preference in *Limia.* (2) The species we used rely upon multimodal signals to evaluate potential mates. (3) Females are mating randomly which counterintuitively selects for males who invest minimally into reproduction. (4) Cryptic female preferences are undermining fecundity selection. (5) The genus *Limia*'s unique distribution throughout the Caribbean has favoured deviant mate preferences. By highlighting potential avenues of research, we aim to better determine the relationship between reproductive investment in male preference.

4.1. Mate preference is too costly? Edward and Chapman suggest male choosiness is likely to evolve when females vary in quality and the cost of choosiness is less than the cost of evaluating mates for males (2011). Our study verified that female *L. perugiae* and *L. zonata* vary in quality, finding that female size is positively correlated with fecundity. It is worth noting *L. zonata*'s size-fecundity relationship is not as strong as *L. perugiae,* which is likely a result of *L. zonata*'s slim-bodied morphology. However, it is possible the fitness benefit of mating with more fecund females is not enough for male preference to be an evolutionarily stable strategy. Thus, the additional costs of pre-copulatory reproductive investment may be too high relative to the fecundity benefits to justify the evolution of preference. Selection may then favour a more effective alternate use of male resources such as using ornaments and courtship to reduce opportunity costs by attracting more mates.

4.2. Multiple sensory cues for mate evaluation We also considered that our results may have been influenced by the nature of our methodology. For example, many animals rely upon a suite of visual, chemical, and mechanical cues to evaluate potential mates. Because previous studies demonstrated visual cues are fully sufficient to elicit male mate preference (Arriaga and Schlupp, 2013; Jeswiet and Godin, 2011; Plath et al., 2008; Deaton, 2009) and the elimination of these additional factors would simplify our statistical models, we elected to present males with only

variations in female size (i.e., a visual cue). However, it is possible that *Limia* males may use alternative visual cues, mechanosensory cues, chemical cues, or a combination of sensory cues to evaluate mates. Multimodal signals have been commonly described as requirements to elicit preference in other Poeciliids (Haines and Gould, 1994; Rosenthal et al., 2002; Morris, 1998; Wong et al., 2005; Makowicz et al., 2016). If this is the case, future studies should allow for multiple modes of communication or manipulation of female traits outside *Limia*'s natural spectrum. Similarly, it is important to consider that *Limia* males may express preferences that deviate from those of other livebearing fishes. *Xiphophorus birchmanni* females also initially seemed to not be choosy when exposed to live stimuli, yet a preference for males with small dorsal fins and large bodies was found when females were exposed to animations that manipulated these characteristics (Fisher et al., 2009). Thus, *Limia*'s seeming lack of preference might simply indicate they prefer traits that are not naturally present within females. Moreover, with studies of male mate preference being a relatively new field, association time's accuracy as a proxy for preference has not been tested. Previous work on male mate preference in livebearing fishes has often tested a suite of behaviours including association time (Jeswiet and Godin, 2011; Plath et al., 2008; Deaton, 2009). We elected to use association time because of its prevalence in both male and female and male mate preference studies. However, we recognize direct analysis of association time is crucial for further male mate preference research. Additionally, future studies are encouraged to allow males to assess a multitude of behavioural traits, including olfaction and lateral line information.

4.3. Randomly mating females and cryptic female choice Male preference often evolves alongside female preference within polygynous systems. We assumed here that pre-copulatory female preference is present within both populations due to the prevalence of the phenomenon

within livebearing fishes (Rios-Cardenas and Morris, 2010). However more recent work suggests female pre-copulatory preference is absent in *L. perugiae* and *L. zonata* (Spikes et al. *in review*). If females mate randomly, selection would favour males that invest minimally into ornamentation. Coercive males are longer lived, enter the breeding population earlier, and are less burdened by the costs of secondary sexual characters than courting males (Weinstein et al., 2019; Mangel and Stamps, 2001). These qualities would suggest coercive males copulate more frequently than courting males. Assuming males are still limited in the number of times they can copulate, coercive males can more readily reject matings because the likelihood of another mating opportunity is high. Together, these factors may sufficiently select for preference in males that invest minimally into reproduction. Our results would suggest that the reduced burden of reproductive investments favours the evolutiona of mate choice may be what is occurring in *L. zonata.* This would raise the critical question: Why are males evolving these expensive ornaments if they reduce fitness? A potential answer might be cryptic female choice, which encompasses behaviours or traits that occur during or after copulation that bias male paternity (e.g. biasing fertilization of eggs toward preferred males) (Birkhead and Pizzari, 2002). Evidence of cryptic female preferences is documented in various Poeciliid species (Evans and Pilastro, 2010; Gasparini and Evans, 2018, Gasparini et al., 2010). If cryptic female choice is present in a polygynous population, selection would favour males that maximize use of their sperm by females. For instance, males may invest in traits that increase paternity, such as sperm characteristics, like velocity, vitality, or volume, or behaviours, like mate guarding and multiple copulations. Assuming paternity is biased toward males investing heavily into reproduction, those males that invest heavily into reproductive traits, like *L. perugiae*, yield little benefit in being choosy, because of the higher percentage of fertilization in each mating event. Conversely,

because fertilization events are biased against coercive males, selection should favour males that not only mate often but also prefer highly fecund females that can yield them more total offspring. Coercive males' ability to readily reject copulations would further reinforce selection for mate preference in low investing males. Together with our results, it is probable cryptic female preference may be present in *Limia* and likely have played a large role of their evolution of preference.

4.4. Atypical preference due to unusual distribution Finally, we must note *L. perugiae*'s and *L. zonata*'s unusual preferences are perhaps due to them occurring in sympatry with several other *Limia* species. *Limia* is a young genus endemic to the Greater Antilles islands of Cuba, Grand Cayman, Hispaniola, and Jamaica. One unique species of *Limia* inhabits each island except Hispaniola, which is inhabited by more than 19 species, including both *L. perugiae* and *L. zonata* (Weaver et al., 2016). Both species are widely distributed on the island, often sympatric with other *Limia* species. Therefore, we posit the species tested here may possess male preference but use preference to discriminate between conspecifics and heterospecifics, like sailfin mollies discriminate against Amazon mollies (*Poecilia formosa*) (Schlupp et al., 1991, 1994). Preferences that encourage sexual isolation reduce an individual's likelihood to produce costly hybrids (Servedio and Noor, 2003). Moreover, the development of preferences that ensure intraspecific matings have been described in many taxa (Nosil et al., 2006). Because of this genus' unique distribution and seeming lack of within species preferences, they provide a unique opportunity for comparative testing of this hypothesis. If supported, these results would provide substantial support for the reinforcement of speciation through mate choice in *Limia*. Individuals that lack preference for conspecifics are therefore more likely to produce unfit hybrids. Additionally, such a result may suggest a limited cognitive bandwidth for mate evaluation, as

suggested by Phelps et al. (2006). To determine if this is the case, more in-depth studies of preference in the entire genus are required to understand the preferences and predisposition of preference in *Limia.*

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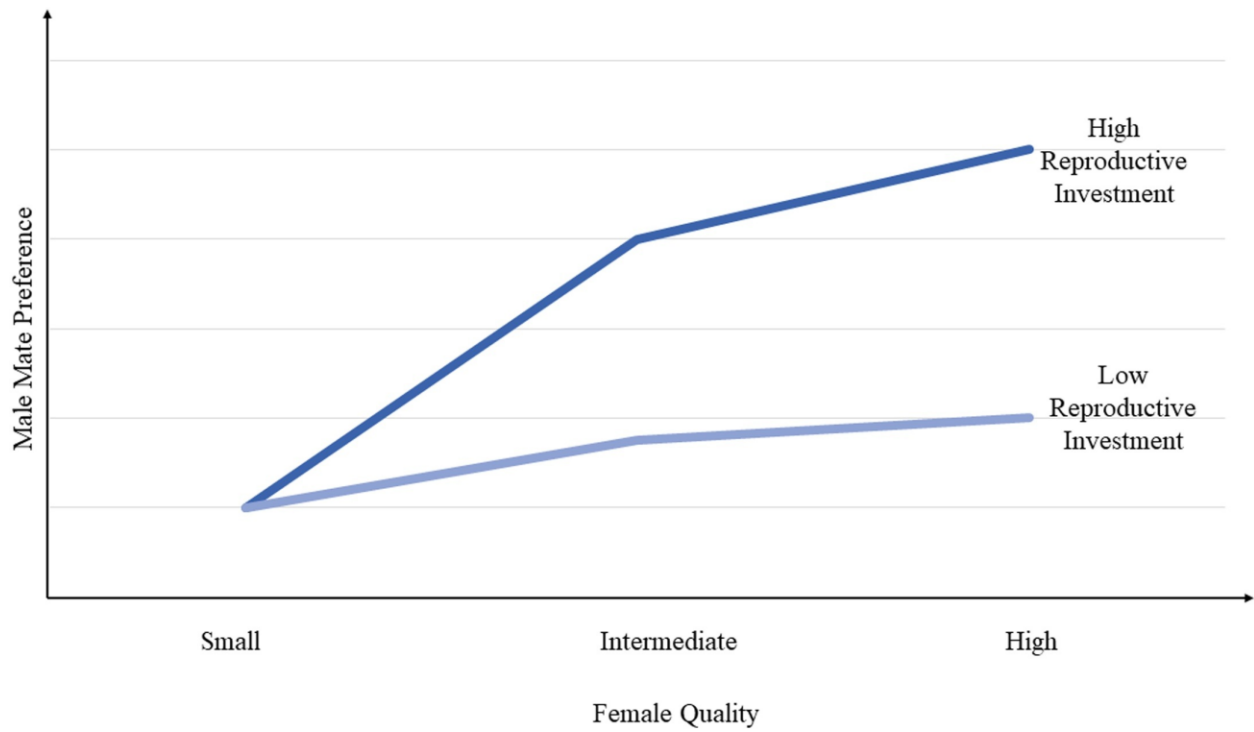
Figures:

Figure 1: Hypothesized effect of male reproductive investment on male preference for female quality. We hypothesized that increased male reproductive investments should select for exaggerated preference for female quality.
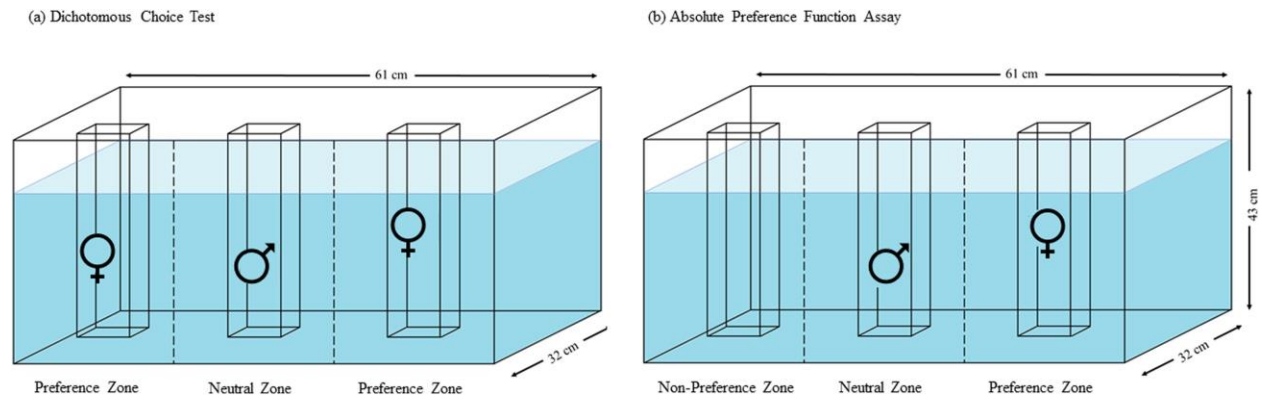
Figure 2: Experimental setup. Schematic illustration of the experimental setup of (a) dichotomous choice test and (b) absolute preference function assays. The illustration is not to scale.
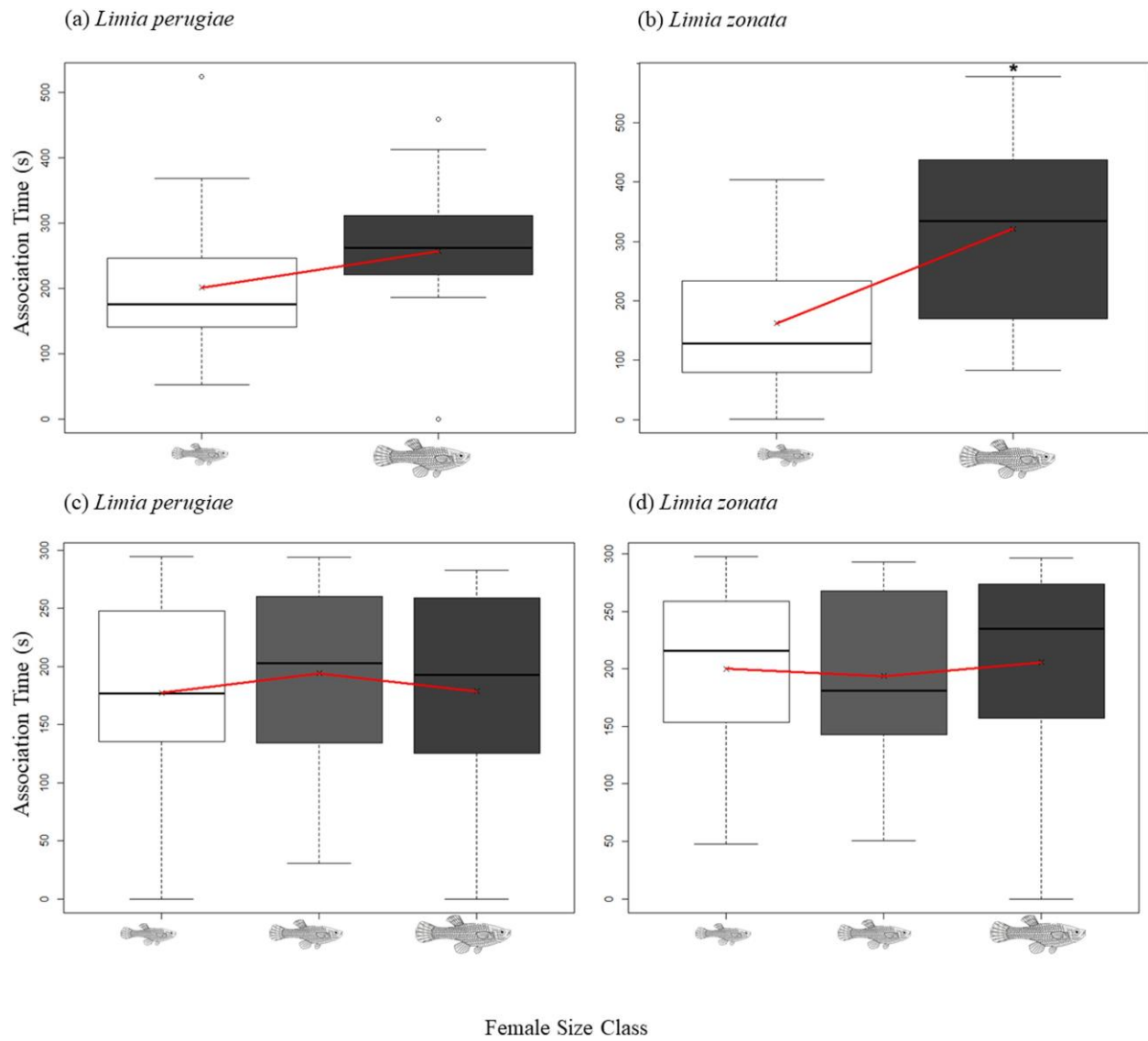
Figure 3: *Limia zonata* males choose large females over small females in dichotomous choice

tests. (a) *L. perugiae* males show no preference for female size, whereas (b) *L. zonata* males

choose large females over small females. The mean association time is indicated by the "×" and

the central bar of the boxplots indicates the median. (c) There was no significant relationship

between male preference and female size in *L. perugiae* or (d) *L. zonata*. Reproductive

investment has no significant effect on male preference in *Limia*. There is a large amount of

variation in preference amongst all individuals. The mean association time is indicated by the

"×" and the central bar of the boxplots indicates the median.

CHAPTER 2: Male secondary sexual traits do not predict female preference in Caribbean livebearing fishes (*Limia*)

Formatted for publication in *Ethology*

Short Title: Female preference in Caribbean fishes

Authors: Montrai Spikes*[1], Sophia Huebler[1], and Ingo Schlupp[1]

*Corresponding Author
[1]Department of Biology, University of Oklahoma, 730 Van Vleet Oval, Norman, Oklahoma USA 73019 spikesm@ou.edu

Abstract

Female preference is widely described in various taxa, and the underlying mechanisms shaping

preferences remain a major focus of sexual selection studies, particularly in species where males

contribute minimally to offspring. Female preference is associated with maintaining male

secondary sexual traits (SST). However, how male SST impact female preference is less

understood. We hypothesized the strength of female preference should scale with the expression

of male SST. To test this prediction, we compared female preference for male body size (an easily

quantifiable trait that scales with other SST) in three species of *Limia* (Poeciliidae) varying in

secondary sexual traits: *L. perugiae, L. dominicensis*, and *L. zonata.* The degree of SST was

assessed based on the amount of ornamentation and the presence of courtship in the species. *Limia*

perugiae, *L. dominicensis,* and *L. zonata* were designated as possessing high, intermediate, and

low male SST, respectively. Female preference was quantified as the relative amount of time females associated with males of various size classes: small, intermediate, and large. Therefore, we predicted because *L. perugiae* males have the most SST, females would associate more strongly with large males. *Limia perugiae* females were the only species to display female preference in relation to male body size, but they preferred small males. Although preference was observed, the direction of preference was unexpected. Moreover, the lack of preference for large male size and thereby other SST in the species suggests pre-copulatory female preference is unimpacted by male SST. We suggest cryptic female choice (i.e., preference enacted during or after copulation) may maintain costly male traits. However, future work remains necessary. The present study provides foundational behavioural work on *Limia* and examines the ubiquity of the evolution of female preference in poeciliids.

Keywords: female sexual preference, mate choice, secondary sexual traits, absolute preference functions, *Limia*

Introduction

Sexual selection is a mechanism of evolution in which fitness is determined by an individual's ability to secure matings. Mate choice is one of the primary drivers of sexual selection (Andersson, 1994; Rosenthal, 2017) and is typically defined as any behaviour or trait that leads to non-random mating (Edward, 2015). Pre-copulatory female preference is among one of the most well-studied and prevalent aspects of mate choice (Ryan and Keddy-Hector, 1992; Varela et al., 2018; Wilson et al., 2017). Although female preference is widely described, the underlying mechanisms that shape preferences are not fully understood, hence these are still a major focus of sexual selection studies (Candolin et al., 2007; Kirkpatrick & Ryan, 1991; Mitoyen et al., 2019). Our lack of

knowledge is most apparent when examining species where males contribute little or nothing to the offspring beyond sperm to fertilize eggs (Achorn and Rosenthal, 2020).

In species where males contribute minimally to offspring, males more readily re-enter the breeding population after mating than females causing a male-biased operational sex ratio (OSR) (Ahnesjö, 1996). The abundance of males allows females to mate selectively, with female preferences selecting for elaborate male secondary sexual traits (Clutton-Brock, 2007). Often secondary sexual traits take the form of ornaments such as fluorescent colours, movement patterns, and body size (Maynard-Smith et al., 2003). Although it is well-established male secondary sexual traits are a product of female preference (Fisher et al., 2009; Mitoyen et al., 2019), the impact of male secondary sexual traits on female preference is less well-understood (Ptacek and Travis, 1997). Given that the intensity of male secondary sexual traits is often attributed to Fisherian runaway selection, whereby male SST become more exaggerated due to directional female choice, we hypothesized that female preference should scale with the intensity of male secondary sexual traits as well (Fisher, 1915).

We determined if female preferences scaled with male secondary sexual traits by comparing female preferences between three species of *Limia*. *Limia* is a genus of livebearing fishes in the family Poeciliidae, endemic to the Greater Antilles (Weaver et al., 2016). The species of *Limia* are closely related and share similar life histories (Cohen et al., 2015); however, they vary considerably in male sexual strategies (Farr, 1984). Specifically, species differ in their degree of ornamentation and the use of courtship displays. Here, we compared female preference between *L. perugiae*, *L. dominicensis*, and *L. zonata.* The species serve as treatment groups: high, intermediate, and low secondary sexual traits, respectively. Species degree of secondary sexual traits was based on ornamentation indices using dorsal fin size as described by Goldberg et al. (*L.*

perugiae 0.36, *L. dominicensis* 0.09, and *L. zonata* -0.11) (Goldberg et al., 2019) as well as the presence of courtship, which is only present in *L. perugiae* (Erbelding-Denk et al., 1994). These differences in mating strategies make them ideal for our comparative study.

However, comparing mating preferences between species presents challenges. The primary challenge being mate preference data gathered via mate choice designs (i.e., binary choice tests) cannot be directly compared between species (Wagner, 1998). We circumvented this problem by using absolute preference functions to measure preferences in lieu of binary choice tests. Preference functions describe the correlation between a phenotypic character expressed by males and the resource(s) expended by females (Lande, 1981). Here we elected to measure female preference for male body size in *Limia* because preference for large more ornate males is widely described in poeciliids. For example, female guppies (*Poecilia reticulata*) prefer highly ornamented males (Houde and Endler, 1990), and swordtails (*Xiphophorus pygmaeus*) females show strong preferences for large male body size (Hankison and Morris, 2002; Morris, 1998). Moreover, other male secondary sexual traits, such as ornamentation and courtship, are known to scale with male size in poeciliids, including *Limia* (Farr, 1984). Therefore, by using male body size as a SST as well as a proxy for ornamentation, we were able to easily ascertain female preference in *Limia*.

Because we hypothesize female preference should scale with male secondary sexual characters, we predicted 1) *L. perugiae* females possessed the most pronounced preferences for male body size of the three species. 2) *L. zonata* to display the least exaggerated preference, 3) while *L. dominicensis* showed an intermediate preference. However, we expected females of all species to show a preference for large males because of the purported indirect genetic benefits associated with mating with large males (Hankison and Morris, 2002; Morris, 1998). Support for the null

hypotheses was determined as female preference being indistinguishable between species and a lack of preference for larger males. In addition to testing our hypothesis, our results provide some of the first descriptions of female preference in the *Limia*.

Materials and Methods

Collection and Size Classification

For this study, we used three species of closely related Caribbean livebearing fishes: *Limia perugiae*, *L. dominicensis,* and *L. zonata* (Weaver et al., 2016). The fishes we used were descendants of wild-caught populations from the Dominican Republic on Hispaniola. *Limia perugiae* were collected in 2014 in a small ditch near the south shore of Lake Enriquillo (18°24'4.61"N, 71°34'16.61"W). *Limia dominicensis* were also caught in 2014 in a ditch east of Polo, Barahona Province (18°19'6.93"N, 71°34'14.24"W). *Limia zonata* were caught in 2012 in the shallows of the Río Yuna near Bonao (18°57'33.5"N, 70°24'32.1"W). All specimens were transported to a greenhouse (now the International Stock Center for Livebearing Fishes) at the Aquatic Research Facility at the University of Oklahoma, where they were kept under common garden conditions. The *L. perugiae* and *L. dominicensis* populations were kept in 1000-l flow-through stock tanks. *Limia zonata* were housed in a similar 500-l tank due to their smaller population size compared to *L. perugiae* and *L. dominicensis*.

We haphazardly collected 40 individuals (20 females and 20 males) of each species (N = 120) from these stock tanks, moved them into an indoor fish room, divided them by sex, and placed them in 37 L holding tanks. All individuals were acclimated to the laboratory environment for 14 days prior to any subsequent handling. All individuals were kept in a climate-controlled room with 26ºC (±3º) on a 12-hour day-night cycle. The fishes were fed *ad libitum,* a mixture of frozen brine

shrimp naupliae, *Daphnia,* bloodworms (mosquito larvae), and Tetra Min flakes twice daily before and after behavioural assays.

A laminated grid was used to measure the standard length of each individual (to the nearest 0.1 mm). Fishes were then photographed (Nikon D5200 camera with a Nikon AF-S DX NIKKOR 18-200mm f/3.5-5.6G ED VR II Standard Zoom Lens). Fish were gradually cooled to anesthetize them for photographs; they were held in ice water until they became still enough to be photographed (Klontz and Smith,1968; Collymore, et al. 2014). We elected to cool fishes because preliminary studies of *L. dominicensis* found that mortality increased when alternative methods of anaesthesia, such as tricaine methanesulfonate (MS-222), were used (Spikes Obs). Immediately afterward, individuals were placed in a recovery tank and were given two days to recover before beginning the behavioural tests. No mortality was associated with this procedure. Females of each species were randomly assigned an ID number and then placed in individual 5 L tanks under identical conditions in a climate-controlled fish room. To ensure individuals were reproductively mature, we used only fish with a standard length (measured from the tip of the snout to the end of the spinal column) greater than 16 mm (Arriaga and Schlupp, 2013). All females less than 16 mm were classified as juveniles and returned to their respective stock populations. Males with fully developed gonopodia, the intromitting organ typical for the family, were determined to be reproductively mature.

We used male size as a likely female-preferred trait in *Limia* because female preference for large males is well-documented in other Poeciliid species (Ríos-Cardenas and Morris, 2011). Within each species, we sorted males into discrete size categories based on the measured population distribution of male standard length. Using median standard length as an anchor, we divided the size distribution into equal quartiles. We classified males as small if they were in the lowest

quartile (five males), intermediate if they were within the two middle quartiles (10 males total), and large if they fell into the upper quartile (five males). *Limia perugiae* size classes were defined as small (14 mm – 20 mm), medium (21 mm – 26 mm), and large (27 mm <). Similarly, *L. dominicensis* size classes were defined as small (14 mm – 23 mm), medium (24 mm – 26 mm), and large (27 mm <). *L. zonata* size classes were defined as small (14 mm – 20 mm), medium (21 mm – 24 mm), and large (25 mm <). The males of each species were then placed into three 37-l aquaria (9 aquaria total), according to size classifications.

Experimental Setup

We used an absolute preference function assay to measure female preference in all three species. Assays were conducted in a 76 L tank that was divided lengthwise into three equal zones (Figure 2). Two vertical lines were drawn on the front pane of the tank to designate zones. The centre zone was deemed the neutral zone, with the two outer zones designated preference and non-preference zones. In each zone, we placed a clear Plexiglas tube (8.5 cm x 8.5 cm rectangular prism), which was used to restrict a male within its assigned zone and to reduce the transmission of mechanosensory and chemical signals (Figure 2). In the preference zone, one male fish was placed in a Plexiglas tube, and in the non-preference zone, there was no male present (i.e., an empty clear Plexiglas tube to control for any bias the female might show toward the tube). The preference and non-preference zones were randomized to control for side bias.

During absolute preference function assays, females of each species (N = 20 per species) were sequentially presented with three conspecific males of varying size (small, medium, and large) in a randomized order. Females were randomly selected by ID number and placed in a clear Plexiglas tube in the centre of the neutral zone after her assigned male was first placed into the preference zone. Both individuals were then given 300 seconds to acclimate to the environment. To begin the

trial, we released the female from the tube, and after she was swimming freely around the tank (i.e., showing no signs of stress-like behaviours), we recorded for 300 seconds the amount of time (s) the female spent in the preference or non-preference zone. Females were defined to be in the preference or non-preference zone if their entire head passed the vertical line on the front pane of the tank. After the 300 seconds passed, we gently placed the female back in the tube in the centre of the neutral zone, and the male was removed. Then a male of another size class was presented to the focal female. The process was repeated until the female was exposed to one male from each of the three size classes. Once a female was exposed to three males, she was returned to her home tank, and males were placed in size-specific recovery tanks labelled 'used.' Males remained in recovery tanks for 3 days before haphazardly being used in another trial with a different conspecific female. After a trial, the testing tank received a 50% partial water change to reduce any lingering chemical signals from affecting future trials. All trials for any given species were conducted over the course of 30 days. Trials began in November 2017 and were concluded in January 2018.

Data Analysis

All analyses were conducted in R using the nlme and BayesFactor packages (R, v.3.0.2 R Core Team, 2019; Pinheiro et al., 2019; Morey and Rouder, 2018). A linear mixed-effects model was used to determine if the intensity of male secondary sexual traits influenced female preference. Specifically, we loaded species and male size class as explanatory variables and association time as the response variable. Presentation order, female size, and female identity were considered as random factors to control for order effects and prevent pseudo-replication in the analyses, respectively. However, to preserve non-singularity, both presentation and order female size were removed from the model. If a significant effect was observed, we conducted a *post-hoc* Tukey test

to determine the direction of preference. After running the full model, including all species, we used independent species-specific linear mixed-effects models on each species to compare female association time as it relates to male size within species, loading the same factors excluding species.

Because *Limia* has previously demonstrated atypical behaviour as compared to other poeciliids (Spikes & Schlupp, 2021), we elected to conduct a Bayes factor *post-hoc* analysis. The Bayes factor analysis uses Bayesian inference to computes an integer known as a Bayes factor (BF), which can be interpreted using the cut-offs posited by Morey et al. (2016). The Bayes factor - like the *p*-value - is then indicative of how much support one has for the hypothesis. Unlike the *p*-value, however, the Bayes factor allows for interpretation of the support for the null hypothesis. Hence the analysis is particularly advantageous when interpreting insignificant results. Particularly if a study yields negative data that requires the rejection of the hypothesis, Bayes factors will indicate if the results are inconclusive or if the null hypothesis should be accepted.

Results

The variance between species had the largest impact on the amount of time females associated with males (Table 1). *Limia zonata*, the low male SST species, associated with males more than both *L. perugiae*, the high male SST species ($p = 0.03$), and *L. dominicensis*, the intermediate male SST species ($p = 0.0002$; Figure 1). Species-specific linear mixed-effects models revealed *L. perugiae* females possess preference. Using a *post-hoc* Tukey-test, we observed *L. perugiae* females prefer small males over both intermediates and large males ($p = 0.02$; $p = 0.04$; Figure 2a). Neither *L. zonata* nor *L. dominicensis* displayed a preference for male body size ($p = 0.94$; Figure 2b; $p = 0.90$; Figure 2c). In all three species, neither presentation order, female identity, nor female size significantly influenced female preference. The Bayes factor analysis revealed

substantial evidence for *L. perugiae*'s preference for small males (BF = 4.81), as well as anecdotal evidence for no relationship between female preference and male size in both *L. dominicensis* (BF = 0.51) and *L. zonata* (BF = 0.35).

Discussion

When comparing female preference for male size, we expected female preference to scale with male secondary sexual traits. Female *L. zonata* associated with males more than the other two species; however, they did not demonstrate a preference for male size. *Limia perugiae*, the species with the most secondary sexual traits, were the only species to display female preference, providing support to our hypothesis that female preference should scale with male SST. However, *L. perugiae* females preferred small males, which tend to be minimally ornamented and rely on coercion to secure matings (Farr, 1984). Together with our results, this suggests that pre-copulatory female preferences are not maintaining male ornaments in these species.

The results of the present study may have been influenced by the design of our experiment. Absolute preference function assays introduce males sequentially to females and remove social information females may use to evaluate mates. However, we aimed to understand the preferences underlying female mating decisions, and by controlling for social information via sequential exposure of males, we removed the element of choice present in other methodologies. Our experiment would have benefited from allowing sensory cues other than only visual. Poeciliiids such as *Poecilia chica* and *Poecilia sphenops* use chemical and tactile feedback, respectively, when assessing mates (Brett and Grosse, 1982; Schlupp et al., 2010). Although, the bulk of poeciliid studies have found very strong responses using only visual cues. Indeed, one study found that visual information was the strongest in a study comparing multiple sensory channels

(Makowicz et al., 2016). Finally, live males introduce variation in individual stimuli which is difficult to control. For example, *L. perugiae* females' preference for small males may be indicative of an increase of reproductive effort on behalf of males via courtship in an environment where predation risk is minimal. Despite this possibility, studies using live individuals in preference assays have demonstrated live individuals do not compromise the integrity of preference studies (Fisher et al., 2009).

In the bigger picture, it is well-documented in livebearing fishes and beyond that females tend to prefer large male body size and heavily ornamented males (Bisazza and Marin, 1991; Fernandez and Bowser, 2010; Hughes, 1985; Plath et al., 2004). Moreover, females across many taxa show a consistent pattern of preference for larger mates (Ryan and Keddy-Hector, 1992). Females of a closely related species, also from Hispaniola, *Limia nigrofasciata*, show a clear preference for large males (Holz, 2015), which makes our result stand out as very unusual. The lack of pre-copulatory female preference for larger males within the *Limia* species studied here raises the important question: How are male ornaments seemingly uninvolved in female preference evolved and maintained? We offer two hypotheses for the maintenance of ornamentation in the absence of female pre-copulatory preference. First, females may exhibit cryptic preferences, as opposed to pre-copulatory choice, wherein females seemingly mate indiscriminately and bias male paternity toward preferred males via selecting sperm from preferred males. Secondly, male secondary sexual characters could be the product of alternative selective forces or may not even be adaptive.

Cryptic female choice in livebearing fishes tends to be expressed as a bias toward the sperm of the preferred male being retained in the female reproductive tract (Firman et al., 2017). The higher retention of preferred male sperm leads to biases in offspring paternity toward preferred mates. This mating strategy is particularly effective in species where female pre-copulatory preferences

are suppressed due to male harassment and forced copulations, like in many livebearing fishes (Plath et al., 2007). We hypothesize that cryptic female choice is a mechanism maintaining male secondary sexual traits in *Limia.* Initial work by Schartl and colleagues have provided find *L. perugiae* non-dominant males are more reproductively successful than dominants (1993). However, it is inconclusive whether this is a product of cryptic female preference or male behaviour. Therefore, we suggest an experimental design that tests if male secondary sexual traits are positively correlated with a bias in sperm retention in the female reproductive tract in *Limia.*

Given the relatively recent diversification of *Limia* found on Hispaniola (Weaver et al., 2016), we would be remiss in not acknowledging that there are a variety of other evolutionary alternatives we are unable to mention here, which could result in sexual dimorphism in *Limia* (Bisazza, 1993). Our study assumed, as with many other taxa, that females were evaluating male secondary sexual characters. Therefore, male secondary sexual characters are maintained through mate choice. Our results suggest this may not be the case. Instead, the sexual dimorphism found in *Limia* could be a means of hybrid avoidance, male-male competition, or even a vestigial trait (Berglund et al., 1996; Liou and Price, 1994). Further study of female *L. perugiae's* preference for small males, in particular, may shed light into the unusual preferences we find. It is worth noting that the consideration of these alternative research opportunities would not be possible without the use of an animal system where there is limited knowledge. Future work in *Limia* provides an excellent opportunity to assess the ubiquity of the phenomena described in well-researched taxa.

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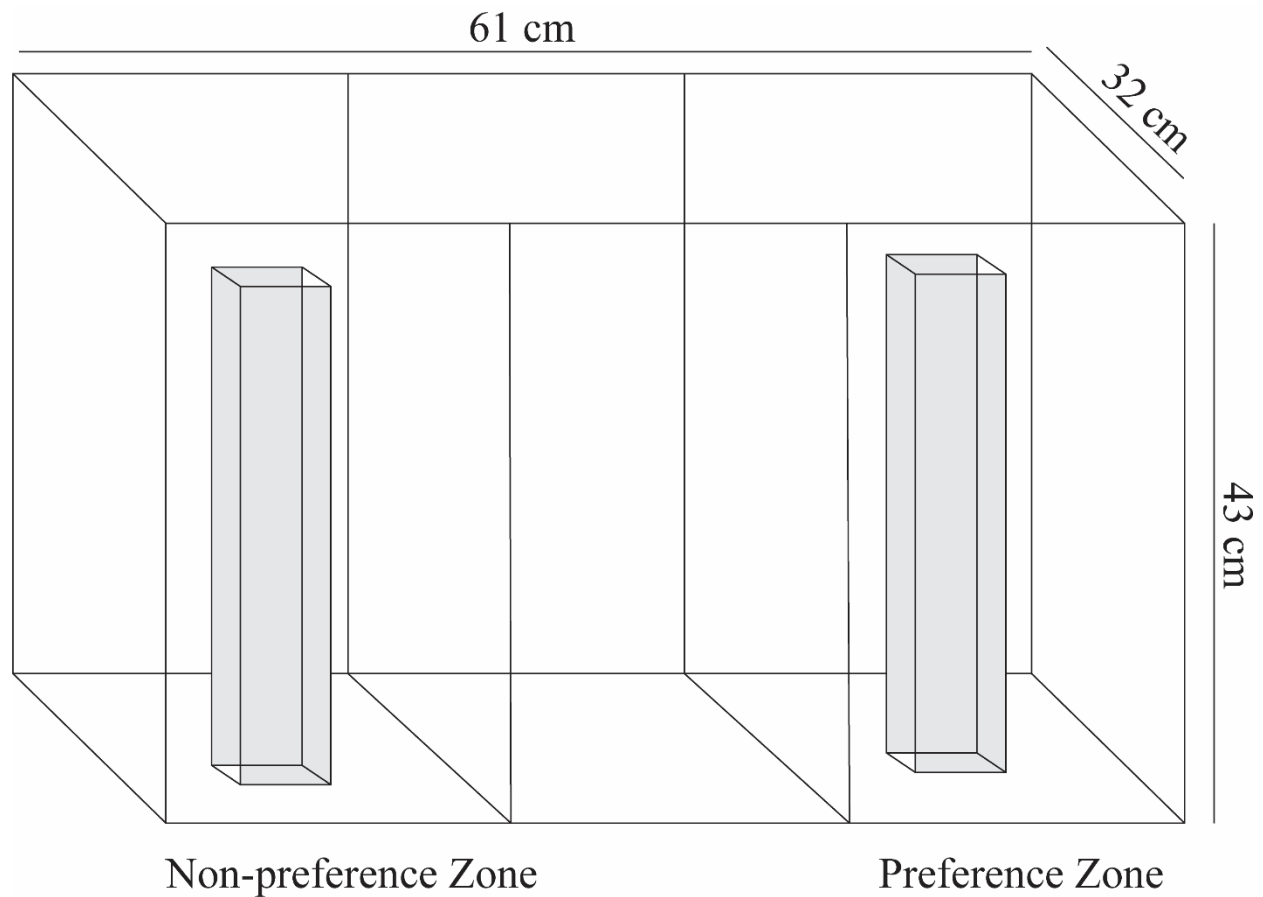
Figures:

Figure 1: Absolute preference function assay setup. A 76-liter tank was divided into three zones, marked with drawn lines on the tank's exterior. A female was initially placed in the middle zone within a plexiglass tube. Three males were presented sequentially in a plexiglass tube on a randomly selected side of the tank, deemed the preference zone. The zone on the opposite side of the tank was then deemed the non-preference zone. During assays, females were able to swim freely throughout the tank for 300 seconds. Time (s) spent in each preference zone was recorded.

(a) *Limia perugiae* (ornamentation and courtship)

(b) *Limia dominicensis* (ornamentation)

(c) *Limia zonata* (minimal ornamentation)
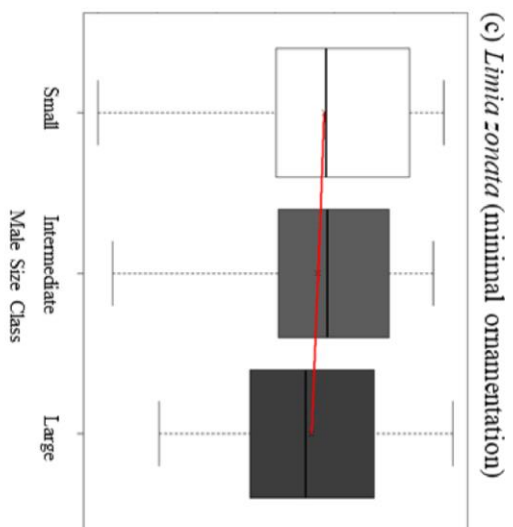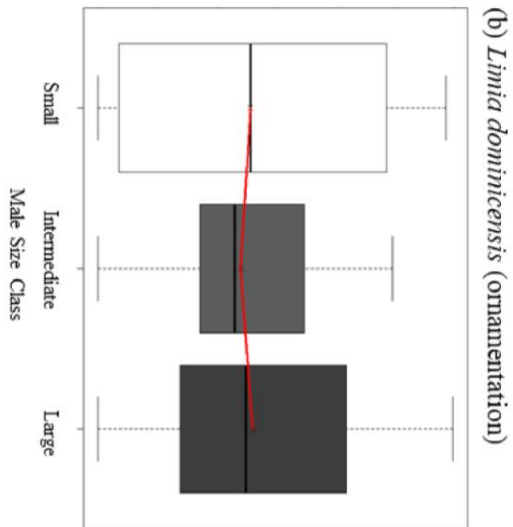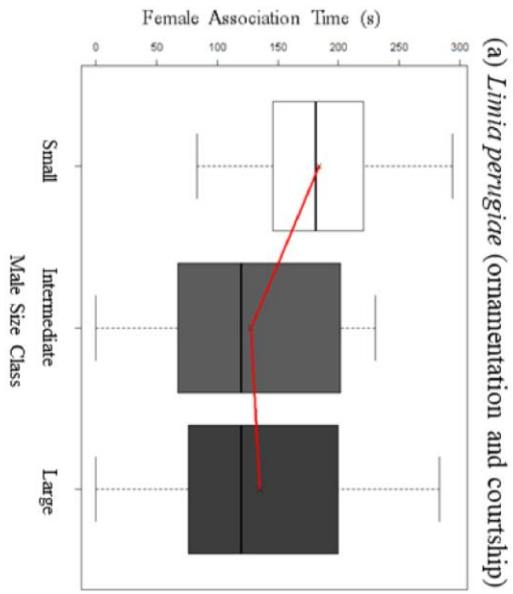
Figure 3: Comparison of preference functions of three species varying in secondary sexual traits (SST). *Limia perugiae* (a), *L. domenicensis* (b), and *L. zonata* (c) males have high, intermediate, and low SST, respectively. Females of each species were shown males of various size classes, and the total time females associated with each male was recorded. The red dot indicates the mean time females associated with each male size class. The black line indicates the preference function of each species, with a flat line suggesting no preference.

CHAPTER 3: The comparison of choosiness and mating behaviors between three male

morphotypes of *Limia perugiae* (Poeciliidae)

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Authors: Montrai Spikes*[1], and Ingo Schlupp[1]

*Corresponding Author
Montrai Spikes
[1]Department of Biology, University of Oklahoma, 730 Van Vleet Oval, Norman, Oklahoma,
73019, USA
Email address: spikesm@ou.edu

Abstract

Historically, mate choice was assumed to occur primarily in females; however, evidence of male

mate choice has accumulated in various animal systems. Theory posits that males should

selectively breed with large, fecund females to maximize fitness. However, additional costs

imposed on males, such as energetically (?) expensive secondary sexual traits (SST), can make

fecundity benefits negligible. Therefore, we postulate the costs of elaborate SST are negatively

correlated with male choosiness. To test our hypothesis, we use *Limia perugiae*, which possesses

three male morphotypes; sneakers that invest minimally into SST, courters that invest heavily,

and intermediate males that invest moderately. We expected sneaker males to mate selectively,

courter males to mate randomly, and intermediates - being by definition in the middle - to show

weak choosiness, if any. We found that regardless of morphotype, male *L. perugiae*, unlike many

livebearing fishes, are not choosy when it comes to female size. The lack of variance in

choosiness between the male morphs provides further evidence of the unique mating system

found in *L. perugiae.* This atypical mating system raises many questions, chiefly what aspect of

L. perugiae has inhibited the expression and evolution of male mate choice. To learn (?) why

divergent preferences have evolved in *Limia*, future studies should prioritize 1) direct comparisons of SST costs between male morphs, 2) cryptic mate choice, and 3) habitat composition.

Introduction

Mate choice can be defined as an individual choosing amongst multiple potential mates based on environmental cue(s) (Andersson, 1994; Candolin, 2003; Edward, 2015). Historically, mate choice was assumed to mainly occur in females, except in extreme cases like sex-role reversal (Rosenqvist, 1990; Trivers, 1972). The assumption that mostly females were choosy was likely due to the relative abundance of elaborate male secondary sexual traits (SST), such as the dewlap in *Anolis* (Andrews, 1985), acoustic signals in Orthoptera (Morris & Luca, 1998), and ornamentation in Poeciliidae (Bisazza & Marin, 1991). Theory predicts that female choice evolved in response to a combination of selection pressures. The classical argument posits that, because oocyte production is energetically costly, females should mate with males who confer the most benefits (Bateman, 1948; Hill, 1991; Kose & Møller, 1999), either in the form of direct benefits (such as nuptial gifts) or indirect benefits via good genes. Females also typically spend less time than males in the breeding population because of gestation periods and parental care, causing male-biased operational sex ratios (OSR) (Kvarnemo & Ahnesjö, 1996). The bias in the OSR lowers the opportunity cost for females who reject potential mates. Therefore, females can selectively mate with attractive males who possess costly SST without jeopardizing their fitness (Andersson, 1994; Andersson & Simmons, 2006). Because female choice can maintain ostentatious SST, female choice remains a well-studied field within sexual selection (Andersson & Simmons, 2006; Birkhead & Pizzari, 2002; Edward, 2015; Rosenthal, 2017).

Although there is abundant evidence for female choice, evidence of male mate choice has accumulated in various animal systems relatively recently (Côte & Hunte, 1989; Jones et al., 2001; Orrell & Jenssen, 2002; Preston et al., 2005; Schlupp, 2018, 2021). Unlike female preference, male choice is more restrained in its expression (Edward & Chapman, 2011; Fitzpatrick & Servedio, 2017, 2018). Males tend to prefer traits indicative of female receptivity and fecundity, such as female size (Jones et al., 2001; McLennan, 1995; Méndez-Janovitz & Macías Garcia, 2017). The convergent evolution of male preference for female size is due to male fitness being directly dependent on female fitness (Edward and Chapman 2011). Selectively breeding with large, fecund females maximizes male fitness. Females presented simultaneously also reduce opportunity costs, further increasing the benefits to mate preference (Head et al., 2015). However, the cost of evolving mate choice is impacts males differentially. Specifically, additional expenses imposed on some male, such as expensive SST, might reduce fecundity benefits, thus favoring random mating (Edward & Chapman, 2011). Therefore, male choice theory also predicts that male choice is most likely to evolve in individuals with low costs for SST, although empirical tests of this prediction are few (Furness et al., 2020; Ptacek & Travis, 1997; Spikes et al., n.d.; Spikes & Schlupp, 2021). Taxa such as salmon (Salmonidae) (Horth & Dodson, 2004), sunfish (Centrarchidae) (Taborsky, 2001), dragonflies (Telephlebiidae) (Futahashi, 2016), salamanders (Salamandroidea) (Pierson et al., 2019), and livebearing fishes (Poeciliidae) (Erbelding-Denk et al., 1994), where males adopt alternative mating strategies (Oliveira et al., 2008), present ideal opportunities to test empirically how the costs of SST impact choosiness.

Livebearing fishes, in particular, offer an ideal system for studying mate choice because of their diversity of sexual strategies and because they are easily maintained in lab settings. Poeciliids are

a robust family of livebearing fishes that occur in mixed-sex shoals (Evans et al., 2011). Within

shoals, males and females mate multiply throughout their lifespan (Evans et al., 2011).

Alternative mating strategies have evolved within this family multiple times (Farr, 1984; Furness

et al., 2020; Ptacek & Travis, 1997; Ryan et al., 1992). Sailfin mollies (*Poecilia latipinna*),

Panuco swordtails (*Xiphophorus nigrensis*), and *Limia perugiae* all possess three male

morphotypes: sneakers, courters, and intermediates (Farr, 1984; Farr et al., 1986; Ryan et al.,

1992). Sneakers tend to be small, with limited ornamentation, and they typically force

copulations (Ryan et al., 2001). Because sneakers do not invest heavily into secondary sexual

traits, they reach sexual maturity much more quickly than the other male morphs (Weinstein et

al., 2019). Courters attract females with their large size, ornamentation, and courtship displays,

but the development of these traits is relatively expensive; thus, courters enter the breeding

population relatively late (Erbelding-Denk et al., 1994; Fisher et al., 2009; Gabor, 1999).

Additionally, to achieve the increased size, courter males increase their predation risk (Godin &

McDonough, 2003), sacrifice immune function (Devigili et al., 2015), and reduce their ability to

respond to parasitic and physiological stressors (Weinstein et al., 2019). Intermediates adopt a

mixture of both courter and sneaker strategies, yielding some of the benefits and costs of both

sneaker and courter males (Lange et al., 2021). Often, the various male mating strategies confer

roughly equal fitness, indicating a balanced polymorphism (Ryan et al. 1992).

The trade-offs associated with male size can influence male mate choice (Ptacek & Travis,

1997). In Ptacek & Travis's study, the largest Sailfin males were choosiest. However, other

studies of how male morphotype impacts male choosiness are lacking. In mating systems with

multiple male morphs, like the Sailfin molly and *Limia perugiae*, male fitness for all

morphotypes may be roughly equal which may be indicative of a balanced polymorphism (Ryan

et al. 1992). However, the potential differences in costs between the male morphs can also lead to predicting that male morphotypes (or size-classes) could differ in female preferences. Earlier studies of *L. perugiae* studies did not account for their alternative reproductive strategies and their impacts on male choosiness (Schlupp, 2018; Spikes & Schlupp, 2021).

In this study, we sought to address the limitations of previous studies of *L. perugiae* by determining if the lack of male choosiness observed is due to differences in male mating strategies. We hypothesized the costs of elaborate secondary sexual traits should be negatively correlated with male choosiness. We reasoned that the costs associated with elaborate SST combined with the costs of evolving choice would make the benefits of selectively mating negligible (Spikes &Schlupp 2021). Therefore, we expected courter males to mate randomly, sneaker males to mate selectively, and intermediates - being by definition in the middle - to show weak choosiness, if any. Conversely, the absence of preferences may indicate a balanced polymorphism.

Material and Methods

We collected *Limia perugiae* in the Dominican Republic from a small ditch off the south shore of Lake Enriquillo (18°24'4.61"N, 71°34'16.61"W) in 2014. After collection, fishes were transported to the International Stock Center for Livebearing Fishes at the University of Oklahoma. We kept the fish under common garden conditions. Fishes used in this study were a minimum of four generations removed from wild-caught populations. We conducted behavioral trials in the fall of 2020 between August and September at the University of Oklahoma Norman Campus.

Sixty males and 60 females, 120 individuals total, were collected from the International Stock Center. Fishes were collected haphazardly using a small seine. We then transported the fishes to

an indoor climate-controlled room kept at (26°C±3) on a 12-hour day-night cycle. We placed all

fishes in 37 L holding tanks separated by sex. Fishes acclimated to the lab environment for 14

days before any additional handling. We fed fishes twice per day, a mixture of TetraMin Flake

food, frozen brine shrimp, *Daphnia*, and bloodworms *ad libitum*.

Size and phenotype classes for females and males, respectively, were determined using the

standard length (the tip of the snout to the last vertebrae before caudal fins). All individuals were

measured using a laminated millimeter grid, then photographed with a Nikon D5200 camera with

a Nikon AF-S DX NIKKOR 18-200mm f/3.5-5.6G ED VR II Standard Zoom Lens. We

administered 100 mg of the anesthetic tricaine methane sulfonate (MS222) in a 1 L tank to

reduce individuals' mobility during measurements and photographs. Immediately after fishes

were measured and photographed, they were placed in a 1 L recovery tank and monitored for a

minimum of 20 minutes. Fishes recovered for an additional week before subsequent handling.

Using standard length, we divided the females into two equal groups. Half of the females, those

greater than 26 mm, were deemed large, and those females 25 mm or less were considered small.

Males were separated by phenotype into courter, intermediate, and sneaker categories (Figure 1).

Courters were classified as having standard lengths greater than 30 mm, ornate dorsal fins, and

iridescent scales. Sneakers were identified by standard lengths less than 23 mm and little to no

ornamentation. Intermediate males were between 29 and 24 mm long and showed some

ornamentation.

Experimental setup

We conducted behavioral tests in a 76 L tank (61 x 30 ½ x 40 ½ cm) containing 2.5 mm of multi-

colored gravel. The tank was divided into three equal sections lengthwise, denoted by two

vertical lines drawn on the tank's front pane. Except for the front pane, white plastic panels

covered all sides of the tank. Two Plexiglas unperforated prisms (tubes) were placed within the center section. Tubes restricted the stimuli transmitted between (?) stimulus females and focal males and eliminated chemical and mechanosensory signals during acclimation periods. Set 1 m away was a Nikon D5200 camera on a tripod directly in front of the test tank.

Behavioral test

At the beginning of any testing, a 50% water change of the test tank removed lingering chemical cues from previous trials. Afterward, a randomly selected focal male and stimulus female were placed individually into the two tubes in the tank's center section. Both fish acclimated to the test tank for 5 minutes. The fish were then released and allowed to swim freely throughout the test tank. The behaviors of the two individuals were video-recorded for 5 minutes using the Nikon D5200 camera. After the 5 minutes elapsed, the recording was paused, and the male was placed back in the tube in the center of the tank. The female was removed and replaced with a new stimulus female of the opposing size class in the tank's center in a tube separate from the male. Using a table generated in Microsoft Excel, the order of females' size class presentation was computationally randomized. After the trial elapsed, all fish were removed and placed in recovery tanks, and a 20% water change removed any remaining chemical cues.

Males were used only once and were returned to their stock population at the International Stock Center for Livebearing Fishes after the experiment. However, due to unexpected mortality, some females were used in a second trial. Females were given a minimum of 48 hours before they were used in a second trial. Focal males were not paired with the same combination of stimuli females that another male was exposed to. Females used again were used in a maximum of two trials before being returned to their stock population.

Scoring behaviors

A total of 120 videos were saved on two 64 GB SanDisk ultra-drives. The videos were then evenly divided and randomly assigned to four scorers. An additional five videos were randomly assigned that overlapped with the previously given videos of other scorers to control for scorer biases. The scorers then used Behavioral Observation Research Interactive Software (BORIS) (Friard & Gamba, 2016) to measure the following suite of preference and copulatory related behaviors: association time, gonopore nibbles, gonopodial swings, mating attempts, and sigmoid displays. Males were considered to be in the preference zone if they inhabited the same section of the tank as the female stimuli. Fishes were defined as being in a section when their head (snout to operculum) completely passed the divisionary lines on the aquarium's front pane. In addition to association time, we also tracked a suite of male mating behaviors: gonopore nibbles, gonopodial swing, mating attempts, and sigmoid displays (Erbelding-Denk et al., 1994; Farr, 1984, 1984; Rosen & Tucker, 1961). Gonopore nibbles were logged when males bit at female gonopores (Farr et al., 1986; Sumner et al., 1994). Gonopodial swings were when the male moved his gonopodium towards his head (Bisazza, 1993; Greven, 2005; Rosen & Bailey, 1963). If the male also attempted to mate with the female, the behavior counted as both a swing and mating attempt (Bisazza, 1993). Finally, sigmoid displays were scored when males undulated their body in the shape of an "S" (Bisazza, 1993; Farr, 1989; Rios-Cardenas & Morris, 2011). Because *L. perugiae* have displayed irregular preferences compared to other poeciliids (Spikes & Schlupp, 2021), we elected to analyse a suite of behaviours associated with preference.

Statistical analysis

All statistical analyses and data visualizations were conducted in R and RStudio using tidyverse, rstatix, nlme, BayesFactor, mvabund, and ggplot2 packages (Morey & Rouder, 2018; Pinheiro et al., 2020; R Core Team, 2017; Wang et al., 2020; Wickham, 2016). We ran a principal

component analysis (PCA) to identify which male mating behaviors best explained the variance among the male morphotypes. We then used the results of the PCA to inform a generalized linear mixed model (GLMM). Using a negative binomial distribution in our GLMM, we compared the number of times males of the three morphotypes performed various mating behaviors (i.e., sigmoid displays, gonopore nibbles, gonopodial swings, and mating attempts). We also compared differences in how males directed mating behaviors toward the females in each size class. A two-way repeated-measures ANOVA and a linear mixed model were used to compare how the different male morphotypes allocated time between the females in the two size classes. We ran these analyses to account for the two explanatory factors (male morphotype and female size) as well as to prevent pseudo-replication due to each male providing data points from small and large females.

Because a lack of preference has been previously described in *L. perugiae* (Spikes & Schlupp, 2021), we elected to run a Bayes factor analysis on the linear mixed model. Bayes factor analyses compute an integer, known as a Bayes factor (BF), by determining and comparing the best of all possible models given the explanatory and response variables (Morey et al., 2016). The BF can then be used to interpret the support for or against the hypothesis. For example, in instances where there is no significant support for the thesis, the BF can then be used to determine if the data provide evidence of an absence of a trend or if the analysis's current parameters failed to capture conclusive support. Thus, BFs allow extrapolation from statistically insignificant data, which is particularly beneficial when working with atypical species like *L. perugiae*.

Results

The PCA revealed that PC1 and PC2 explained 81.2% and 8.3% of the variance, respectively. Gonopore nibbles contributed the most to PC1, and mating attempts contributed the most to PC2. When we attempted to group male morphotype or female stimuli size within the PCA, distinct clusters did not form (Figure 2). The generalized linear model revealed a similar pattern where male morphotype and female size did not affect the occurrence of male mating behaviors ($p = 0.615$; Figure 2). When the occurrence of sigmoid displays, gonopore nibbling, gonopodial swings, and mating attempts was analyzed separately, models also showed that males of all morphotypes directed the same number of mating behaviors to each of the female size classes ($p = 0.95$, $p = 0.865$, $p = 0.652$, and $p = 0.667$, respectively; Figure 4). Neither female size nor male morphotype impacted the amount of time males spent with females ($p = 0.085$, $p = 0.199$, $p = 0.139$, respectively; Figure 5). The Bayes factor analysis revealed weak to moderate support for the null hypothesis that male morphotypes allocate time equally between females of two size classes ($BF = 0.236$).

Discussion

We found that regardless of morphotype, male *L. perugiae,* unlike several livebearing fishes (Schlupp 2018), are not choosy when it comes to female size. These results contradicted our hypothesis that male choice was more likely to arise in males with minimal secondary sexual characters. Our analyses revealed that of all the behaviors recorded, gonopore nibbling occurred significantly most often. This result was unsurprising, as gonopore nibbling is often the first behavior males perform when initiating a copulation attempt in livebearing fishes (Farr, 1984; Ptacek & Travis, 1997). Interestingly, the other behaviors did not vary between morphotypes. The lack of variance in choosiness between the male morphs provides further evidence of the unique mating systems found in *L. perugiae* (Spikes et al., n.d.; Spikes & Schlupp, 2021). This

atypical mating system raises many questions, chiefly what aspect of *L. perugiae* has inhibited the expression and evolution of male mate choice within this species. Here, we suggest and discuss some of the most plausible explanations of our data.

Balanced polymorphism in L. perugiae

The present results suggest courter males incur similar costs to their sneaker counterparts, or the difference is negligible. Our hypothesis assumes that courter males suffer higher metabolic, predation, foraging, and opportunity costs due to their investment in increased size and ornamentation. We based our predictions on previous studies of *Limia* and other taxa, which found less ornate males tended to be choosier (Devigili et al., 2015; Godin & McDonough, 2003; Weinstein et al., 2019). However, sneaker males in this species may bear previously unaccounted costs, such as increased intrasexual and intersexual aggression (Bildsøe, 1988). If males within this species are overall incurring similarly high costs regardless of their phenotype, they would all possess similar fitness (Oliveira et al., 2008). This balanced polymorphism would result in similar selection pressure for choice, or lack thereof, regardless of phenotype. Balanced polymorphism could also be attained if male choosiness does not confer enough benefits to to provide sufficient selection pressure for it to evolve. In both male and female *L. perugiae,* preference for larger mates is absent (Spikes et al., n.d.; Spikes & Schlupp, 2021). Because females and males are mating randomly and multiple times in wild populations, all male phenotypes are equally likely to sire offspring. Future studies should examine the direct costs and benefits of the various mating strategies in *Limia.*

Cryptic preference in Limia

The lack of pre-copulatory choosiness in *L. perugiae* could also be evidence of cryptic mate choice, in which females and males bias parentage to preferred mates after or during copulation (Gasparini & Evans, 2018). The evolution of cryptic female mate choice could respond to the polygynous mating systems where sexual harassment depresses female pre-copulatory preference (Gasparini & Evans, 2018; Ryan et al., 2001). Similarly, because females mate multiple times, males may devote disproportionate amounts of high-quality sperm toward attractive females (Gasparini et al., 2010, 2013). This alternative hypothesis does not wholly explain why there is male polymorphism in *Limia.* Therefore, identifying the reason for the lack of choosiness observed in *Limia* and the evolution of polymorphism in *L. perugiae* remains a priority in sexual selection and Poeciliid research.

Habitat influences male mate choice

Environmental factors such as predator abundance, food availability, and social environment significantly impact choosiness and preference in livebearing fishes (Rosenthal, 2017; Schlupp, 2018). *Limia perugiae* occur throughout Hispaniola and inhabit multiple environments, from hypersaline lagoons to freshwater streams (Haney & Walsh, 2003; Weaver, Cruz, et al., 2016). The fishes used in this study were collected from one site in Hispaniola, near Lake Enriquillo, where they are the dominant Poeciliid species (Weaver, Tello, et al., 2016). In many other localities across the island, *L. perugiae* occurs in sympatry with other *Limia* species and different types of livebearing fishes (Weaver, Cruz, et al., 2016). In a similar study comparing male choosiness between Sailfin molly morphs, habitat played a substantial role in the expression of choice (Ptacek & Travis, 1997). Future research should consider habitat composition analyses paired with comparative choice assays.

Conclusions

In this study, we sought to add more nuance to previous studies of *L. perugiae* by determining if the observed lack of male choosiness is due to differences in male mating strategies. We hypothesized the costs of elaborate secondary sexual traits should be negatively correlated with male choosiness. We found that secondary sexual traits did not impact male choosiness. In fact, in *L. perugiae,* males adopt the same mating behaviors, regardless of morphotype. Our study provides further evidence of the peculiarity of mating behaviors found in this species. To understand the evolution of *Limia's* unique preferences, future studies should prioritize 1) direct comparisons of secondary sexual trait costs between male morphs, 2) cryptic mate choice, and 3) habitat composition.

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Figures:

Figure 1: Three male morphotypes in *Limia perugiae*. *Limia perugiae* males adopt one of

three mating strategies: courters (top), intermediates (middle), sneakers (bottom). Courter males

invest heavily into secondary sexual traits such as ornamentation note the bright yellow color,

iridescent and black scales, as well as the large size. Intermediates adopt a similar morphology,

albeit to a less extreme degree, as courters. Sneakers forgo elaborate ornamentation and large

size; instead, they enter the mating population as soon as possible.
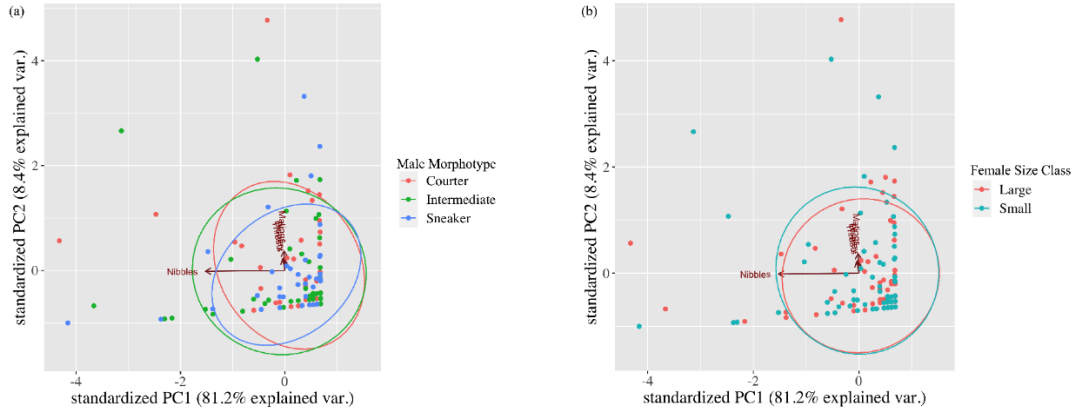
Figure 2: Gonopore nibbling contributed most to PC1. The following male mating behaviors were scored using Behavioral Observation Research Interactive Software: gonopore nibbles (nibbles), gonopodial swings (thrusts), mating attempts (matings), and sigmoid displays (displays). In a principal component analysis, PC1 and PC2 accounted for 81.2% and 8.4% of the variance, respectively. Gonopore nibbling contributed most to PC1. When grouped by male morphotype (a) or female size class (b), no distinct clusters formed.
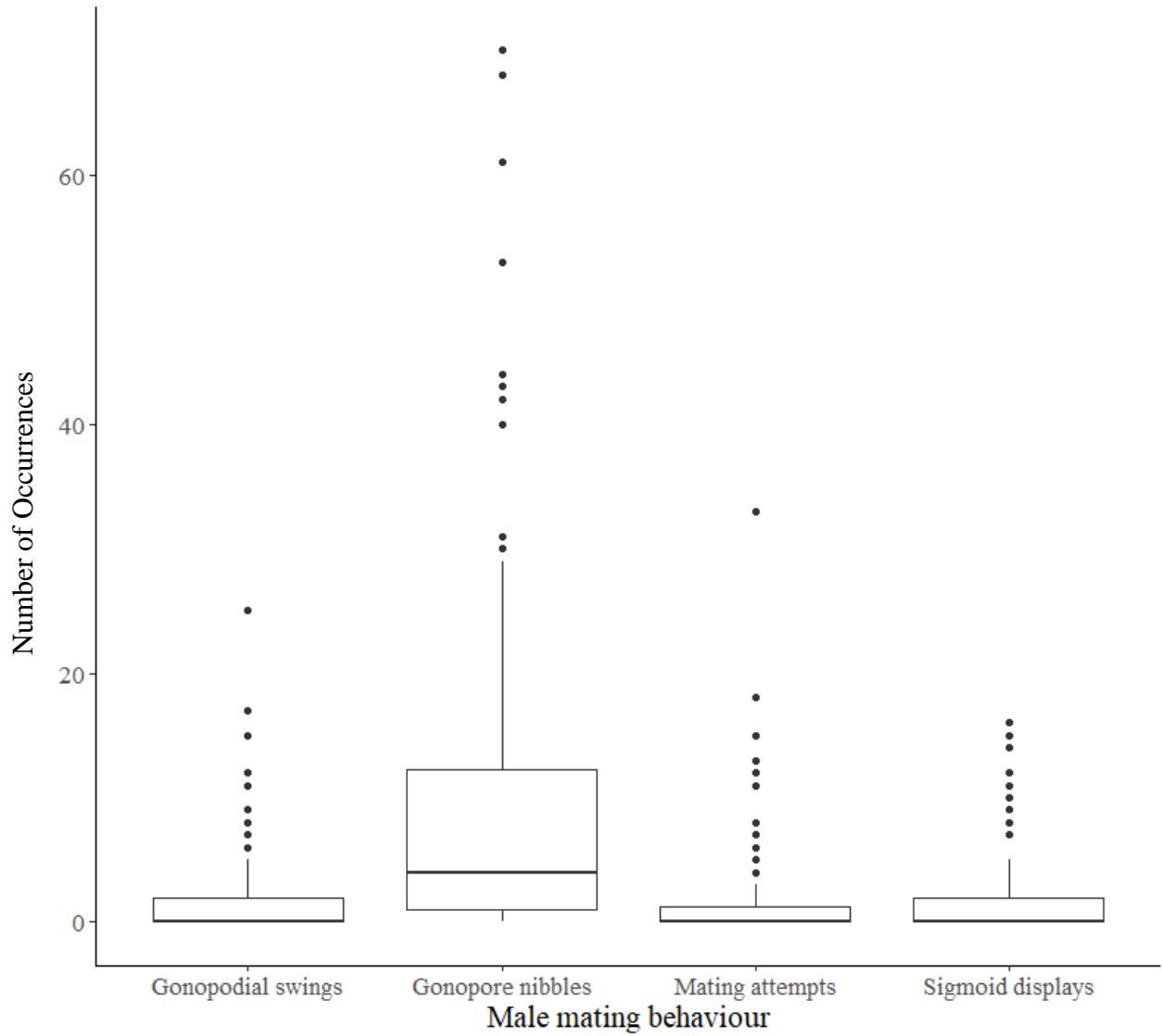
Figure 3: Mating behaviors were performed with equal frequency. When the total number of occurrences of male mating behaviors were compared (i.e., gonopore swings, gonopore nibbles, mating attempts, and sigmoid displays), disregarding male morphotype and female size class, no differences were found.
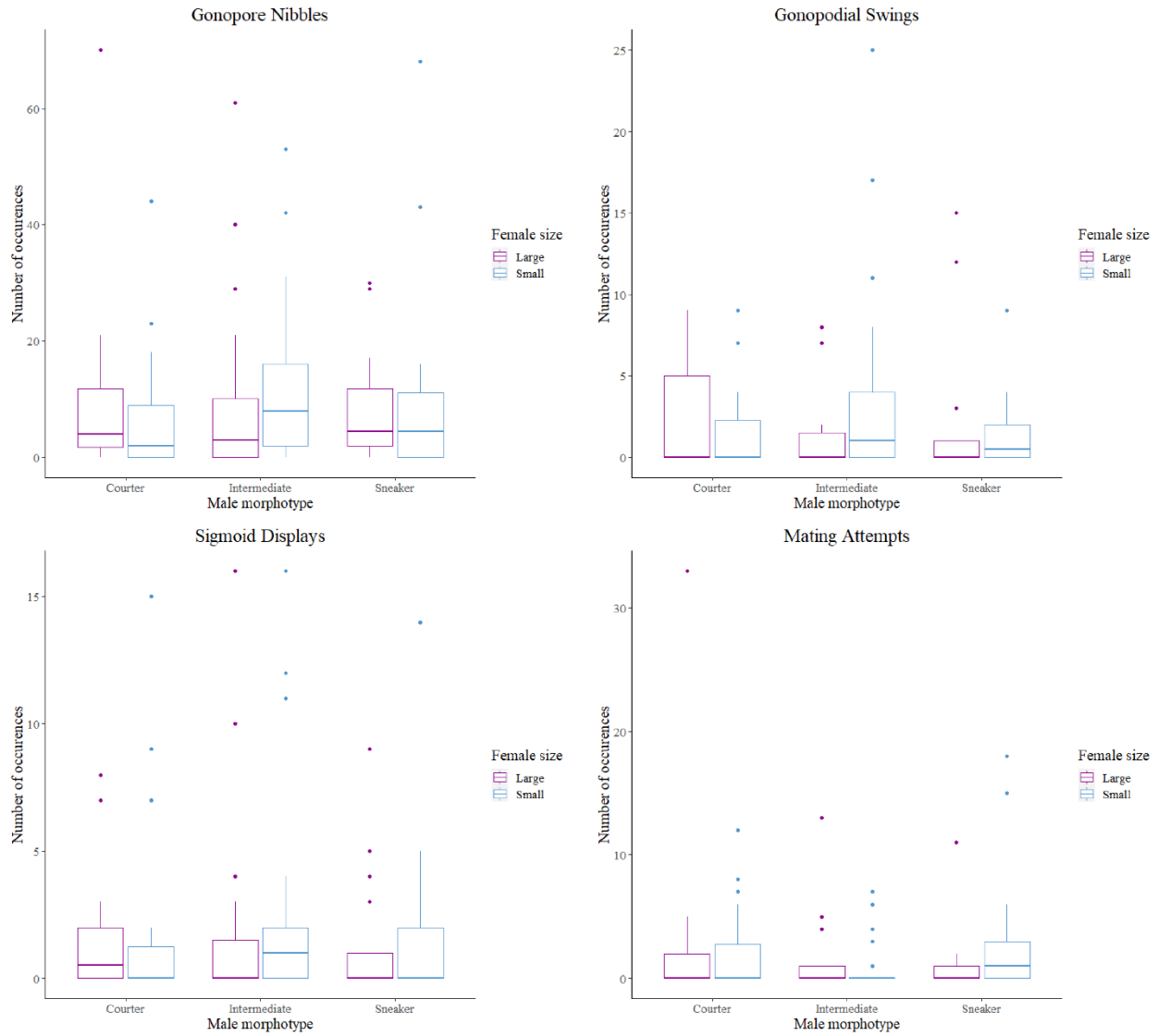
Figure 4: Male mating behaviors were not impacted by female size or male morphotype.
When male mating behaviors (i.e., gonopore swings, gonopore nibbles, mating attempts, and sigmoid displays) were analyzed separately, neither male mating strategy nor female size impacted how often males performed any behavior.
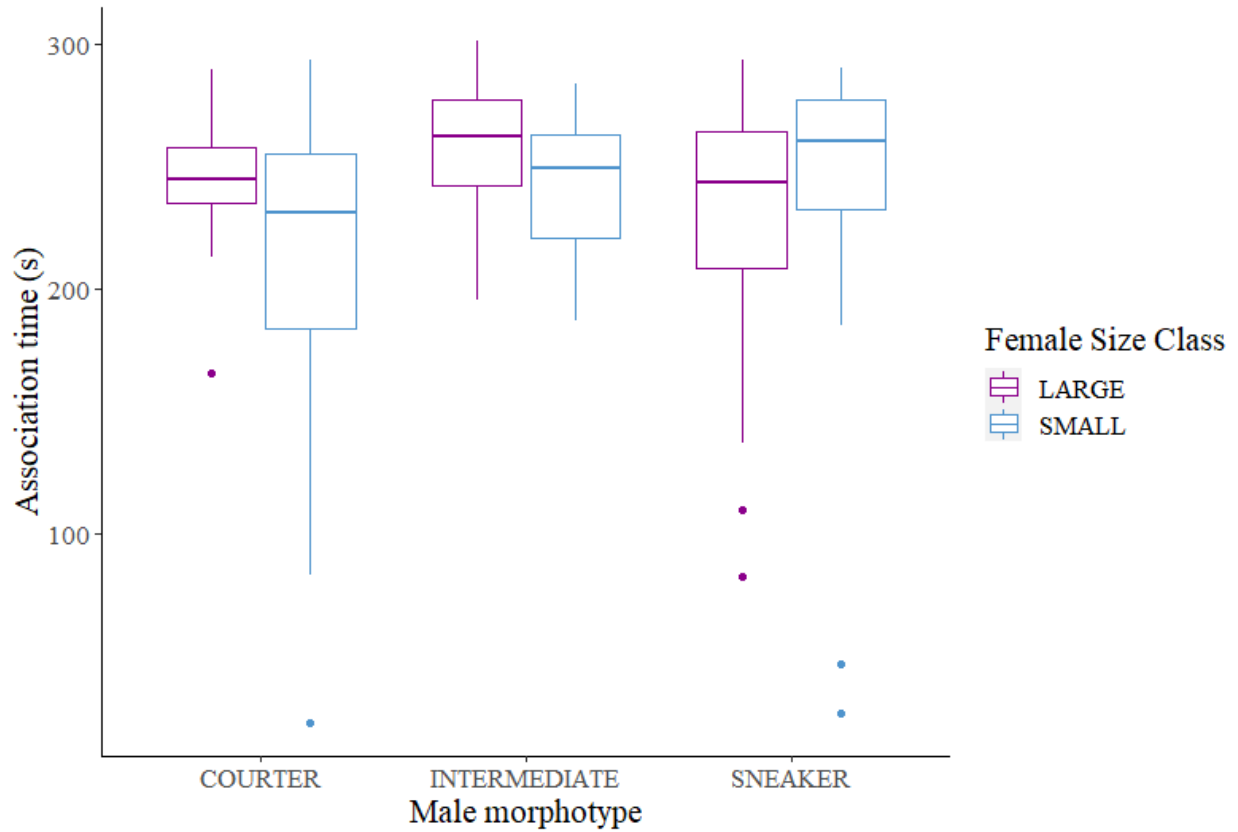
Figure 5: Males spent the same amount of time with females regardless of male morphotype and female size class. We found that males did not spend significantly more time with large females or small females. Similarly, males with the three mating strategies did not differ in association time with large and small females.

CHAPTER 4: A phylogeny of the genus *Limia* (Teleostei: Poeciliidae) suggests a single-lake radiation nested in a Caribbean-wide allopatric speciation scenario

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Montrai Spikes[1,6], Rodet Rodríguez-Silva[1], Kerri-Ann Bennett[2], Stefan Bräger[3], James Josaphat[4], Patricia Torres-Pineda[5], Anja Ernst[6], Katja Havenstein[6], Ingo Schlupp[1,6], Ralph Tiedemann[6*]

[1] University of Oklahoma, Department of Biology, 730 Van Vleet Oval, Norman OK 73019, USA.

[2] The University of the West Indies (Mona Campus), Department of Life Sciences, Kingston 7, Jamaica

[3] German Oceanographic Museum (DMM), Katharinenberg 14-20, 18439 Stralsund, Germany

[4] Caribaea Intitiative and Université des Antilles, Guadeloupe

[5] Museo Nacional de Historia Natural Prof. "Eugenio de Jesús Marcano", Avenida Cesar Nicolás Penson, Santo Domingo 10204, Santo Domingo, República Dominicana.

[6] Unit of Evolutionary Biology/Systematic Zoology, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Straße 24-25, Haus 26, 14476 Potsdam, Germany

Abstract (300 words)

The Caribbean is one of the most important biodiversity hotspots on the planet due to the high level of species diversity and endemism in plants and animals. As elsewhere, adaptive radiations in the Caribbean have led to many speciation events within a limited period and hence are particularly prominent biodiversity generators. The general prediction from Island Biogeography that relates species richness to island size is valid for livebearing fishes in general in the Greater Antilles, where larger islands have higher numbers of species mainly due to *in situ* speciation. A prime example of this speciation process can be seen in the genus *Limia*, endemic to the Greater Antilles. Within Hispaniola, nine species have been described from a single isolated site, Lake Miragoâne, pointing towards extraordinary sympatric speciation in *Limia*. Few studies have examined the evolutionary history of the fishes found in Lake Miragoâne. Here, we address the gaps in knowledge by providing a preliminary phylogeny of *Limia* and testing whether the species found in Lake Miragoâne may originated from an *in situ* radiation. We targeted the mitochondrial cytochrome *b* gene, a well-established marker for lower-level taxonomic relationships for which we obtained almost complete sequences for 13 species. The general topology of the phylogenies we produced are in concordance with other published phylogenies of *Limia*. There is also strong support [evidence instead of support?] that the species found in Lake Miragoâne in Haiti are indeed monophyletic (BS=97; PP=1.0), confirming the hypothesis of a recent local radiation. Within Lake Miragoâne, speciation is likely extremely recent, leading to incomplete lineage sorting in the mtDNA. Future studies are needed using multiple unlinked genetic markers to disentangle the relationships within the Lake Miragoâne clade.

Introduction

The Caribbean is considered to be one of the most important biodiversity hotspots on the planet due to the high level of species diversity and endemism in plants and animals (1). Among the islands of the Caribbean basin, the largest biodiversity is found in the Greater Antilles (Cuba, Hispaniola, Jamaica and Puerto Rico), where a remarkable diversification is observed in freshwater fishes (2–5), amphibians (6,7), reptiles (8,9), several groups of invertebrates (10–12), and multiple (?) families of plants (13,14). The complex geological history of the archipelago of the Greater Antilles, coupled with other factors such as island size, environmental heterogeneity, and the tropical climate, have fostered the evolution of an extraordinary number of species in certain groups (15).

Adaptive radiations lead to many speciation events within a limited period of time and are hence particularly prominent biodiversity generators. They typically occur when a set of open niches becomes available because of a key innovation or the arrival of a founder species, which subsequently differentiates to occupy these niches (16). Many of the classical examples are linked to islands. For example, Darwin's Finches on the Galapagos islands formed a group of species, all descended from a single ancestor (17–19). Research on Darwin's Finches also highlighted the role of hybridization in speciation (20). Other well-explored radiations include the Hawaiian silverswords (21–23) and the Hawaiian honeycreepers (24). Probably the best-known examples from the Caribbean region are *Anolis* lizards (25) and *Eleutherodactylus* frogs (26). Both taxa experienced convergent evolution, as the same ecotypes evolved to occupy specific types of vegetation. These lizards and frogs by no means represent the total radiations in the region, as the Caribbean is home to many such radiations, both in plants and other animals. In all these examples, molecular evidence has played an important role in understanding the evolutionary processes of speciation.

Aquatic organisms of Middle America, especially freshwater fishes, are receiving renewed attention for their adaptive radiations. Poeciliidae are a group of freshwater livebearing fishes that has experienced the highest radiation in aquatic environments of the West Indies, with three endemic genera (*Girardinus*, *Quintana* and *Limia*) distributed in the Antilles (3,4,27,28). The Caribbean is also the site of two lesser known radiations in isolated inland lakes, both of which involve fishes of the genus *Cyprinodon*. One radiation was reported from the Laguna Chichancanab in Yucatan, Mexico (29–31) and the other from the Bahamas (32). These radiation of Caribbean fishes share many characteristics with arguably the most prominent example of rampant radiation in freshwater fishes, the cichlids in the lakes of the Rift Valley of East Africa, where each lake has selected for a distinct cichlid fauna (33–35). One of the important drivers for speciation in these fishes seems to be feeding specializations (32,36,37). Furthermore, as generally predicted from the theory of island biogeography (38) and recently empirically confirmed for island birds (39,40), the number and diversity of species in both the Rift Valley lakes and Greater Antilles correlates with the size of the habitat.

Island Biogeography theory states that generally larger islands have higher numbers of species mainly due to *in situ* speciation (37). While this theory is valid for livebearing fishes in general, in the Greater Antilles, the origin of the different lineages and species composition within each genus may show peculiar patterns (39). Evidence of this divergent speciation process can be seen in the genus *Limia*, which is part of the unique freshwater fish fauna found in the Greater Antilles. *Limia* is found in most freshwater habitats in Hispaniola, ranging from hypersaline lagoons to relatively cool mountain streams (42,43). *Limia* species are generally considered to be feeding generalists (2,44,45). The biogeographical distribution indicates a radiation on the island of Hispaniola (2,46),

with 19 of the 23 known species found on this island (45,47) (Figure1). By stark contrast, Cuba, Jamaica, and Grand Cayman, each have only one species (27,43,48).

Within Hispaniola, nine *Limia* species have been described from a single site, Lake Miragoâne (*Étang de Miragoâne*). This lake is one of the largest freshwater lakes in the Caribbean and is located in the southwestern part of Haiti. Lake Miragoâne is considered an isolated, endorheic drainage (49), which suggests sympatric speciation at the site. This radiation was hypothesized by Rivas (2) for Lake Miragoâne and has received renewed attention through the description of two new species from the lake (43,45). Although *Limia* presents a prime opportunity to further our understanding of radiations in freshwater systems, few studies have examined the evolutionary history of the fishes found in Lake Miragoâne.

Though the potential radiation event in Lake Miragoâne has received little attention, some studies of *Limia* have resolved the general phylogeny of the genus. Current literature suggests *Limia* forms a monophyletic group with the genera *Pamphorichthys*, *Mollienesia*, *Micropoecilia*, and *Poecilia*, with *Limia* as sister taxon to *Poecilia* (50–52). *Limia melanogaster* is the most basal species, branching off early and colonizing Jamaica (2). *Limia melanogaster*'s divergence was followed by the inhabitation of Hispaniola, where the species diverged into over 20 recognized species (43). Nested within the species native to Hispaniola are *L. vittata* and *L. caymanensis* (2,53) which are the only species native to their respective islands, Cuba and Grand Cayman (27,43). While these general patterns are maintained in the few phylogenies of *Limia,* most analyses use only a few species (51,52,54). The most robust species sampling and reliable phylogeny to date used nine species of *Limia*, among them only two native to Lake Miragoâne, *Limia nigrofasciata* and *Limia islai* (2,43,45). Such limited species sampling has hampered our understanding of the evolutionary history of this genus, specifically of those species found in Lake Miragoâne. Rivas hypothesized

a local radiation within Lake Miragoâne in his original description of the (1) species, but this hypothesis remained to be tested.

Our study further examines the evolutionary history of the genus *Limia*. Prior to this study, we could reasonably estimate the evolutionary relationships of only nine of the over 20 *Limia* species. The phylogeny constructed here consists of 13 species of *Limia*. It is particularly novel regarding its more comprehensive sampling of Lake Miragoâne, including five of its native species. We expected that if a local radiation event did occur in Lake Miragoâne, those species native to the lake would be monophyletic. Our study appends to the phylogeny of *Limia* – for the first time - the evolutionary history of four additional species, two of which were only recently described (45,47). Summarily, we address the gaps in present knowledge by providing a preliminary phylogeny of *Limia* and testing whether the species found in Lake Miragoâne may represent a local radiation.

Materials and Methods

Sampling

Samples of muscle tissue from the caudal peduncle were taken from *Limia* species from Cuba, Hispaniola, and Jamaica. We used four to five individuals per species, except in cases where sampling was limited (Table 1). Ingroup sampling consisted of 62 individuals representing 13 species of *Limia* (Table 1). Outgroup sampling consisted of eight individuals representing three species of *Poecilia,* the sister taxon to *Limia* (27,43,54). We use *P. dominicensis* (44) and *P. hispaniolana* (55), both of which are endemic to Hispaniola, and *P. mexicana*, which is from the Atlantic side of Mexico.

Molecular methods

We targeted the mitochondrial cytochrome *b* gene, a well-established marker for lower-level taxonomic relationships as in recent radiations across vertebrates (see (56) for a fish example). We obtained an almost complete sequence from the 60 individuals we sampled.

Sequences used in our analyses were obtained from NCBI GenBank where available (52) (57) and complemented by novel sequences collected for our wild-caught specimens (Table 1). Genomic DNA was extracted from muscle tissue using a cetyl trimethylammonium bromide (CTAB) protocol (58). DNA concentration was measured using a NanoDrop ND-1000. Using polymerase chain reaction (PCR), we amplified a 1,127 bp fragment of the mitochondrial cytochrome *b* gene. Primers are listed in Table 2 and were modified from Hrbek et al. (50). Genomic DNA isolate concentrations ranged from 2.7 ng/ μl to 120 ng/ μl. One microliter of DNA isolate was used during amplification, except if the DNA concentration was less than 20 ng/ μl. When DNA isolate concentrations were low, we increased the amount of DNA isolate by 1 μl. The mitochondrial cytochrome *b* gene was amplified using 0.12 μl of 5U/μl MyTaq mtDNA polymerase (Bioline), 0.5 μl of each 10 μM primer, 5 μl of 5x MyTaq reaction buffer and 17.88 μl of HPLC H_2O for a final reaction volume of 50 μL. Primer combinations and amplification temperature profiles can be found in Table 2. Except for *L. vittata*, *P. dominicensis*, and *P. hispaniolana*, all species used the primer combinations L14725 and H15981 (Table 2). Cycle sequencing reactions were run using Applied Biosystems[TM] BigDye™ Terminator v3.1 Cycle Sequencing Kits (ThermoFisher). PCR products were purified with ExoSAP (Exonuclease I (59) and Antarctic Phosphatase (60)) according to manuals provided by New England Biolabs. Sequences were run on an Applied Biosystems[TM] 3500 sequencer. Mitochondrial DNA sequences were manually edited and assembled using BioEdit v.7.2 (61).

Phylogenetic and Population Genetic Analyses

Sequences were aligned with ClustalW in BioEdit v.7.2 (61,62) and 1,127 bp of cytochrome *b* were used in phylogenetic analyses. We conducted Maximum Likelihood analyses using RAxML GUI v.2.0 (63,64) and assessing clade support via 10,000 rapid bootstrap pseudoreplicates. Separately, we conducted Bayesian analyses in MrBayes v.3.2.7. (65). In MrBayes we ran four Markov chains for 1,000,000 iterations, sampling every 1,000 iterations, with three heated chains and one cold chain and default parameters unlinked across partitions. Convergence was assessed using Tracer v.1.7, and all parameter estimates were verified to have been sampled sufficiently (ESS>200). Although all samples showed patterns consistent with stationarity after 1,000,000 iterations we conservatively removed the first 25% of our trees as burn-in, such that 3,002 trees were considered. We determined that a node was highly supported when the bootstrap support values (BS) and the Bayesian posterior probability (PP) was greater than 70 and 0.95, respectively (66,67). Moderate support was assigned to nodes that fell between 75-95 (BS) and 0.75-0.95 (PP). To better visualize the relatedness of closely related species a haplotype network was constructed within PopArt (68) using a median joining network (69). Genetic distances between taxanomic groups were calculated using the proportion of sites with transitional and transversional nucleotide differences to total nucleotides in MEGA (70).

Results

The Maximum Likelihood and Bayesian Inference trees revealed nearly identical topologies for interspecific relationships (Figure 2). In both trees, there is strong support that the species found in Lake Miragoâne in Haiti are monophyletic (BS=97; PP=1.0). However, within Lake Miragoâne, *L. mandibularis* is the only species to form a monophyletic group, while the phenotypically described species *L. islai, L. immacualata, L. miragoanensis*, and *L. nigrofasciata* form a polytomy (Figure 2).

For the majority of taxa outside Lake Miragoâne, both species monophylies and respective taxonomic relationships were well supported. In both phylogenies, however, *L. yaguajali* is sister to a clade consisting of *L. perugiae*, *L. dominicensis,* and all the species found in Lake Miragoâne, but this node has moderate support (BS=61; PP=72). We also found strong support for significant genetic divergence of two populations of *L. melanogaster* found on Jamaica (BS=100; PP=1.0). The haplotype network (Figure 3) generally confirms divergent *Limia* evolution among the different Caribbean islands. Within Lake Miragoâne and in the *L. perugiae* group, mitochondrial haplotypes occurred that were shared among morphological species.

Discussion

Our study is the first to use molecular techniques to explicitly test the hypothesis that the *Limia* species in Lake Miragoâne represent an *in situ* radiation. Our samples from Lake Miragoâne include three of the species originally described by Rivas (2) that had not previously been used in a phylogenetic analysis. We also included two recently described species from the lake, *Limia islai* and *Limia mandibularis* (43,45,47). These five species from Lake Miragoâne are recovered as part of a well-supported clade in all phylogenetic analyses; however, with the exception of *L. mandibularis*, analyses failed to recover *L. islai, L. immacualata, L. miragoanensis*, and *L. nigrofasciata* as reciprocally monophyletic clades consistent with recognized species boundaries. Our inability to completely resolve these taxonomic relationships is due to the limited loci and specimens sampled. We hypothesize these species have diverged too recently for complete lineage sorting and reciprocal monophyly to evolve at a single maternally inherited locus like cytochrome *b*. Alternatively, the observed pattern could be due to species hybridization, phenotypic species misassignment, or introgression (71).

Most other relationships supported in phylogenetic analyses are consistent with the findings of other recent phylogenetic studies (27,43). Within *Limia*, the basal group is *L. melanogaster* from Jamaica (Figure 2). *Limia melanogaster*'s basal placement further corroborates the findings by Weaver and colleagues (41) that *Limia* initially colonized Jamaica before radiating across other islands in the Greater Antilles, lending additional support to the GAARlandia hypothesis (72,43). Moreover, we find that the two populations of *L. melanogaster* sampled show significant genetic divergence. This divergence within *L. melanogaster* may be the first evidence of a further cryptic speciation event in *Limia* (Supplemental Table 1). *Limia vittata* from Cuba and *L. caymanensis* from Grand Cayman group within the Hispaniola clade. These two species are likely the sister taxa to *L. yaguajali*, which is found in the north of Hispaniola. Also, there is geological evidence that eastern Cuba and north-central Hispaniola were likely connected as a single magmatic arc during the Paleocene-Eocene (73) until the Oligocene (74). Together, the biogeographic and geological evidence suggests that an *L. vittata* ancestor reached Cuba from the north of Hispaniola, and subsequently an *L. caymanensis* ancestor reached Grand Cayman from Cuba. Alternatively, they may have reached Cuba via open ocean migration, which has occurred in other freshwater fishes (5).

The sister group to all species in Lake Miragoâne is the *L. perugiae* group. This group also exhibits a shallow phylogeny with relatively short branches and one haplotype shared across species. Again, this may indicate a recent divergence or incomplete lineage sorting, suggesting *L. perugiae* populations are differentiating. *Limia perugiae* shows a remarkable plasticity in habitat requirements. It is found from hypersaline lagoons to cool freshwater streams and dominates another large lake on Hispaniola, Lake Enriquillo. *Limia perugiae* are also widely distributed throughout Hispaniola, with many populations located far from each other, so the possibility of

gene flow between them is low. The combination of *L. perugiae*'s diverse life history strategies and fragmented populations may promote cryptic speciation. However, given our inability to genetically resolve this group, phenotypic plasticity could be an alternative explanation.

We present initial evidence for a potential radiation in Lake Miragoâne, yet we recognize the limitations of a single-gene phylogeny. Our preliminary findings are supported by morphometric data that show distinct phenotypic differences between multiple *Limia* populations (1,43,45). The use of multiple unlinked markers, such as microsatellites or SNPs, along with increased population sampling, are imperative to understand the radiation event within this clade, as is true for the *L. perugiae* group as well. The analyses of nuclear and additional mitochondrial loci may also resolve the relationship of *L. yaguajali* with *L. vittata* and *L. caymanensis.* Clarifying the evolutionary history of this group will provide insights into the biogeography of the Greater Antilles. If our results are confirmed by additional analyses, the radiation in Lake Miragoâne would represent the first significant radiation within the family Poeciliidae. We acknowledge that 13 species represent only a subset of the 23 known species of *Limia*. Future studies should continue to increase species sampling.

So far it is still unclear whether natural selection, which may be reinforced by sexual selection, or sexual selection alone has been the main driver of speciation in Lake Miragoâne. Even though ecological data remains limited, it is known that *Limia* species from Lake Miragoâne all inhabit extremely similar niches (75) and they likely possess similar life histories, perhaps with the exception of *L. mandibularis*. This species has evolved to utilize particular food resources [missing words here?] well-developed and anteriorly projected lower jaw, which deviates from that of other poeciliids. *Limia mandibularis*' modified jaw may offer some advantages or specializations in relation to diet (47). Marked sexual dimorphism, with males such as *L.*

nigrofasciata being extremely ornamented, suggests that sexual selection is also present. Therefore, it is plausible that both natural and sexual selection might – independently or in concert - act as drivers in the potential radiation event of *Limia* in Lake Miragoâne.

Geographically restricted species flocks are not only uniquely important to our understanding of speciation, but they may also be particularly vulnerable to environmental threats. For freshwater taxa worldwide, human-induced climate change is just one of many threats. The cichlids of the Rift Valley Lakes are famously influenced by human-induced turbidity (76). Similarly, throughout the Caribbean habitat alteration, human activities and invasive species especially are threatening the native fauna (77,78). Conservation actions protecting endemic species within these unique habitats are urgently required to preserve hotspots of biodiversity, such as Lake Miragoâne.

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Table 1. Taxon list including collection localities, country of origin, coordinates, date of collection, and accession numbers. 'NA' denotes the information is either unavailable or inapplicable.

| Species	No. of Individuals	Locality	Latitude	Longitude	Date of Collection	Accession Number	Reference
Limia caymanensis*	1	NA, Grand Cayman Island	NA	NA	Sep-1991	KJ696810.1	(50)
Limia dominicensis	6	Puerto Escondido, DR	18.319	−71.570	Mar-2014	MW355516 - 21	NA
Limia garnieri*	1	NA, DR	NA	NA	NA	KJ696811.1	(50)
Limia immaculata	4	Lake Miragoâne, Haiti	18.426	−73.049	Jun-2019	MW355522 - 25	NA
Limia islai	5	Lake Miragoâne, Haiti	18.426	−73.049	Jun-2019	MW355526 - 30	NA
Limia mandibularis	2	Lake Miragoâne, Haiti	18.426	−73.049	Jun-2019	MW355531 - 32	NA
Limia melanogaster	4	Caymanas Spring, Jamaica	18.042	−76.910	Jun-2017	MW355533 - 36	NA
Limia melanogaster	4	Roaring River, Jamaica	18.283	−78.056	Jun-2017	MW355537 - 40	NA
Limia melanonotata*	1	Callon Papio, DR	NA	NA	Apr-1949	KJ696813.1	(50)
Limia miragoanensis	2	Lake Miragoâne, Haiti	18.426	−73.049	Jun-2019	MW355541 - 42	NA
Limia nigrofasciata	4	Lake Miragoâne, Haiti	18.426	−73.049	Jun-2019	MW355543 - 46	NA
Limia pauciradiata*	1	Lake Miragoâne, Haiti	NA	NA	Jan-1986	KJ696815.1	(50)
Limia perugiae	4	Río Verde, DR	19.312	−70.524	Dec-2018	MW355547 - 50	NA
Limia perugiae	3	La Azufrada, DR	18.561	−71.700	Jun-2003	MW355551 - 53	NA
Limia rivasi*	1	NA, DR	NA	NA	NA	KJ696817.1	(50)
Limia sulphurophila*	1	NA, DR	NA	NA	NA	KJ696818.1	(50)
Limia versicolor*	1	Río Haina, DR	NA	NA	Jun-1977	KJ696819.1	(50)
Limia vittata	4	Guanimar, Cuba	22.694	−82.651	Aug-2018	MW355554 - 57	NA
Limia yaguajali	7	Río Cana, DR	19.496	−71.281	May-2019	MW355558 - 64	NA
Limia zonata	5	Río Yaguajal	19.472	−70.342	Dec-2018	MW355565 - 69	NA
Poecilia dominicensis	3	Río Yasica Abajo, DR	19.633	−70.594	2018	MW355570 - 71	NA
Poecilia hispaniolana	4	Río Cana, DR	19.496	−71.280	May-2019	MW355572 - 75	NA
Poecilia mexicana*	1	Cueva Luna Azufre, Mexico	17.442	−92.773	Jan-2006	EU269065.1	(56)

* Sequences acquired from GenBank

Tables:

Table 2. List of cytochrome *b* PCR primer pairs and temperature profiles. Primer sequences are listed from 5' to 3' ends. References are provided where applicable.
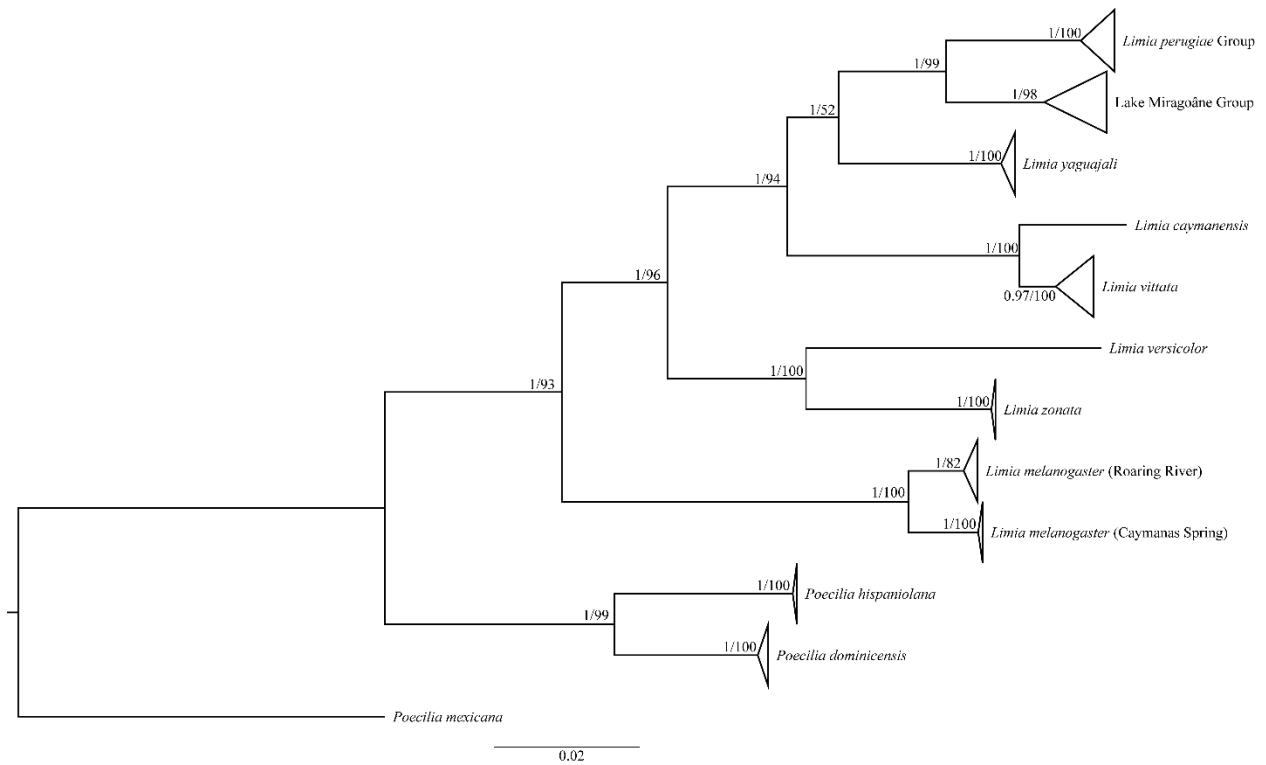
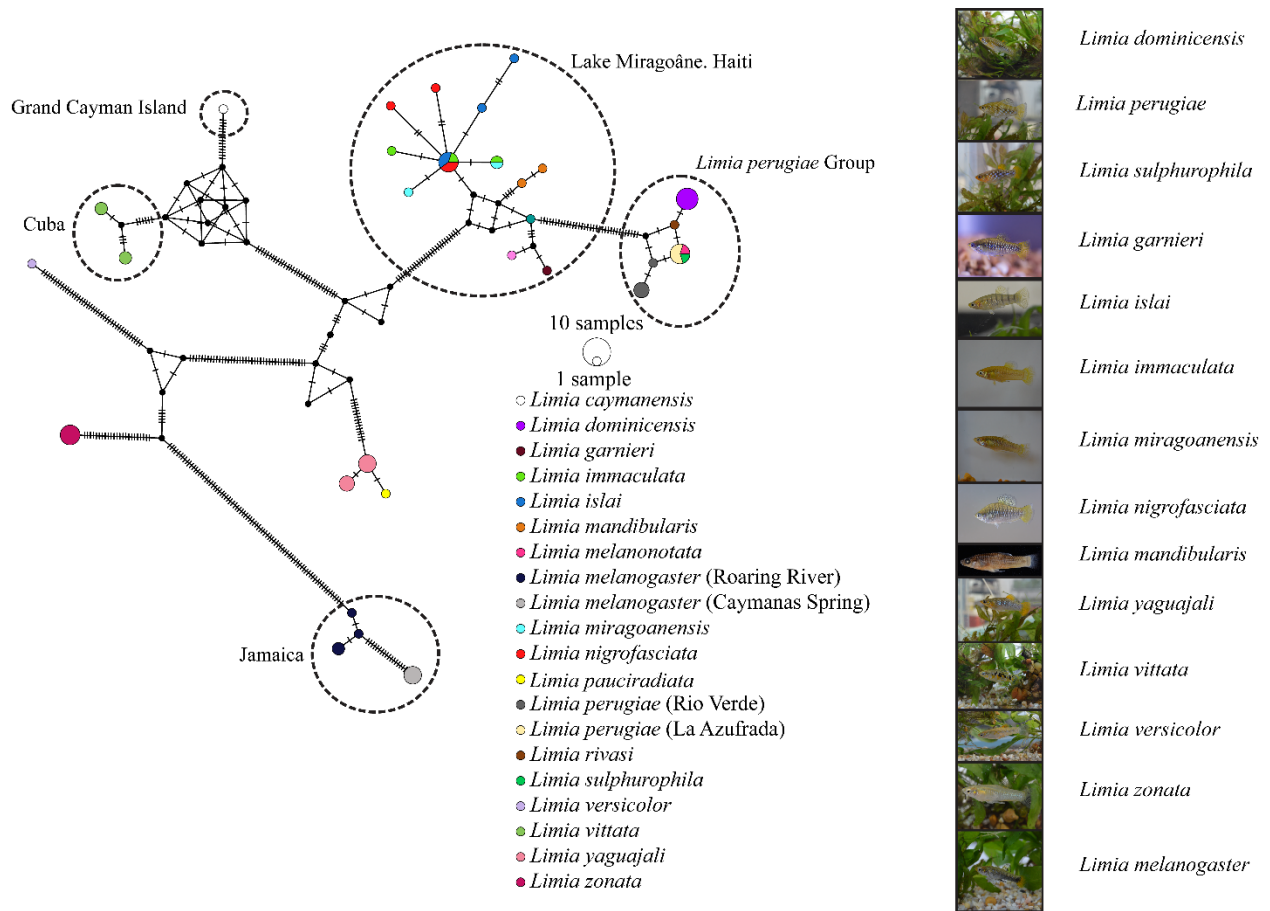| Primer pairs	Sequence	Temperature profiles	Reference
Forward: L14725 Reverse: H15981	5'-GAYTTGAARAACCAYCGTTG-3' 5'-GAATYCTAGCTTTGGGAGYTAG-3'	1 cycle of 1 min at 95 °C 40 cycles 25 s at 95 °C 20 s at 46 °C 25 s at 72 °C 1 cycle 10 min at 72 °C	(48)
Forward: Limia L14740 Reverse: Limia-Hint	5'-CCGTTGTWAYTCAACTCACAAG-3' 5'-GGTGAAGTTTTCTGGGTCTCC-3'	1 cycle of 1 min at 95 °C 40 cycles 25 s at 95 °C 20 s at 46 °C 25 s at 72 °C 1 cycle 10 min at 72 °C	(48)
Forward: Limia Lint Reverse: Limia H15981	5'-GCCTYGTMCAATGAATCTGAGG-3' 5'-GAATYCTAGCTTTGGGAGYTAG-3'	1 cycle of 1 min at 95 °C 40 cycles 25 s at 95 °C 20 s at 46 °C 25 s at 72 °C 1 cycle 10 min at 72 °C	-
Forward: Limia Lint Reverse: Limia H16249	5'-GCCTYGTMCAATGAATCTGAGG-3' 5'-GCCGACTTTCGATTTACAAGAC-3'	1 cycle of 1 min at 95 °C 40 cycles 25 s at 95 °C 20 s at 46 °C 25 s at 72 °C 1 cycle 10 min at 72 °C	-
Forward: Limia L14763 Reverse: Limia Hint	5'-GACCTACGAAAATCYCACCCCC-3' 5'-GGTGAAGTTTTCTGGGTCTCC-3'	1 cycle of 1 min at 95 °C 40 cycles 25 s at 95 °C 20 s at 50 °C 25 s at 72 °C 1 cycle 10 min at 72 °C	-

Figures:
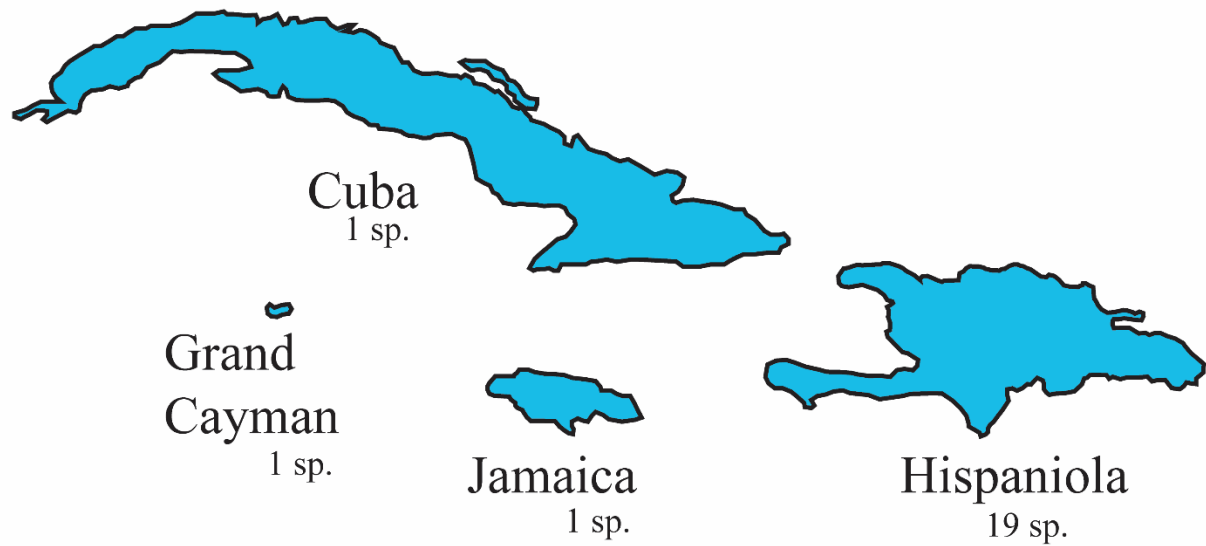
Figure 1. Biogeographical distribution of *Limia* suggests a radiation event on Hispaniola. Island Biogeography theory suggests species diversity should scale with an island's landmass. We observe a differing pattern in *Limia,* where the greatest diversity is not found on Cuba, the largest island of the Greater Antilles, but rather on Hispaniola.

Figure 2. Maximum Likelihood and Bayesian Inference trees both recover species endemic to

Lake Miragoâne originated from an *in situ* radiation. The Maximum likelihood bootstrap values

are placed above Bayesian inference posterior probabilities on each node. Both phylogenetic

analyses show identical topologies. Both methods had difficulty resolving species in Lake

Miragoâne.

Figure 3. The mitochondrial haplotype network shows shared haplotypes in both the Lake Miragoâne group and *L. perugiae* group. A median joining network was used to visualize haplotypes between closely related species in Lake Miragoâne and in the *L. perugiae* group.