Divergent Mating Behavior and the Evolution of Reproductive Isolation

by

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Sexual selection can cause rapid co-divergence of mating traits and mate preferences, generate reproductive barriers among individuals bearing divergent mating traits, and potentially lead to speciation. In my dissertation, I focused on two emerging topics that challenge this traditional speciation-by-mate-choice paradigm. First, sexual selection encompasses both mate preferences and intrasexual competition, yet speciation research disproportionally focused on the role of the former. Second, sexual behaviors are usually assumed to be genetically inherited, but they may often be shaped by learning instead, which can generate very different evolutionary trajectories for traits and preferences. Using studies of the highly polymorphic strawberry poison frogs (*Oophaga pumilio*), I demonstrated how incorporating (i) male-male competition and (ii) behavioral learning can enhance our understanding of the potential for speciation to be driven by sexual selection. I first characterized behavioral patterns across a natural contact zone between color morphs and showed that coloration (the divergent mating trait) mediates both female choice and male-male competition. Females often prefer males of their own (local) color over a novel color, and males, when defending territories, are more aggressive against their own color morph. I then tested how these color-mediated female preferences and male aggression biases interact to determine mating patterns. I conducted a controlled breeding experiment in which male-male competition and female mate choice act either in same or in opposing directions. In this study, females reproduced more often with the territorial male over the non-territorial male, regardless of the males' coloration. This challenges the common assumption that knowledge of female preferences for male mating traits is sufficient to predict mating patterns. Finally, I discovered that

learning from mothers during the tadpole stage shapes both female mate preferences and male aggression biases in *O. pumilio*. Based on this finding, I built a population genetic model and used it to demonstrate a simple and elegant mechanism by which sexual selection alone has the potential to initiate speciation. My research highlights the importance of considering interactions between mate choice, intrasexual competition, and behavioral learning, for studies of mating trait evolution and sexual selection's role in speciation.

Table of Contents

List of Tables

List of Figures

Preface

First and foremost, I would like to express my deepest gratitude to my advisor Corinne Richards-Zawacki for her patient support and mentorship during my six years in her lab, for letting me think and work independently but lending a hand when I need it, and for always being more confident in me than I am in myself. I thank all my scientific mentors that have helped me during my PhD study and made me a better researcher. To Matthew Dugas, for showing me the ropes during my first field season in Panama, and for pushing me to write clearly and efficiently. To Maria Servedio, my external dissertation committee member, who hosted me for a month in her lab at UNC Chapel Hill and patiently guided me to build my first population genetic model. To my present dissertation committee (Walter Carson, Mark Rebeiz, Martin Turcotte) and all former members (Nathan Morehouse, Jonathan Pruitt, Elizabeth Derryberry, Jordan Karubian), who have always urged me to "find the big question" and place my research in a broader framework in ecology and evolutionary biology. Thank you to the Pitt E&E community for the vibrant energy, scientific feedback, and career development advice. Thank you to the staff at Smithsonian Tropical Research Institute in Bocas del Toro, especially Plinio Gondola, for their logistical help and occasional fieldwork assistance during my field seasons in Panama.

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1.0 Chapter 1: Introduction

Understanding how new species are formed is a fundamental goal in evolutionary biology. Sexual selection can cause rapid co-divergence of mating signals and mate preferences, and can restrict gene flow between the divergent phenotypes (Lande, 1981; Kirkpatrick, 1982; West-Eberhard, 1983). This process represents the core mechanism of speciation-by-sexual-selection theory (Panhuis *et al.*, 2001; Ritchie, 2007), and numerous empirical examples of co-divergence in mating traits and preferences among populations have lent it support (Scordato *et al.*, 2014). However, sexual selection encompasses both female mate choice and male-male competition, and the role of male-male competition has been largely ignored in speciation research (Qvarnström *et al.*, 2012; Tinghitella *et al.*, 2017; Lipshutz, 2018). Moreover, despite the established importance of learning in the development of behavior, mate preferences are traditionally considered genetically inherited in speciation models (Ritchie, 2007; Verzijden *et al.*, 2012; Servedio & Boughman, 2017). It is now increasingly clear that both **intrasexual competition** and **behavioral learning** can be important components of sexual selection, but their implications for the evolution for population divergence and speciation remains unclear.

My dissertation examines how mate choice and intrasexual competition interact to effect reproductive isolation, and the importance of behavioral learning in mediating the process. I integrate field, laboratory, and mathematical approaches to examine the mechanisms that shape divergent sexual behaviors, and the evolutionary and ecological consequences of such divergence. The strawberry poison frog (*Oophaga pumilio*) shows evidence of recent, rapid divergence in a sexually selected trait (color) among populations (**Figure 1-1**; Summers *et al.*, 2003). Using field and lab behavioral assays, I showed that coloration is under selection due to both mate choice and

intrasexual competition. Females often prefer males of their own (local) color over novel color morphs (i.e. assortative preference, **2.0**); and males, when defending territories, often bias their aggression toward other males of their own color morph (i.e. assortative aggression, **3.0**). Taking advantage of a natural contact zone, I also showed that these assortative behaviors can break down when divergent populations come into secondary contact. This suggests that the hypothesis that divergent preferences restrict gene flow upon secondary contact (Panhuis *et al.*, 2001; Kraaijeveld *et al.*, 2011) may not always be accurate, and we need to further investigate the social and environmental factors that dictate these behavioral patterns.

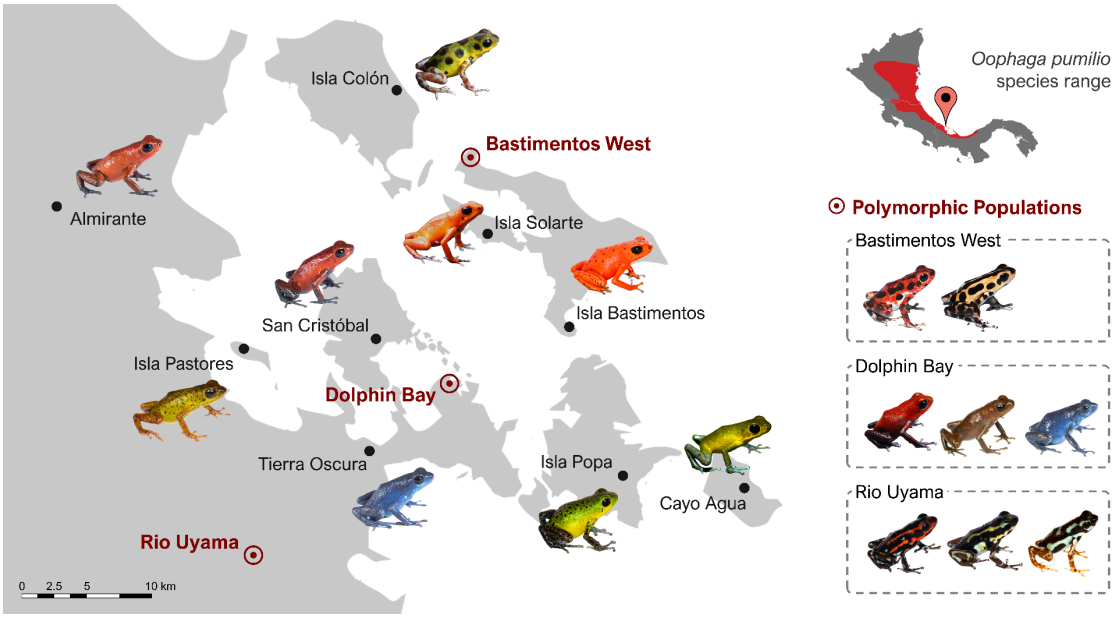

Figure 1-1 Color pattern diversity among Bocas del Toro *O. pumilio* populations

Top right, species range of O. pumilio across Nicaragua, Costa Rica and Panama. The location pin indicates the Bocas del Toro archipelago. Left, color-monomorphic populations are labelled with a solid dot and an exemplar of the color morph is shown next to the respective dot. Sympatric color- polymorphic populations are labelled with a circled dot, and exemplars of the color variants are shown on the right. Color variation at Bastimentos West (Richards-Zawacki & Cummings, 2011) is discrete, whereas color variation at Dolphin Bay (Dugas *et al.*, 2015; Yang *et al.*, 2016) and Rio Uyama (Summers *et al.*, 2003) is continuous. Photographs by V. Prémel, J. P. Lawrence, S. A. Echeverri, I. J. Wang and Y.Y. Map data 2018, Google.

Recent studies have proposed two main roles of male-male competition in the evolution of reproductive isolation: i) maintaining sexual trait and preference polymorphism in the face of gene flow, and ii) limiting assortative mating when males of the non-preferred phenotype are superior competitors. Both scenarios rely on the assumption that male-male competition can limit the expression of divergent female mate preferences. I used a controlled breeding experiment to test this critical but rarely tested assumption (**4.0**). I housed females with two differently-coloured males, and compared reproductive patterns when the more attractive male was the territory holder *versus* when he was the non-territorial male. Females mated primarily with the territory winner, regardless of his coloration, suggesting that when a choice must be made between the two, the results of male-male competition overrides female preferences for male mating traits. This challenges the common assumption that knowledge of female preferences is sufficient to predict mating patterns, and further emphasizes the importance of male-male competition the evolution of reproductive isolation.

Finally, I tested the potential for learning to shape male and female behavior (**5.0**). The biparental care exhibited by *O. pumilio* provides ample opportunity for tadpoles to observe their parents' colors. Using a controlled rearing experiment, I showed that maternal imprinting shapes both female mate preferences ('sexual imprinting') and male aggression biases ('rival imprinting') in *O. pumilio*. Tadpoles likely learn their mother's coloration while begging for her to deposit trophic eggs (the tadpoles' only food source) and use it as a template for mate preference and rival aggression biases expressed in adulthood. This constitutes the first evidence of imprinting in an amphibian. To explore the evolutionary implications of these imprinted behaviors, I built a population genetics model, and showed that imprinted male aggression biases can help maintain the coexistence of different color morphs in sympatry while imprinted female preferences reduce

gene flow among the color morphs. Contrary to previous work suggesting that the conditions needed for speciation driven by sexual selection are unlikely to occur, my work presents a simple and elegant mechanism by which sexual selection alone has the potential to initiate speciation.

My dissertation work highlights the interacting roles of mate choice, intrasexual selection, and behavioral learning in mating trait evolution and speciation. While my research has focused on *O. pumilio*, this frog is just one of the many species representing a diverse set of animal taxa where sexually selected traits are used for both mate choice and intrasexual competition (Andersson, 1994; Berglund *et al.*, 1996; McCullough *et al.*, 2016). Imprinting has also been shown to shape behavioral biases in both contexts in many taxa (Hansen & Slagsvold, 2003; Dijkstra *et al.*, 2008a; Verzijden *et al.*, 2009, 2012). Thus, my work likely has broad implications for understanding the role sexual selection in speciation.

2.0 Chapter 2: Poison Frog Color Morphs Express Assortative Mate Preferences in Allopatry but not Sympatry

The contents in this chapter are adapted from the following publication:

Yang, Y., Richards-Zawacki, C.L., Devar, A. & Dugas, M.B. 2016. Poison frog color morphs express assortative mate preferences in allopatry but not sympatry. *Evolution*. **70**: 2778–2788. doi: 10.1111/evo.13079

2.1 Chapter Summary

The concurrent divergence of mating traits and preferences is necessary for the evolution of reproductive isolation via sexual selection, and such coevolution has been demonstrated in diverse lineages. However, the extent to which assortative mate preferences are sufficient to drive reproductive isolation in nature is less clear. Natural contact zones between lineages divergent in traits and preferences provide exceptional opportunities for testing the predicted evolutionary consequences of such divergence. The strawberry poison frog (*Oophaga pumilio*) displays extreme color polymorphism in and around the young Bocas del Toro archipelago. In a transition zone between red and blue allopatric lineages, we asked whether female preferences diverged along with coloration, and whether any divergent preferences persist in a zone of sympatry. When choosing among red, blue and phenotypically intermediate males, females from monomorphic red and monomorphic blue populations both expressed assortative preferences. However, red, blue, and intermediate females from the contact zone all preferred red males, suggesting that divergent

preferences may be insufficient to effect behavioral isolation. Our results highlight the complexity of behavioral isolation, and the need for studies that can reveal the circumstances under which divergent preferences do and do not contribute to speciation.

2.2 Introduction

Isolated populations of the same species can differ markedly in behavior, morphology, and physiology (Kraaijeveld *et al.*, 2011; Miller & Svensson, 2014; Brodersen *et al.*, 2015). One potential consequence of such differentiation is a reduction in the probability that lineages will interbreed, an initial step in the process of speciation (Rundle & Nosil, 2005; Kraaijeveld *et al.*, 2011). The evolution of traits important in mate choice and acquisition may be especially likely to effect reproductive isolation (Panhuis *et al.*, 2001; Gage *et al.*, 2002; Arnqvist & Rowe, 2005; Ritchie, 2007; Kraaijeveld *et al.*, 2011), and numerous studies have demonstrated the co-evolution of mate preferences and courtship traits (Scordato *et al.*, 2014). While such divergence in preferences and traits is necessary for the evolution of behavioral reproductive isolation (Panhuis *et al.*, 2001; Arnqvist & Rowe, 2005; Ritchie, 2007; Kraaijeveld *et al.*, 2011), differentiation alone may not be sufficient to drive behavioral isolation (Dougherty & Shuker, 2015; Edward, 2015). When lineages are sympatric, gene flow between lineages can occur when preferences are plastic and altered by experience (Jennions & Petrie, 1997), and when the costs of choosing constrain the extent to which mate preference actually determines mate choice (Irwin & Price, 1999; Hebets & Vink, 2007; Rodríguez *et al.*, 2013). Preferences for hybrid phenotypes and/or the mating decisions of any hybrids can similarly drive gene flow between lineages (Culumber *et al.*, 2014).

Lineages that are polytypic in sexual communication traits provide exceptional opportunities to test for the co-evolution of traits and preferences and to test the hypothesis that such co-evolution can drive reproductive isolation (Panhuis *et al.*, 2001; Ritchie, 2007; Twomey *et al.*, 2016). The strawberry poison frog (*Oophaga pumilio*) is remarkably polytypic in and around the Bocas del Toro Archipelago of Panama (**Figure 1-1**), which reached its current conformation 1 – 9 kya (Gehara *et al.*, 2013). This region is largely shaped by the rise and fall of sea-level, and hence *O. pumilio* populations have likely experienced several periods of connectivity and vicariance. This frog displays a red body with blue or black limbs throughout most of its range, but in Bocas del Toro, isolated populations display coloration spanning the visual spectrum (Summers *et al.*, 2003; Pröhl *et al.*, 2007; Wang & Shaffer, 2008; Hauswaldt *et al.*, 2011). While minor variation may be present within islands, the most striking color variation (e.g., distinct 'morphs' of different dominant color) occurs among even the most recently-isolated island populations, supporting the hypothesis of rapid divergence in allopatry (Gehara *et al.*, 2013).

As in other poison frogs (Dendrobatidae), coloration of the toxic *O. pumilio* is hypothesized to function as an aposematic signal (Darst, 2006; Saporito *et al.*, 2007), but may also serve in intersexual communication (as similar signals do in other systems: Jiggins *et al.*, 2001, 2004; Nokelainen *et al.*, 2011; Twomey *et al.*, 2014, 2016). Females from most *O. pumilio* populations tested spend more time associating with males displaying coloration typical of the female's population (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008, 2009; Richards-Zawacki & Cummings, 2011). Because there is no evidence of post-mating reproductive isolation among Bocas del Toro *O. pumilio* lineages (Summers *et al.*, 2004; Dugas & Richards-Zawacki, 2015), these preferences seem the most likely mechanism to prevent gene flow (Panhuis *et al.*, 2001; as is common in young divergences: Arnqvist & Rowe, 2005; Ritchie, 2007;

Kraaijeveld *et al.*, 2011). However, remaining untested is the extent to which courtship preferences drive reproductive isolation in sympatry, the key prediction of a speciation-by-sexual-selection argument.

While most distinct *O. pumilio* color morphs occur only in allopatry, there are a few reported cases of sympatry (Summers *et al.*, 2003; Dugas *et al.*, 2015), and such populations allow for tests of the hypothesized role of female mate choice in driving and/or maintaining phenotypic diversity (Twomey *et al.*, 2014, 2016). In one polymorphic population, there is some evidence for asymmetric reproductive isolation in the wild, and female preference patterns suggest reinforcement (Richards-Zawacki & Cummings, 2011; Richards-Zawacki *et al.*, 2012). However, because the morphs from this polymorphic population do not occur in allopatry today, a full comparison of traits and preferences across a transition zone is not possible (Richards-Zawacki *et al.*, 2012). Here, we address this by comparing female preferences through a phenotypic transition zone in which a polymorphic population occurs between two allopatric, phenotypically distinct, populations of *O. pumilio* (**Figure 2-1**). We began by testing the prediction that females in allopatric populations would prefer males with local coloration. We then asked whether and to what extent morphs are behaviorally isolated in the transition zone by characterizing female preferences of both "pure" phenotypes (individuals phenotypically similar to those from allopatric populations) and the co-occurring phenotypic intermediates. Together, these results will increase our understanding of how phenotypic diversity is maintained and the conditions under which phenotypic divergence does and does not lead to reproductive isolation.

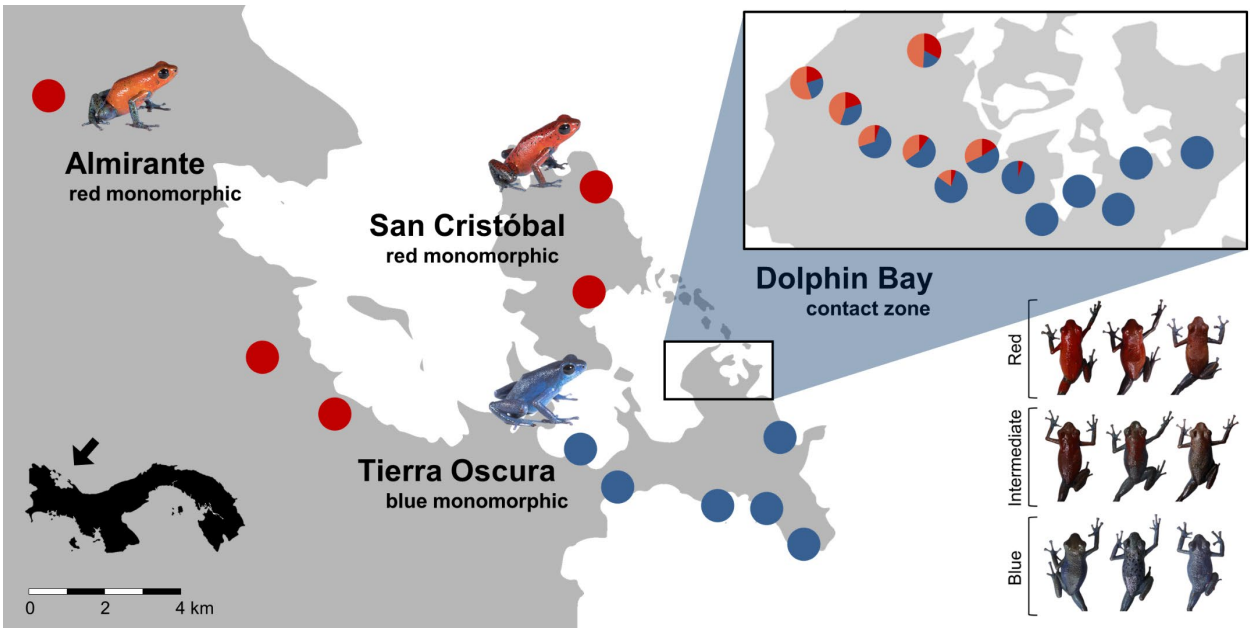

Figure 2-1 Transition zone between red and blue morphs

Map showing the transition zone between red and blue populations of *Oophaga pumilio* in the Bocas del Toro Archipelago, Panama. Pie charts show the relative red, blue and phenotypically intermediate morph frequencies at each location.

2.3 Methods

2.3.1 Study Species

Oophaga pumilio is a small (~2cm snout-vent length), diurnal terrestrial frog that occurs in lowland forests along the Caribbean side of Central America from Nicaragua to Panama. Males defend territories from which they court females, and females sample males in and around their own, larger, home ranges; both sexes mate multiply (Pröhl & Hödl, 1999). Following successful courtship, females lay a clutch of ~5 eggs in the leaf litter, where males may tend the clutch, moistening it daily. Once eggs hatch, one parent, typically but not always the female (Weygoldt, 1980; Killius & Dugas, 2014), transports tadpoles to water-filled leaf axils. The female then

provisions her tadpoles with unfertilized eggs throughout their development (Dugas *et al.*, 2016; Dugas, 2018).

2.3.2 Study Animals

In May–July 2011 and 2012, we collected male and female *O. pumilio* from three mainland populations in the Bocas del Toro region of Panama (**Figure 2-1**). As is the norm for *O. pumilio*, males and females at each site are similar in coloration (Summers *et al.*, 2003). In a monomorphic population near Almirante (09°19’16.3”N, 82°29’49.5”W), frogs are phenotypically similar to ancestral populations (Wang & Shaffer, 2008), with red dorsal and ventral coloration and blue legs (**Figure 2-1**). Near Tierra Oscura, on the north face of the Aguacate peninsula (09°10’37.9”N, 82°16’00.4”W), frogs are monomorphic and entirely blue (**Figure 2-1**). Near Dolphin Bay, on the northern tip of the Aguacate peninsula (9°13'15.70"N, 82°13'5.60"W), both red and blue frogs are present, along with a range of phenotypic intermediates (**Figure 2-1**). A mark-recapture survey of 255 frogs at Dolphin Bay indicated that the population contains 6% red, 22% blue and 72% intermediate frogs (M. B. Dugas unpublished data). Although the genetic architecture of coloration in *O. pumilio* remains unknown, captive breeding of several color morphs has demonstrated that coloration in this species is heritable (Summers *et al.*, 2004; Dugas & Richards-Zawacki, 2015). The presence of phenotypic intermediates in the red-blue transition zone suggests that coloration is an additive trait in this case; in another polymorphic population, red vs. yellow coloration seems most likely to be controlled in large part by dominant/recessive alleles at a single locus (Richards-Zawacki *et al.*, 2012). From Dolphin Bay, we collected frogs that were, by eye, at the extremes of red and blue or most ‘intermediate’. Differences among these by-eye categories are perceivable in the frog's visual system and by-eye categorizations are equivalent to more quantitative methods in

this (Dugas *et al.*, 2015) and other (Richards-Zawacki *et al.*, 2013) polymorphic *O. pumilio* populations.

We immediately transported field-collected *O. pumilio* to the Smithsonian Tropical Research Institute's Bocas del Toro field station, where we maintained frogs in plastic enclosures (37 x 22 x 25 cm), separated by sex. Each enclosure housed at most 3 frogs, each of a different color, to allow identification of individuals. Frogs fed on insects (mostly *Drosophila spp.*) attracted to fruit placed in their enclosures, and were supplemented with vitamin-dusted termites. Enclosures also contained locally collected vegetation, and were misted daily to maintain humidity. So that we could match males for body size in behavioral assays (see below), we measured mass to the nearest 0.01g and snout-vent length to the nearest 0.1 mm within one day of capture. We released all individuals following the completion of the experiment.

2.3.3 Experiment Design and Protocol

Following previous studies in *O. pumilio* (Maan & Cummings, 2008; Richards-Zawacki & Cummings, 2011), we tested the prediction that females would prefer to associate with males from the same population and/or of the same color using a three-way choice design under laboratory conditions. Each female was simultaneously presented with a male from Almirante (red with blue legs), a male from Tierra Oscura (entirely blue) and a male with intermediate coloration from Dolphin Bay.

The experimental arena was modified from a similar three-way choice test in Richards-Zawacki & Cummings (2011). The entire arena was a plastic container (60 × 60 × 45 cm) opaque on the sides and covered on top with plastic mesh to allow behavioral observations from above (**Figure 2-2A**). The three stimulus males were individually restricted under clear plastic domes (r

= 3.5 cm H = 4.5 cm) placed equidistant from each other; the position of males from each population was determined haphazardly. During behavioral observations, the focal female was allowed to move freely through the entire arena. All observations were conducted in a dark room, with arenas illuminated by two 60-W halogen bulbs (A19, GE Reveal, USA) and four 75-W UV lights (A19 Blacklight, Koninklijke Philips N.V., Netherlands) covered by two green-blue gel filters (Lee 728 + CyanGel 4315) to generate lighting conditions similar to that on the forest floor (*sensu* Maan & Cummings, 2008). Males presented simultaneously in trials were matched for snout-vent length (within 1 mm). Males were used in multiple behavioral assays to decrease the total number of animals used in the experiments, but were swapped for newly caught individuals every 7 days to alleviate the possibility of color change in captivity (Summers *et al.*, 2003). Size-matched trios of males were formed upon the day of capture, and remained together throughout the week of experiment. On each experimental day, a male trio was chosen at random, used for assays of four consecutive females, then swapped for a new, randomly selected trio. Unfortunately, records of trio identity were not retained.

We cannot exclude the possibility that male traits other than color influenced the expression of female preference. We can, however, exclude body size, call, tactile, or chemical information, the other traits most likely to shape preference (Dreher & Pröhl, 2014), as we size-matched males, no males called during these trials (if they had, we would have excluded these trials from analysis *sensu* Richards-Zawacki & Cummings, 2011), and males were confined under domes.

We placed males in the arena immediately prior to introducing a female. We then placed the female in the arena, equidistant from all three males (Fig. 2), isolated under a dome that was covered with a black visual barrier to prevent her from seeing the males. After 5 minutes, we removed the visual barrier (but not the transparent dome) for 2 minutes before finally lifting the

dome from over the female. We observed female behavior for 15 minutes after she first demonstrated interest in a male, operationally defined as approaching within a 4 cm (~2 body lengths) interaction zone while also facing the male (to distinguish interactions from non-courtship movement, *sensu* Maan & Cummings, 2008). If a female failed to demonstrate interest in a male within 15 minutes of her dome being lifted, we terminated the trial and did not include this 'non-responsive' female in further trials or further analyses (*sensu* Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008, 2009; Richards-Zawacki & Cummings, 2011). To avoid any bias introduced by the placement of males, we ran each responsive female through a second trial immediately after the first, rotating the position of males. If a female showed interest in the first but not the second 15 minutes trial, we terminated the experiment and re-tested the female on a different day.

During the total 30 minutes of observation, we quantified i) association time, defined as the cumulative time the focal female spent in each of the interaction zones surrounding each male's dome, and ii) approaches, defined as the number of times the focal female oriented towards and entered each interaction zone. These two female behaviors we recorded are typically predicted to be positively associated with the probability that a female will mate with a male in the wild (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008, 2009; Richards-Zawacki & Cummings, 2011); consistent with this assumption, *O. pumilio* courtship in the wild is more likely to result in mating if the female stays in close physical proximity to the male (Yang *et al.*, 2019a). We used total association time and approaches during two 15 minute trials for all analyses. Our total sample of responsive females included 30 from Almirante, 29 from Tierra Oscura, and 90 females from Dolphin Bay (30 each of red, blue, and intermediate phenotypes).

2.3.4 Statistical Analyses

To test for female preferences among the three males presented to her simultaneously, we used generalized linear mixed models (GLMM) with female identity included as a random effect (details below). We tested the main effects of female population-of-origin (Almirante, Tierra Oscura, Dolphin Bay), stimulus male color (red, blue, intermediate) and the interaction between these two terms. When the interaction term was significant, we separated the three female populations for further analysis, testing only the effect of male color for the two monomorphic populations (Almirante, Tierra Oscura) and the effects of male color, female color and their interaction in Dolphin Bay models. All analyses were performed in R 3.6.2 (R Core Team, 2019).

We fit 'approaches' to a GLMM with negative binominal error structure (data were overdispersed when fitted with Poisson) using the glmmADMB package (Skaug *et al.*, 2011). We tested the significance of main effects using the 'Anova' function in the car package, which compares overall model fit with and without a particular effect. We used Tukey's post hoc tests for pairwise comparisons of approaches to the three stimulus male colors. We fitted 'association time' to a linear mixed model (LMM) using the lme4 package (Bates *et al.*, 2014). Because of non-normality of residuals, for hypothesis testing we bootstrapped estimated 95% confidence intervals of the fixed effects and their interaction terms using 5000 iterations. We applied a semiparametric bootstrapping approach using the 'bootMer' function in the boot package (Canty & Ripley, 2012). The LMM was fitted with the Nelder Mean option in 'lmer' to improve performance of the semiparametric bootstrap routine. A main effects or interaction was considered significant if the bootstrapped 95% confidence interval did not overlap zero. For post hoc comparisons of the stimulus male colors, we repeated the semiparametric bootstrapping process with a zero intercept

model to generate confidence interval for all three levels; in this case, groups were considered significantly different if the 95% confidence intervals did not overlap.

Finally, we asked whether the strength of female approach and association preferences differed among populations. We first determined the preferred male color for females in each of the three populations (see Results). For each individual female, we modeled her interest in the male of the population-preferred color, including her 'overall preference' (sum or interest in all three males) as an offset term, as some females may be overall more interested in males than others. Approach preference strengths were compared using a generalized linear model (GLM) with quasi-Poisson error structure, including total approaches made by females as an offset term. To avoid normality assumptions, association preference strengths were compared by applying a permutation based linear model using the 'lmp' function of the lmPerm R package (Wheeler & Torchiano, 2010), including total association time as an offset term. Significance was determined using p-values calculated from 5000 iterations.

2.4 Results

In our initial model that included all observations, we found a significant interaction between female population-of-origin and male phenotype with respect to both approaches (**Table 2-1**) and association time (**Table 2-2**). Because of this significant interaction, we then considered female behaviors separately for each female population-of-origin.

Almirante (red) females spent unequal amounts of time interacting with red, blue, and intermediate males. They spent significantly more time interacting with red males than with blue or intermediate males (bootstrapped 95% CI, red [242.42, 383.01], intermediate [45.84, 109.65],

blue [-44.55, 103.12], **Figure 2-2**). Almirante females also approached the three stimulus males with unequal frequency (GLMM, $\chi2 = 16.95$, df = 2, $p < 0.001$), approaching red males more often than blue males (Tukey post hoc comparisons, red – intermediate: $p = 0.212$, red – blue: $p < 0.001$, intermediate – blue: $p = 0.171$, **Figure 2-2**).

Tierra Oscura (blue) females also spent unequal amounts of time interacting with red, blue, and intermediate males. They spent significantly more time interacting with blue males than with red or intermediate males (bootstrapped 95% CI, red [6.99, 137.16], intermediate [-16.22, 125.10], blue [167.83, 300.07], **Figure 2-2**). Tierra Oscura females also approached the three stimulus males with unequal frequencies (GLMM, $\chi2 = 17.15$, df = 2, $p < 0.001$). They approached blue males more often than red or intermediate males (Tukey post hoc comparisons, blue – intermediate: $p = 0.020$, blue – red: $p = 0.015$, intermediate – red: $p = 0.995$, **Figure 2-2**).

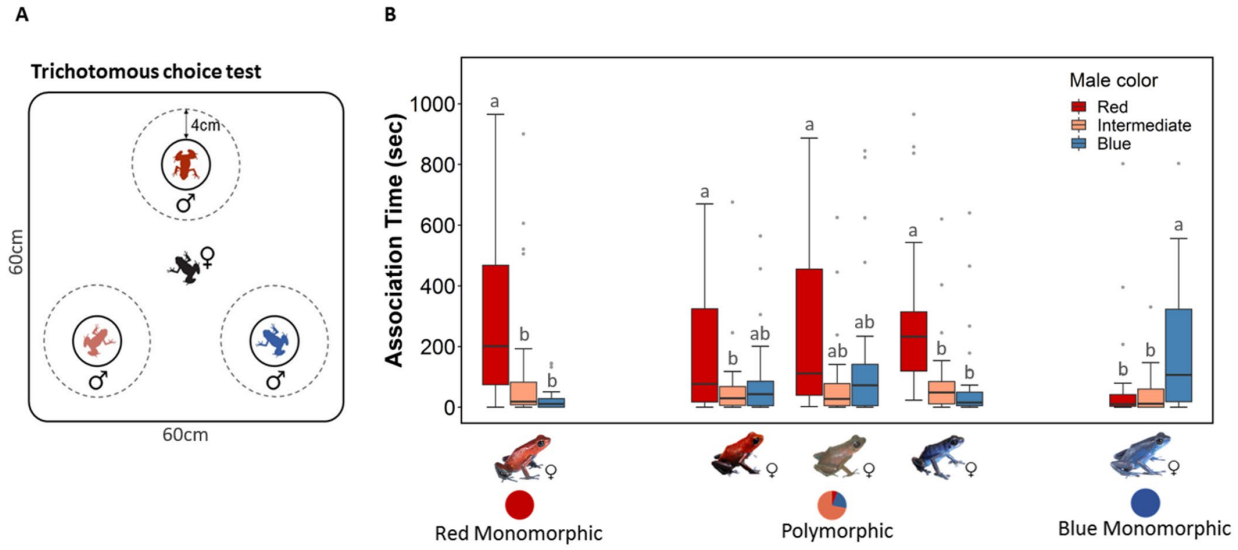

Figure 2-2 Color-mediated female preferences

A: Experimental apparatus used in assays of female preference. During behavioral observations, the three stimulus males were confined under clear plastic domes, and the female was al- lowed to move freely in the arena. **B**: Time female *O. pumilio* from monomorphic red (Almirante, left), monomorphic blue (Tierra Oscura: right), and polymorphic (Dolphin Bay, middle) populations spent with red, blue, and intermediate stimulus males. Letter codes denote statistical significances (see main text for details).

Table 2-1 GLMM: number of approaches among all populations

Generalized linear mixed model evaluating the effects of stimulus male phenotype, female's population-of-origin and the interaction between the two terms on number of approaches. Stimulus male population-of-origin: red = Almirante, blue = Tierra Oscura, intermediate = Dolphin Bay.

Parameters	χ^2	*df*	**p-value**
Male Phenotype	11.12	2	0.004
Female population-of-origin	15.17	2	< 0.001
Male Phenotype × Female population-of-origin	31.06	4	< 0.001

Table 2-2 GLMM: association times among all populations

Linear mixed model evaluating the effects of stimulus male phenotype, female's population-of-origin and the interaction between the two terms on association time. Confidence intervals (CI) were 95% percentile bootstrapped. Significance of a term was determined by if the bootstrapped confidence interval overlapped 0. Stimulus male population-of-origin: red = Almirante, blue = Tierra Oscura, intermediate = Dolphin Bay.

Parameters	**2.5% CI**	**97.5% CI**
Male Phenotype[1]		
intermediate	-292.23	-72,29
red	-277.27	-48.02
Female population-of-origin[2]		
Dolphin Bay	-222.80	-40.12
Almirante	-312.30	-84.74
Male Phenotype × Female population-of-origin		
intermediate × Dolphin Bay	51.20	298.14
red × Dolphin Bay	197.38	466.99
intermediate × Almirante	109.68	417.42
red × Almirante	292.42	598.66

[1]male phenotype 'blue' is the baseline [2]population 'Tierra Oscura' is the baseline

In Dolphin Bay, the female color × male color interaction term was significant for comparisons of association time (**Table 2-3**), so we tested association preference of each female phenotype separately. Despite a significant interaction term, females of all three colors expressed similar preferences. They spent more time associating with red males than with blue or intermediate males, though only for red and blue females were the 95% CIs non-overlapping for

red versus other male phenotypes (bootstrapped 95% CI, red females: red [128.40, 241.07], intermediate [15.03, 125.68], blue [35.06, 146.73]; intermediate females: red [198.01, 362.13], intermediate [46.49, 208.42], blue [73.84, 237.53]; blue females: red [280.77, 454.86], intermediate [7.17, 182.68], blue [-6.38, 170.09], **Figure 2-2**). The female color × male color interaction term was non-significant for comparisons of approaches (GLMM, $\chi2 = 0.952$, df = 4, $p = 0.917$), and there was no main effect of female color for approaches (GLMM, $\chi2 = 0.32$, df = 2, $p = 0.852$). Dolphin Bay females did, however, approach males of the three colors with different frequencies (GLMM, $\chi2 = 9.55$, df = 2, $p = 0.008$). They approached red males more often than blue males, and approached intermediate males with frequencies between the two (Tukey post hoc comparisons, red – intermediate: $p = 0.214$, red – blue: $p = 0.005$, intermediate – blue: $p = 0.736$, **Figure 2-2**).

Table 2-3 LMM: association times at Dolphin Bay

Linear mixed model evaluating the effects of stimulus male phenotype, female phenotype and the interaction between the two terms on association time. Confidence intervals (CI) were 95% percentile bootstrapped. A term was considered significant if the bootstrapped confidence interval did not overlap 0. Stimulus male population-of-origin: red = Almirante, blue = Tierra Oscura, intermediate = Dolphin Bay.

Parameters	**2.5% CI**	**97.5% CI**
Male phenotype[1]		
intermediate	-101.60	126.62
red	174.97	401.91
Female phenotype[2]		
intermediate	-37.28	195.49
red	-103.74	129.09
Male phenotype × Female phenotype		
intermediate × *intermediate*	-209.09	128.07
red ×*intermediate*	-329.80	-2.80
intermediate × *red*	-197.44	125.50
red × *red*	-357.23	-32.52

[1]male phenotype 'blue' is the baseline [2]female phenotype 'blue' is the baseline

Females from the three populations did not differ in the strength of their preferences for their most preferred male color. The Dolphin Bay females' association preference for red is not significantly different in strength from the Almirante females' association preference for red or the Tierra Oscura females' association preference for blue (permutation based linear model, df = 2, p = 0.24). Similarly, the Dolphin Bay females' approach preference for red is not significantly different from the Almirante females' approach preference for red or the Tierra Oscura females' approach preference for blue (GLM, LRχ2 = 0.837, df = 2, p = 0.658).

2.5 Discussion

The divergence of mating signals and preference for these signals are necessary for behavioral isolation between differentiated lineages (Panhuis *et al.*, 2001; Ritchie, 2007). We found evidence suggesting that coloration and female preferences have indeed diverged in concert in the monomorphic *O. pumilio* populations: females from the pure blue and red populations spent more time with males from their own population and approached them more often. In the transition zone, however, instead of favoring males similar to their own color, red, blue, and intermediate females all preferred red males, and this preference was similar in strength to the preferences observed monomorphic populations. While divergent preferences may or may not have contributed to the initial divergence in coloration between morphs, they would be critical to limiting gene flow in sympatry. At least for these two color morphs, assortative preferences are expressed in allopatry but not in sympatry, and are thus unlikely to bring about reproductive isolation. Similar breakdowns of preferences expressed in allopatry have been documented in grasshoppers (*Chorthippus parallelus parallelus*, *C. p. erythropus*: Ritchie *et al.*, 1989), wood rats

(*Neotoma bryanti*, *N. lepida*: Shurtliff *et al.*, 2013) and fruit flies *(Drosophila subquinaria, D. recens*: Bewick & Dyer, 2014), indicating that divergent traits and preferences often do not contribute to reproductive isolation in sympatry.

Although allopatric divergence in coloration is the norm in *O. pumilio* (Gehara *et al.*, 2013), distinguishing between primary divergence and secondary contact in a polymorphic zone is notoriously difficult (Barton & Hewitt, 1985). It is possible that the red-blue polymorphic population we studied resulted from i) secondary contact between red and blue lineages that diverged in allopatry, ii) the blue phenotype first arising in a monomorphic red population in the Northern tip and spreading to fixation on the rest of Aguacate peninsula, or iii) the red phenotype arising from within the monomorphic blue population in Northern Aguacate, independent of the Almirante red phenotype (i.e., a reversion to a phenotype similar to the ancestral one). Given the likely alternating periods of connectivity and vicariance in this archipelago, along with current distribution of distinct *O. pumilio* color morphs, secondary contact seems the most parsimonious explanation for the red-blue transition zone. Regardless of how the transition zone arose, the global preference for red in the polymorphic population suggests that color and color preference have evolved at different speeds in red and blue lineages. We cannot exclude the possibility that male traits other than color cause transition zone females to preferred red stimulus males (although our methods allow us to exclude body size, call and tactile or chemical cues). If this is the case, although unlikely, our results would similarly suggest that the unknown trait and corresponding preference evolved at different speeds.

Assuming secondary contact, it remains unclear why blue females in the polymorphic population expressed a preference different from blue females in the allopatric population, and why female preference in the polymorphic population converged on red. Myriad mechanisms can

initiate the co-divergence of traits and preferences in isolated populations (e.g. sensory drive, ecological adaption, genetic drift), but the association between preference and trait loci can easily be broken down by recombination (Otto *et al.*, 2008; Servedio & Bürger, 2014). Recombination is likely in *O. pumilio* given that female preferences are clearly not absolute (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008; Dugas & Richards-Zawacki, 2015). The preference for red displayed by females from the polymorphic population may be advantageous if females accrue direct or indirect benefits from mating with a red male in Dolphin Bay, for example if red males are better brood tenders, or sire healthier tadpoles (a possibility suggested by among-morph differences in male reproductive success and tadpole performance: Dugas & Richards-Zawacki, 2015, 2016).

A universal preference for red in the transition zone could also emerge without any changes in the genetic mechanism underlying mate preferences. Within populations, *O. pumilio* females prefer brighter males over duller ones (Maan & Cummings, 2009), a pattern that might explain a universal preference for the brighter red males over the duller blue and intermediate ones in the transition zone (Rudh *et al.*, 2011; Maan & Cummings, 2012; Dreher & Pröhl, 2014). However, this explanation is inconsistent with the finding that females from several *O. pumilio* lineages (including the Tierra Oscura females tested here) prefer males from their own population even when offered males from brighter allopatric populations (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008). It remains possible that color-based female preferences are shaped by the integration of independent assessments of brightness and color (i.e., hue) or that color is simply important along a gradient of familiar to unfamiliar. Mate preferences might also be plastic, with convergent female preferences in the polymorphic population reflecting shared natural and/or social environments (Svensson *et al.*, 2010; Kozak *et al.*, 2011); rather than

selection on preferences driving the preference for red, this scenario allows females to learn that some phenotypes make better mates (Rodríguez *et al.*, 2013). Sexual imprinting based on social interactions can create positive frequency dependent selection that results in females shifting to prefer the most common male type (Laland, 1994; Rodríguez *et al.*, 2013). However, red is the rarest phenotype in Dolphin Bay (~6%), suggesting alternative mechanisms. Females may also simply learn to prefer the rarest male phenotype; while this 'rare-male effect' has been documented in several taxa, the proximate mechanism is poorly understood (Singh & Sisodia, 1999; Eakley & Houde, 2004). The effect of behavioral learning on population divergence is dependent upon the cue for learning, and the tutor from whom females learn (Verzijden *et al.*, 2012; Yeh & Servedio, 2015). Asymmetric responses to diverged sexual signals are commonly observed between young lineages (Hardwick *et al.*, 2013; Martin & Mendelson, 2013; Shurtliff *et al.*, 2013). Identifying the mechanisms shaping female preferences will be key to predicting the speed and direction of evolution when divergent lineages interact and understanding the full complement of selective pressures shaping and maintaining phenotypic diversity (Verzijden *et al.*, 2005).

The high frequency of intermediate individuals in the red-blue transition zone suggests frequent among-morph matings in the wild, and unless some cost prevents red males from mating with the phenotypically diverse females that prefer them, such gene flow should continue. There is no evidence that between lineage matings are any less productive than within-lineages matings in *O. pumilio* (Dugas & Richards-Zawacki, 2015) or that natural selection penalizes phenotypic intermediates (Richards-Zawacki *et al.*, 2013; Yeager, 2015). If female preferences for red males drive mate choice, the frequency of both red and intermediate phenotypes should increase over time, a possibility consistent with our repeated sampling at Dolphin Bay (Y. Yang and M. B. Dugas *unpublished data*) and one that can be tested with continued monitoring of the entire transition

zone. Equilibrium in morph frequency could be maintained by natural selection against red, for example if local predators must learn to associate the frogs' toxicity with color, red may be a less effective aposematic signal than the more common blue or intermediate morphs (Ruxton *et al.*, 2004). All current evidence suggests that no such natural selection costs occur in *O. pumilio* populations (Hegna *et al.*, 2013; Richards-Zawacki *et al.*, 2013; Dreher *et al.*, 2015; Yeager, 2015). However, it is possible that the results of all these studies are influenced by recent anthropogenic disturbance that has altered the selection regimes on aposematic coloration, resulting in collapse of trait and preference differentiation perhaps driven and/or maintained by natural selection.

The collapse of divergent within-species lineages is much more common than speciation (Rosenblum *et al.*, 2012; Dynesius & Jansson, 2013), and asymmetry in the strength of assortative mating among lineages (Hardwick *et al.*, 2013; Martin & Mendelson, 2013; Shurtliff *et al.*, 2013) has been suggested as a common reason for collapse instead of progression to full reproductive isolation (Arnold *et al.*, 1996; Servedio & Bürger, 2014). While divergent traits and preferences are necessary to drive reproductive isolation, they are not necessarily sufficient to do so or to move lineages towards speciation (Jennions & Petrie, 1997). Studies of Bocas del Toro *O. pumilio* lineages can continue to contribute to our understanding of the role of female preferences in driving behavioral isolation, in particular if focus is directed towards: i) identifying the proximate mechanisms by which female mate preferences are shaped, including the relative contribution of genetics and plasticity, ii) identifying the factors that shape and constrain the relationship between preferences expressed in the lab and actual mate choice in the wild, including the roles not only of female choice but also of male-male competition (Qvarnström *et al.*, 2012; Tinghitella *et al.*, 2017), and iii) continued monitoring of phenotype frequency in contact zones, the natural laboratory that allows the rare but critical test for reproductive isolation in nature. Our findings

highlight the complexity in the evolution of behavioral isolation, and the need for future studies to investigate the circumstances under which divergent preferences do and do not contribute to speciation.

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3.0 Chapter 3: Poison Frog Color Morphs Express Assortative Aggression Biases in Allopatry but not Sympatry

The contents in this chapter are adapted from the following publication:

Yang, Y., Dugas, M.B., Sudekum, H.J., Murphy, S. & Richards-Zawacki, C.L. 2018. Male-male aggression is unlikely to stabilize a poison frog polymorphism. *Journal of Evolutionary Biology.* **31**: 457–468. doi: 10.1111/jeb.13243.

3.1 Chapter Summary

Phenotypic polymorphism is common in animals, and the maintenance of multiple phenotypes in a population requires forces that act against homogenizing drift and selection. Male-male competition can contribute to the stability of a polymorphism when males compete primarily with males of the same phenotype. In and around a contact zone between red and blue lineages of the poison frog *Oophaga pumilio*, we used simulated territorial intrusions to test the non-exclusive predictions that males would direct more aggression toward males of (i) their own phenotype and/or (ii) the phenotype that is most common in their population. Males in the monomorphic red and blue populations that flank the contact zone were more aggressive toward simulated intruders that matched the local coloration. However, males in the two polymorphic populations biased aggression toward neither their own color nor the color most common in their population. In sympatry, the rarer color morph gains no advantage via reduced male-male aggression from territorial males in these *O. pumilio* populations, and so male aggression seems unlikely to stabilize

color polymorphism on its own. More broadly, these results suggest that the potential for divergent male aggression biases to maintain phenotypic diversity depends on the mechanism(s) that generate the biases and the degree to which these mechanisms persist in sympatry.

3.2 Introduction

Phenotypic polymorphism, the coexistence of multiple distinct phenotypes within a species, is widespread in nature (Ford, 1945; Gray & McKinnon, 2007). Enumerating the evolutionary mechanisms that can maintain phenotypic polymorphisms against the homogenizing forces of drift and selection is key to understanding trait evolution and the potential role of polymorphism in speciation (Smith, 1962; Gray & McKinnon, 2007). Assortative mating can facilitate reproductive isolation, and numerous studies of polymorphic taxa have demonstrated assortative female preferences that could result in assortative mating (Jennions & Petrie, 1997). However, assortative mating does not on its own maintain phenotypic polymorphism and may not be sufficient to drive speciation (Kirkpatrick & Ravigné, 2002; van Doorn *et al.*, 2004; Wellenreuther *et al.*, 2014). Male-male competition can also exert selection on phenotype and is often a key component of mate acquisition and thus mating patterns in the wild (Wong & Candolin, 2005; Hunt *et al.*, 2009). The potential for male-male competition to contribute to polymorphism maintenance and reproductive isolation has only recently received much attention (Seehausen & Schluter, 2004; Qvarnström *et al.*, 2012; Tinghitella *et al.*, 2017).

The coexistence of multiple male morphs that differ morphologically and/or behaviorally, has been observed in taxonomically diverse animals (Shuster, 2010). A common scenario is one where most males specialize in fighting, acquiring a territory and/or guarding females, and rarer

'alternative' morphs specialize in sneaking matings and/or mimicking females (e.g. water strider: Hayashi, 1985; bluegill sunfish: Gross, 1991; side-blotched lizard: Sinervo & Lively, 1996). In all these cases, the fitness of an alternative strategy is higher when it is relatively rare (it is under negative frequency dependent selection: Gross, 1991). Frequency dependent selection can also occur even without morphs being restricted to males only or differing in mating strategy, specifically if males of any particular morph direct their aggression non-randomly toward others (Mikami *et al.*, 2004; Seehausen & Schluter, 2004). When competition is stronger among phenotypically similar males, individuals expressing a rare phenotype enjoy the advantage of receiving, on average, less aggression than individuals of the common phenotype (Dijkstra *et al.*, 2010). In an African haplochromine cichlids species complex, males of most color morphs are more aggressive toward rivals with similar nuptial coloration (Dijkstra & Groothuis, 2011). Consequently, this form of male-male competition has been hypothesized to facilitate the invasion of a novel phenotype and potentially stabilize a polymorphism (Dijkstra & Groothuis, 2011).

The opportunity to interact with phenotypically distinct morphs is common when lineages come into secondary contact, and the potential role of this phenomenon in the evolution of reproductive isolation will be especially important when these lineages are newly diverged. Among allopatric, phenotypically diverged populations, males are often more aggressive toward phenotypes typical of their population (Anderson & Grether, 2010; Bolnick *et al.*, 2016). Two potential mechanisms might explain such a pattern, both of which can generate negative-frequency dependent selection among phenotypes upon secondary contact: (i) males act more aggressively toward males of their own phenotype ("own-type", Dijkstra & Groothuis, 2011), and (ii) males act more aggressively toward the most common phenotype in the population ("familiarity", Bolnick *et al.*, 2016). Simulated contact zones or experimental populations can provide valuable insight

into the potential for differentiated male aggression biases to maintain polymorphism upon contact (Dijkstra *et al.*, 2010; MacGregor *et al.*, 2017). However, aggression patterns that arise in allopatry can shift in sympatry via selection (e.g. agonistic character displacement, Grether *et al.*, 2009) and/or non-genetic mechanisms (e.g. cross-generation learning, Verzijden *et al.*, 2008) that may not be expected to operate within short-term experimental assemblages. Natural transition zones provide unique opportunities to test whether and how patterns of aggression bias observed in allopatry are also observed in sympatry, and consequently the extent to which male-male competition might stabilize polymorphisms in nature.

The strawberry poison dart frog (*Oophaga pumilio*) exhibits extreme color polymorphism in and around the Bocas del Toro archipelago of Panama (**Figure 1-1**; Summers *et al.*, 2003). Coloration in males and females is qualitatively similar in this species (Summers *et al.*, 2003; Maan & Cummings, 2009), and is hypothesized to function both as an aposematic signal (Darst, 2006; Saporito *et al.*, 2007) and in inter- and intrasexual communication (Maan & Cummings, 2008; Crothers & Cummings, 2015). Here, we tested the hypothesis that color-mediated male-male aggression could contribute to the stability of this color polymorphism. We took advantage of a phenotypic contact zone between red (with blue legs) and blue colored lineages of *O. pumilio* (Dugas *et al.*, 2015; Yang *et al.*, 2016). We studied two populations in the contact zone that differ in morph frequencies, and two monomorphic populations on either side (**Figure 2-1**). We used simulated intrusions in the field to quantify the aggressiveness of territorial males toward a red or a blue rival, specifically testing whether males (i) bias aggression toward their own color ("own-type", Dijkstra & Groothuis, 2011), and/or (ii) bias aggression toward the most common color in the population ("familiarity", Bolnick *et al.*, 2016). Evidence that either of these mechanisms is

operating in the contact zone would suggest that negative-frequency dependent selection via male aggression biases could contribute to the dynamics of this sympatric polymorphism.

3.3 Methods

3.3.1 Study Species

Oophaga pumilio is a terrestrial frog that occupies lowland forests along the Caribbean side of Central America from Nicaragua to Panama. Throughout most of its range, this frog has a red body with blue or black limbs, but populations in the Bocas del Toro region of Panama display coloration spanning the visual spectrum (**Figure 1-1**; Summers *et al.*, 2003); males and females do not qualitatively differ in color within populations (Maan & Cummings, 2009). Despite dramatic phenotypic differences, *O. pumilio* populations in this young archipelago (1–9 kya) are relatively undifferentiated at neutral microsatellite loci (Hauswaldt *et al.*, 2011) and there is no evidence of postzygotic isolation, and so they are hypothesized to be one biological species (Summers *et al.*, 2004; Dugas & Richards-Zawacki, 2015). Most currently described color morphs occur in allopatry (typically one morph per island), but two morphs are sympatric in a few documented cases (Wang & Shaffer, 2008; Richards-Zawacki & Cummings, 2011; Dugas *et al.*, 2015), and in one, morph frequency varies along a contact zone (Yang *et al.*, 2016).

Male *O. pumilio* are territorial, defending territories through vocalization, visual displays, and physical combat (Bunnell, 1973; Gardner & Graves, 2005). Both sexes mate multiply, with females generally choosing a mate within a home range that overlaps several male territories (Pröhl & Hödl, 1999). Females are unlikely to mate with males not holding a territory (Meuche & Pröhl,

2011; Meuche *et al.*, 2012). Males advertising from higher perches are more likely to attract mates (Pröhl & Hödl, 1999), but all current evidence suggests that no aspect of male territory quality provides a direct benefit to females (Donnelly, 1989; Pröhl & Berke, 2001). If courtship is successful, the female lays a clutch in the leaf litter within a male's territory, where he may moisten it regularly (Weygoldt, 1980; Pröhl & Hödl, 1999). One parent, typically the female (Killius & Dugas, 2014), transports newly hatched tadpoles individually to water-filled leaf axils, and the female provisions her tadpoles with trophic eggs regularly until they complete metamorphosis (~45 d, Weygoldt, 1980; Dugas *et al.*, 2016).

3.3.2 Study Populations

We tested the prediction that males of the rare (polymorphic populations) or novel (monomorphic populations) color morph would experience less male-male aggression than males of the more common phenotype in four *O. pumilio* populations (Yang et al., 2016; hereafter, red monomorphic, high-red polymorphic, high-blue polymorphic, and blue monomorphic; **Figure 2-1**). On Isla San Cristóbal (9°16'25.46"N, 82°15'12.74"W), frogs are similar in coloration to ancestral populations, with red dorsal and ventral coloration and blue limbs (Wang & Shaffer, 2008). On much of the Aguacate peninsula (9°11'47.65"N, 82°15'04.75"W), frogs are entirely blue. However, in the northern portion of the Aguacate peninsula, near Dolphin Bay, red, blue, and phenotypically intermediate frogs coexist. The red and intermediate phenotypes are most common at the tip of the peninsula, near Isla San Cristobal. We studied two populations in this polymorphic zone that differ in the frequencies of red, blue and intermediate frogs. At the 'high-red polymorphic' site, red frogs were almost twice as frequent as blue (33% red, 18% blue, 49% intermediate; 9°13'15.70"N, 82°13'5.60"W), and at the 'high-blue polymorphic' site, blue frogs

were more than three times as frequent as red (16% red, 52% blue, 32% intermediate; 9°12'42.48"N, 82°12'53.17"W). While intermediate morphs were common in both polymorphic populations, more of these intermediate animals fell toward the red end of the spectrum in the high-red population, and more fell toward the blue end in the high-blue population (Y. Yang *unpublished data*; see below and **Appendix A.2** for details). We therefore treated red as the most common coloration in the high-red population, even though the proportion of "intermediate" morphs was greater than that of red.

3.3.3 Experimental Design and Protocol

We used simulated territorial intrusions to test the predictions that wild territorial *O. pumilio* males would be more aggressive toward (i) intruders with coloration similar to their own (Dijkstra & Groothuis, 2011), and/or (ii) intruders displaying the color most common in the population (Bolnick *et al.*, 2016). As model intruders, we used 3D-printed plastic frogs (3D model from Turbosquid.com, printed by Shapeways, Inc., New York, USA; Crothers & Cummings, 2015). We used acrylic paint to match the reflectance of pure red and blue males (**Appendix Figure 1**), and used a clear coat to mimic the reflective properties of moist skin (*sensu* Dreher & Pröhl, 2014; Crothers & Cummings, 2015). Both movement and acoustic signals elicit aggression in poison frogs (Narins *et al.*, 2003; de Luna *et al.*, 2010), so we added full body rotation every 5 seconds by mounting the model on a radio-controlled servomotor hidden within a cylinder modelled to resemble a log, and played back local *O. pumilio* advertisement calls through a Bluetooth-enabled speaker (Omaker M4) (**Figure 3-1**, **online supplemental video**). Call parameters across the red-blue transition zone do not differ among populations or color morphs (M. L. Dye, *unpublished data*), so we used the same call in all trials (**86**). This design enabled us

to control the stimuli and take notes from > 1.5 m away, a distance that does not disturb males (Staudt *et al.*, 2010; Gade *et al.*, 2016). All trials were recorded using a video camera (icools Black Wifi Sports Camera) mounted on the stimulus apparatus.

Figure 3-1 Simulated intrusion setup

Experimental setup used in stimulated intrusion trials to quantify male aggression toward red and blue colored opponents. **A**: Hand painted plastic frogmodels were mounted onto a radio-controlled servomotor hidden in a log-shaped cylinder. Advertisement calls were played through a Bluetooth speaker located behind the model. **B**: Photo of a blue territorial male attacking a red frog model during a trial. See also **online supplemental video**.

We conducted behavioral assays between 700–1300 h, when *O. pumilio* males are most active (Graves, 1999; Graves *et al.*, 2005), locating target territorial males by their advertisement calls (Meuche & Pröhl, 2011). Upon locating a calling male, we placed the intruder stimulus apparatus within one meter of the perch from which the focal male was calling. The color of the intruder stimulus was chosen haphazardly for each trial. Male *O. pumilio* defend ~3 m^2 core territories even in dense populations (Pröhl & Berke, 2001), so this model should always have been perceived as an intruder. We allowed the focal male to acclimate to the presence of the

apparatus for 5 min or until it resumed calling, and then began audio playback and artificial movement of the intruder stimulus. We began behavioral observations (detailed below) after the focal male oriented toward the simulated intruder. If the focal male failed to orient to the model intruder within 15 minutes, we terminated the trial. Of the 372 males tested, 90 % were responsive. When males were responsive ('track', see below for operational definition), we ran trials for 10 minutes, or, if the male escalated the contest to the highest level of aggression ('attack', see below), we extended or shortened the trial to allow 5 minutes of interaction after the first attack. Trials, then, could have ranged from 5 (immediate attack) – 15 (attack at minute 10) mins (mean ± sd: 8.98 ± 2.00 minutes). This approach allowed us to quantify the intensity of the contest in individuals that did attack, but did not bias our likelihood of detecting other aggressive behaviors.

Following trials, we hand-captured the focal male and measured its SVL to the nearest 0.1 mm using a dial caliper. For color quantification (see below), we took digital photographs (Panasonic DMC-TS5, Kadoma, Osaka, Japan) of the dorsum against an 18% grey standard (DGK Color Tools), manually setting white balance for each photograph. We marked each male with a toe clip (Funk *et al.*, 2005) so as to avoid re-sampling (if a male we observed turned out upon capture to be toe-clipped, we discarded this second observation), and released it near the point of capture. *O. pumilio* quickly resume territorial behavior following release (Meuche *et al.*, 2012).

3.3.4 Male Agonistic Behaviors

A typical encounter between *O. pumilio* males involves an intruder approaching and calling to the resident, with the resident responding with calls and approaches. The contest can then either escalate to a physical fight (wrestling and pinning) or be resolved when one male (usually the

intruder) shows submission (freezes in place or retreats and stops calling) before or upon the first attack by the winner (Baugh & Forester, 1994). In the current study, territorial males responded to the simulated intrusions similarly (**online supplemental video**). We recorded whether a male performed each of five aggressive behaviors that typically occurred in order (yes/no, not cumulative): (i) 'track', a male orienting toward and facing an intruder, (ii) 'approach', a male moving towards the model, (iii) 'calling' (typically accompanied an approach, but could occur any time), (iv) 'challenge', if a male continued an approach onto the stimulus model's perch (log), and (v) 'attack', if the male wrestled with the moving model (see **online supplemental video** for examples of each behavior). Since we continued to play calls and move the model frog after a focal male attacked, all trials mimicked a contest in which the intruder did not submit (i.e. an 'escalated contest' with physical fighting, Hsu *et al.*, 2006). For analysis, we treated each of the five behaviors as a binary response (yes/no), and additionally considered the number of attacks a focal male directed to the stimulus model during the 5 minutes that followed the first attack. If the focal male remained in contact with the stimulus model for > 5 seconds (i.e. attempting to pin or subdue the model), we tallied a new attack count every 5 seconds (the frequency at which we moved the model); doing so minimized differences between males that were able to maintain contact when the model was moved and those that were not.

We measured two additional parameters that we included in analyses as covariates. During trials, focal males sometimes interacted with nearby conspecifics (male-male combat or courtship). As these activities lowered the potential time a male could interact with the model, we included whether (yes/no) the focal male engaged in such interactions in all statistical models. Perch height of male *O. pumilio* is correlated with mating success (Pröhl & Hödl, 1999) and could influence the focal male's motivation to defend his territory. To account for this, we measured the height of the

focal male's perch to the nearest cm with a flexible measuring tape and included this as a covariate in statistical models.

3.3.5 Color Quantification

In these polymorphic populations, by-eye categorizations of males as blue, red, or intermediate are equivalent to more quantitative measures of coloration, including visual models of color space that take into account *O. pumilio* visual sensitivities (Dugas *et al.*, 2015). However, we were also interested in testing for nuanced relationships between focal male color and its reaction to blue and red model intruders in the polymorphic population (e.g., asking whether redder males were more aggressive toward red intruders). For these analyses, we used digital photographs to generate objective color scores for each focal male from polymorphic populations. To quantify color in a continuous way, we sampled red (R), green (G), and blue (B) values across five 20 × 20-pixel areas on the frog's dorsum, using the software ImageJ 1.48v (Schneider *et al.*, 2012). We then standardized these values by taking residuals of mean R, G, or B frog color regressed on R, G, or B scores from the 18% gray standard (a 20 × 20-pixel area) in the same photo. We calculated the mean R, G, and B residuals for each frog and used a principal component analysis (PCA) to reduce the number of color parameters (Stevens *et al.*, 2007; methods followed Dugas *et al.*, 2015). Descriptive results are documented in detail in **Appendix A.2**.

3.3.6 Statistical Analyses

In separate analyses, we modelled whether (yes/no) a territorial male (i) tracked, (ii) approached, (iii) called, (iv) challenged, and (v) attacked the model intruder. We tested the main

effect of population (red monomorphic, high-red polymorphic, high-blue polymorphic, and blue monomorphic), intruder model color (red or blue) and their interaction. We used generalized linear models (GLMs) with binomial error structures, and Bonferroni corrected for multiple comparisons after testing the five behaviors. In the subset of males that attacked the frog model (hereafter, 'escalated contests'), we similarly tested the main effect of population, intruder color and their interaction term on the intensity of attack ('attack' count during the 5 min following the first attack). Due to overdispersion when fitted with a Poisson distribution, we modelled attack counts using a negative binomial error structure. The prediction that population morph frequency is associated with aggression towards differently colored intruders would be supported by a significant population × intruder color interaction. When this interaction was significant, we conducted separate post-hoc analyses for each of the four focal populations, testing only the main effect of intruder color; these analyses allowed us to ask whether differences were in the predicted direction.

Observations from polymorphic populations afforded us the additional opportunity to test the prediction that focal males would direct more aggression towards intruders of their own color. We re-ran the follow-up models described above in polymorphic populations, testing (in addition to intruder color) the effect of focal male color (red, blue, or intermediate) and the interaction term. The prediction that males would bias their aggression towards intruders of their own color would be supported by a significant male color × intruder color interaction followed by post-hoc analyses for each male color confirming that the differences were in the predicted direction. Because male color in polymorphic populations is not discrete, we also re-ran the models after replacing the by-eye color categories with quantitative color scores generated from photos (PC1 and PC2, **Appendix Figure 2** & **Appendix Table 1**).

For all statistical models described above, we included perch height and whether the male interacted with nearby conspecifics (yes/no) during the observation period as covariates. Because conspecific interactions were negatively associated with aggression in several models (see Results), we also re-ran all models using a dataset that excluded trials in which the focal male interacted with conspecifics (Supporting Information: Additional results: trials without conspecific interactions).

All analyses were performed in R 3.6.2 (R Core Team, 2019). We used the 'glm' and 'glm.nb' function in the stats package (R Core Team, 2019) to fit the GLMs. We tested the significance of main effects, interaction terms and covariates using a likelihood ratio test with the 'Anova' function in the car package (Fox & Weisberg, 2018), which compares overall model fit with and without a particular effect. *Post hoc* Tukey pairwise comparisons of the significant main effects were done using the 'glht' function in the multcomp package (Hothorn *et al.*, 2008).

3.4 Results

We tested a total of 372 males (after discarding 15 trials due to resampling; see **Appendix Table 2** for a breakdown of sample sizes). Males from all four populations responded aggressively to both red and blue intruder models. Out of the 372 males tested, 335 (90%) tracked, 320 (86%) approached, 312 (84%) called, 252 (68%) challenged, and 142 (38%) attacked the model intruder.

3.4.1 Does Male Aggression Pattern Vary with Population Morph Frequency?

In our initial GLMs modelling whether (yes/no) a territorial male (i) tracked, (ii)

approached, (iii) called, or (iv) challenged the model intruder, the population × intruder color interaction term was always nonsignificant, as were the main effects of population and intruder color (**Table 3-1**), suggesting no effect of color on these aggressive behaviors. However, there was a significant population × intruder color interaction on whether a territorial male attacked the model intruder (**Table 3-1**). In these models, perch height was non-significant (**Table 3-1**). Males that interacted with conspecifics had lower aggression levels, but only with respect to the likelihoods of challenge and attack (yes/no, **Table 3-1**, challenge: $\beta \pm$ SE = -1.01 ± 0.27, attack: $\beta \pm$ SE = -0.82 ± 0.26).

Table 3-1 GLMs: male agonistic behaviors

Generalized linear models evaluating the influence of population (red monomorphic, high-red polymorphic, high-blue polymorphic and blue monomorphic), intruder color (red and blue) and their interaction term on the likelihood of a territorial male to (i) track, (ii) approach, (iii) call at, (iv) challenge and (v) attack the frog model. Perch height and whether the male interacted with nearby conspecifics were included as covariates.

		Track		**Approach**		**Call**		**Challenge**		**Attack**	
Parameters	df	LR χ2	*p*	LR χ2	*p*	LR χ2	*p*	LR χ2	*p*	LR χ2	*p*
Population	3	0.22	0.975	0.30	0.961	0.49	0.920	5.04	0.169	10.64	0.014
intruder color	1	0.05	0.825	0.54	0.464	0.21	0.648	2.30	0.129	1.05	0.305
population × intruder color	3	0.70	0.873	0.63	0.889	1.56	0.669	6.58	0.087	12.87	**0.005**
conspecific interactions	1	3.54	0.060	2.48	0.115	1.64	0.200	17.53	**<.001**	13.03	**<.001**
perch height	1	0.28	0.599	0.77	0.381	0.13	0.720	3.12	0.077	1.33	0.250

Bonferroni correction: alpha level is set to 0.01

When we modeled each population separately and considered all males, regardless of interaction with conspecifics during the trial, territorial males in the monomorphic red population were more likely to attack a red (12/21) than a blue (4/27) model intruder (GLM, $LR\chi^2 = 7.60$, df = 1, $p = 0.006$, **Figure 3-2A**). Similarly, males in the monomorphic blue population were more likely to attack a blue (14/23) than a red (7/22) model intruder (GLM, $LR\chi^2 = 4.16$, df = 1, $p = 0.041$, **Figure 3-2A**). Males in the high-red polymorphic population, on the other hand, were

equally likely to attack a red (23/72) and a blue (26/68) model intruder (GLM, $LR\chi^2 = 1.25$, df = 1, $p = 0.264$, **Figure 3-2A**). Males in the high-blue polymorphic population were also equally likely to attack a red (24/68) and a blue (32/71) model intruder (GLM, $LR\chi^2 = 1.24$, df = 1, $p = 0.266$, **Figure 3-2A**). In none of these four follow up models was perch height associated with the likelihood that a focal male would attack (GLM, all $LR\chi^2 < 2.32$, all $p > 0.128$); interaction with conspecifics (yes/no) was negatively associated with attack likelihood in one population (GLM, high-red: $LR\chi^2 = 9.00$, df = 1, $p = 0.003$, $\beta \pm SE = -1.16 \pm 0.40$) but not the other three (all $LR\chi^2 < 2.71$, all $p > 0.100$).

Considering only the subset of males that attacked the model intruder, there was no effect of population, intruder color or the interaction term on the number of attacks (GLM, population: $LR\chi^2 = 2.00$, df = 3, $p = 0.573$; intruder color: $LR\chi^2 = 1.60$, df = 1, $p = 0.206$; interaction term: $LR\chi^2 = 1.08$, df = 3, $p = 0.781$, **Figure 3-2B**). Both perch height and interaction with conspecifics were non-significant in this model.

When we re-ran the analyses with the subset of observations in which the focal male did not interact with a conspecific during the trial, we found an overall similar pattern to what is presented above (**Appendix Table 3**). The only exception is that in contrast to the original analyses, males in the high-red polymorphic population were more likely to attack a blue model intruder; in the high-blue polymorphic population, this trend was also present but non-significant (see **Appendix A.4** for details).

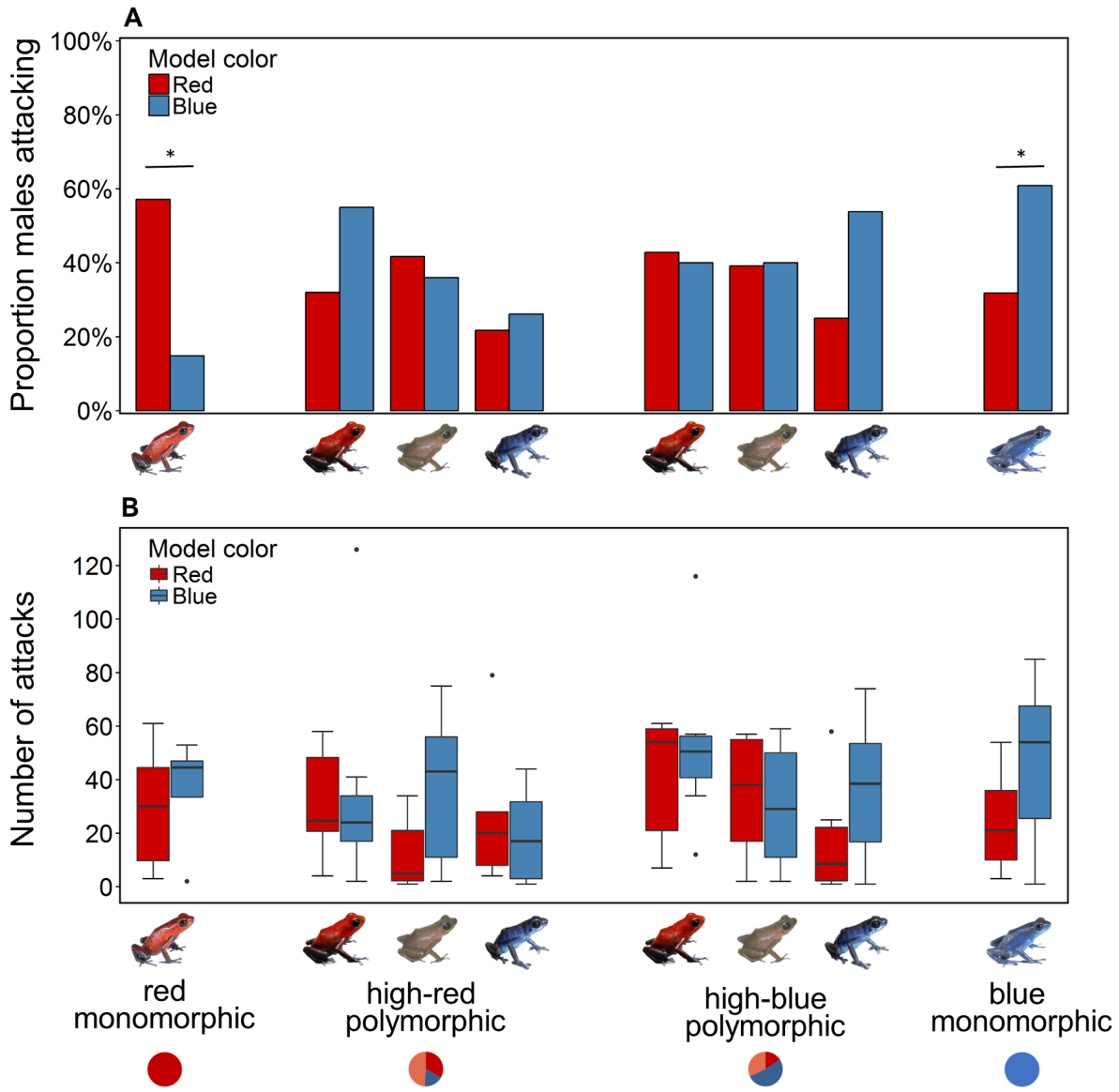

Figure 3-2 Color-mediated male-male aggression biases

Proportion of males that attacked (**A**) and number of attacks on the intruder model (**B**; during 5 minutes following the first attack) when presented with a red or a blue model frog in four populations. Pie charts on the x axis correspond to the morph frequencies in the four populations. Red, blue and phenotypic intermediate males from the two polymorphic populations were plotted separately.

3.4.2 Do Males Bias Aggression toward Intruders of Their Own Color in the Polymorphic Populations?

When considering all territorial males, regardless of interaction with conspecifics during the trial, neither the main effects of male color and intruder color nor their interaction was a significant predictor of the probability of attack in the high-red polymorphic population (**Table 3-2**). We then re-ran the model, replacing by-eye color groups with quantitative color scores PC1 and PC2: there was no significant male × intruder color interaction nor any significant main effects (**Appendix Table 6**). In both models, perch height was unrelated to attack probability (**Table 3-2 & Appendix Table 6**), while interaction with conspecifics (yes/no) was negatively associated with attack likelihood (**Table 3-2 & Appendix Table 6**, by-eye grouping:$\beta \pm$ SE = -1.30 ± 0.42; color PC scores: $\beta \pm$ SE = -1.07 ± 0.42).

In the high-blue polymorphic population, neither the main effects of male color and intruder color nor their interaction was a significant predictor of the probability of attack (**Table 3-2**). When we re-ran the model with male color PC scores, there was no male × intruder color interaction (**Appendix Table 6**). However, the likelihood of attack was positively correlated with PC2 (a hue indicator that increases with male "blueness", **Appendix Table 1**), suggesting that bluer males were more aggressive than redder males. In both models, interaction with conspecifics was unrelated to attack probability (**Table 3-2 & Appendix Table 6**), while focal males on higher perches were less likely to attack in the PC score model (**Appendix Table 6**, $\beta \pm$ SE = -0.024 ± 0.010).

Table 3-2 GLMs: likelihood of attacking in the two polymorphic populations

Generalized linear models evaluating the influence of male color (red, intermediate and blue), model color (red, blue) and their interaction term on the likelihood of a territorial male to attack the frog model in the two polymorphic populations. Perch height and conspecific interactions were included as covariates. GLMs with PC color scores replacing by-eye male color groupings are presented in **Appendix Table 6**. GLMs for the other four variables (likelihood of tracking, approaching, calling and challenging) are presented in **Appendix Table 7**, **Appendix Table 8**, **Appendix Table 9** &**Appendix Table 10**.

Attack (y/n)		**High-red polymorphic**		**High-blue polymorphic**	
Parameters	df	LR $\chi 2$	*p*	LR $\chi 2$	*p*
male color	2	2.84	0.241	1.07	0.585
intruder color	1	1.01	0.316	3.56	0.059
male × intruder color	2	2.44	0.296	2.32	0.314
conspecific interactions	1	10.21	**0.001**	2.47	0.116
perch height	1	0.66	0.416	2.09	0.148

When we re-ran the analyses with the subset of observations in which the focal male did not interact with a conspecific, we again found no significant interaction effect between male color and intruder color (the key prediction of the "own-type" hypothesis) in either polymorphic population, using either by-eye color groups or color PC scores (**Appendix Table 4 & Appendix Table 5**). Different from the analysis of the full data set, our models that included focal male color showed a main effect of intruder color in the high-blue, but not the high-red polymorphic population. Regardless of its own color, males in the high-blue (but not the high-red) population were more likely to attack a blue intruder. When we re-ran these models using color PC scores rather than by-eye category, we found this trend toward a higher likelihood of attacking a blue intruder in both polymorphic populations (see **Appendix A.4** for details).

GLMs for the other four variables (likelihood to track, approach, call and challenge) are presented in **Appendix Table 7, Appendix Table 8, Appendix Table 9 & Appendix Table 10**.

We did not detect any significant main effects or an interaction between male color and model intruder color in any of the models.

3.5 Discussion

Male-male competition is hypothesized to contribute to the stability of a polymorphism when a rare male phenotype has a fitness advantage over a common phenotype (Mikami *et al.*, 2004; Seehausen & Schluter, 2004). Territorial *O. pumilio* males in red and blue monomorphic populations were more aggressive toward the local coloration than the novel (allopatric) coloration, suggesting that color is important in shaping male aggressive interactions. However, this pattern did not persist in sympatry: males in the two polymorphic populations did not bias aggression toward their own coloration ("own-type") nor did they bias their aggression toward the more common color in the population ("familiarity"). Instead, males in both the high-red and the high-blue polymorphic populations were more likely to attack a blue model intruder. While aggression biases may facilitate the initial invasion of a novel *O. pumilio* phenotype, these biases appear to weaken and/or change in direction quickly as a second phenotype becomes more common. A related question remains whether males attend to the color of residents when invading a territory/establishing a new territory as males of the rarer morph might also benefit if their territories are less likely to be invaded. Without any aggression-mediated fitness advantage for the rarer phenotype, male aggression seems insufficient to exert negative-frequency dependent selection on *O. pumilio* color morphs in sympatry.

Identifying the mechanism by which male aggression biases are formed may help to clarify how and why male aggression biases break down in sympatry. If the aggression bias is heritable

(McKinnon & Pierotti, 2010), alternative alleles might have been fixed in each monomorphic population. In this case, the lack of an aggression bias in the two polymorphic populations could be explained by recombination breaking down the association between male coloration and color-biased aggression. Alternatively, if the aggression bias is plastic and shaped by experience (as assumed in the "familiarity" hypothesis), our findings suggest that the threshold for rival recognition is relatively low: a phenotype need not be the most common to be recognized, and any bias disappears long before morph frequencies approach parity. Because *O. pumilio* males interact with conspecific rivals on a daily basis, they are likely to learn from past agonistic interactions and adjust any aggression "bias" accordingly (as in many other animals: Hsu *et al.*, 2006; Reichert & Quinn, 2017). Pertinent to this learning hypothesis, however, male *O. pumilio* do not reduce aggression towards known neighbors like other territorial animals are known to do ("dear enemy": Ydenberg *et al.*, 1988; Bee, 2003; Gardner & Graves, 2005).

A third possibility is that color bias is learned at an early age (i.e. sexual imprinting). Imprinting is common in the context of mate preference (Lorenz, 1935; Verzijden *et al.*, 2012), and may also shape the perception of rivals (e.g. tits: Hansen & Slagsvold, 2003; cichlids: Dijkstra *et al.*, 2008a; but see Verzijden *et al.*, 2009). Depending on the modes of imprinting (e.g., parental vs. social), male color and color-biased aggression can be decoupled (although not as easily as genetic-based behaviors). The degree to which male aggression biases are genetic or plastic/learned in *O. pumilio* remains to be tested, but biparental care in this frog (males tending eggs and females feeding tadpoles, reviewed in Dugas, 2018) provides ample opportunities for tadpoles to see adult colors. Male-male competition was recognized as a potential diversifying force only recently (Qvarnström *et al.*, 2012; Tinghitella *et al.*, 2017), and the evolutionary consequences of whether aggression biases are genetic or learned remained to be explored.

Males from the two monomorphic populations we studied differed in their relative likelihoods of attacking red and blue intruders, but not in the pre-escalation display behaviors ('track', 'approach', 'challenge' and 'call'). This pattern could have arisen because intraspecific communication in *O. pumilio* is multimodal, with both acoustic and visual signals perceived in a sequential order (Dreher & Pröhl, 2014). If males use calls to identify a conspecific intruder from a further distance, while visual signals aid in closer-range assessments (Candolin, 2003), this could explain why aggression biases were only apparent after the focal male hopped on the log ('challenged'); at this point in the interaction the model was not visually shielded by the complex forest environment (Willink *et al.*, 2013). On the other hand, we found that the intensity of attack (number of attacks) was not mediated by the intruder color. In animal contests, aggression biases can influence the probability of contest escalation; however, if coloration does not predict fighting ability (i.e. the color morphs are equally competitive), then color biases may not influence the intensity of the contest once escalated (Hsu *et al.*, 2006). Although the two *O. pumilio* color morphs in this study have not been directly tested for competitive ability, they did not differ in overall aggression level, body size (Y. Yang *unpublished data*) or call characteristics (M. L. Dye *unpublished data*). Nevertheless, the majority of *O. pumilio* agonistic interactions in nature are resolved without escalation (~63% of 19 natural encounters; Meuche *et al.*, 2012), suggesting that color-based differences in the likelihood of attack may be critical to a male's ability to hold/gain a territory. Moreover, females often leave the male they are courting if that male interacts with another male (Yang *et al.*, 2019a). This suggests that even in cases where territories do not change hands, frequent agonistic interactions could be costly.

As in many other animals (Berglund *et al.*, 1996; Rojas, 2017), the dramatic differences in coloration among *O. pumilio* lineages appear also to be important in mediating female mate choice,

with females often (but not always) expressing assortative preferences in laboratory trials (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008, 2009; Richards-Zawacki & Cummings, 2011). Male-male competition in the wild can shape the extent to which these preferences actually contribute to reproductive isolation (Jennions & Petrie, 1997; Wong & Candolin, 2005). In the red-blue contact zone studied here, females from both monomorphic populations spend more time associating with males of the local color morph, while red, blue and intermediate females from the polymorphic region all associate preferentially with red males (Yang *et al.*, 2016). Consequently, when a novel color first invades a monomorphic population, male *O. pumilio* might simultaneously experience two, potentially opposing, selective forces: males of the rare color morph may slip under the radar of competing males and enjoy an advantage in terms of gaining/defending territories, but they may also be less attractive as mates if females preferring the novel color are also rare in the population.

Male-male competition could similarly limit females' access to preferred phenotypes if some male morphs are dominant over others (Dijkstra & Groothuis, 2011; Qvarnström *et al.*, 2012). Allopatric *O. pumilio* color morphs differ in aggression level (Rudh *et al.*, 2013), and brighter males are more aggressive in one monomorphic population tested (Crothers & Cummings 2015). Some of these differences in aggression level are reflected in contest dominance in a laboratory setting (Galeano & Harms, 2016). In this study, males that did not interact with conspecifics during the trial showed a trend toward attacking a blue model intruder more than a red one in both polymorphic populations, and bluer males were more likely to attack a simulated intruder (regardless of its color) in one polymorphic population. While these patterns were only evident in a subset of our analyses, and sometimes with only marginal statistical significance, they suggest that there may be asymmetries between the two color morphs. Whether coloration is

associated with any other measure of competitive ability aside from aggression level in this contact zone warrants further investigation.

While males in monomorphic populations were less aggressive towards novel phenotypes, males in the polymorphic populations bias aggression to neither their own color nor the most common color in the area. Broadly, this result indicates that the potential for divergent aggression biases to stabilize a polymorphism will depend upon whether and how quickly such biases break down as morph frequencies approach parity. Several avenues for future research can offer a more complete understanding of the role of male-male competition in phenotypic divergence: (i) identifying the proximate mechanisms that shape male color-based agonistic behaviors, (ii) quantifying the fitness consequences of differential male aggression, (iii) deciphering the degree to which male-male competition constrains the expression of female preferences, and (iv) exploring how inherited vs. learned male aggression biases may influence the likelihood and persistence of phenotypic divergence.

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4.0 Chapter 4: Male-male Territorial Contest Limits the Expression of Assortative Mate Preferences in a Polymorphic Poison Frog

The contents in this chapter are adapted from from a manuscript currently in review in *Behavioral Ecology*:

Yang, Y. & Richards-Zawacki, C.L. Male-male territorial contest limits the expression of assortative mate preferences in a polymorphic poison frog.

4.1 Chapter Summary

Co-divergence of sexual traits and mate preferences for those traits is hypothesized to lead to assortative mating patterns and subsequently reproductive isolation. However, mate choice rarely operates independently of intrasexual competition, and the effects of the latter on speciation are often overlooked. Recent studies have proposed two main roles of male-male competition in the evolution of reproductive isolation: i) maintaining sexual trait and preference polymorphism in the face of gene flow, and ii) limiting assortative mating when males of the non-preferred phenotype are superior competitors. Both scenarios rely on the assumption that male-male competition can limit the expression of divergent female mate preferences. We tested this critical but rarely tested assumption in the strawberry poison frog (*Oophaga pumilio*), a highly color polymorphic species that has been used as a model system to study the role of mate choice in the early stages of reproductive isolation. Females generally prefer males of the same color morph, and this assortative pattern has been interpreted as support for reproductive isolation evolving

among the divergent phenotypes. However, this inference does not account for male-male territorial competition, which is also mediated by color and can affect male courtship success. In this study, we housed females with two size-matched males, one of an attractive color and one of an unfamiliar color, and compared reproductive patterns when the male of the female's preferred color was the territory holder *versus* when the male bearing the preferred color was the non-territorial male. We found that females mated primarily with the territory winner, regardless of his coloration, suggesting that when a choice must be made between the two, male territoriality overrides female preference for male coloration. Our results highlight the interaction between mate choice and intrasexual competition, and the importance of considering the combined effects of both selective forces in shaping phenotypic divergence and speciation.

4.2 Introduction

Understanding the mechanisms by which sexual selection drives speciation has been a growing focus in evolutionary biology (Ritchie, 2007; Kraaijeveld *et al.*, 2011; Servedio & Boughman, 2017). Sexual selection can cause rapid co-divergence of mating signals and mate preferences, potentially leading to reproductive isolation between divergent phenotypes (Lande, 1981; Kirkpatrick, 1982; West-Eberhard, 1983). However, sexual selection encompasses both female mate choice and male-male competition, and the role of male-male competition has been largely ignored in speciation research (Qvarnström *et al.*, 2012; Tinghitella *et al.*, 2017; Lipshutz, 2018). This is a major oversight because sexual signals often function both in female choice and male-male competition (Andersson, 1994; Berglund *et al.*, 1996; McCullough *et al.*, 2016). Signals or weapons used in male-male contests can convey information on male quality, and drive the

evolution of female preferences on these male traits (e.g. skrraa calls in the *Chlamydera* bowerbirds; Borgia & Coleman, 2000). Traits can also evolve first as courtship signals, and subsequently be co-opted to signal aggression or dominance in male-male contests (e.g. vertical bars in male swordtails; Morris *et al.*, 2007). Because divergence of sexual signals can affect both female choice and male-male competition, without considering both processes, our understanding of speciation by sexual selection is incomplete at best.

Sexual selection arises due to asymmetries in mate limitation (i.e. a skewed operational sex ratio) and/or reproductive fitness gain per additional mating (i.e. the Bateman gradient) between the two sexes (Kokko *et al.*, 2012). In most animal systems, females are choosy because they are able to choose from a large pool of potential mates and have less to gain from additional matings than males. This sexual selection paradigm underlies a mainstream view that female choice and male-male competition should be mutually reinforcing; that high-quality males are both competitively superior and preferred by females (Cox & Le Boeuf, 1977; Berglund *et al.*, 1996; Wong & Candolin, 2005). While this is true in many systems, the pattern is far from universal (Qvarnström & Forsgren, 1998; Wong & Candolin, 2005; Hunt *et al.*, 2009). The reproductive interests of males and females are often at odds, resulting in misaligned or even opposing inter- and intra-sexual selection (Arnqvist & Rowe, 1995; Moore & Moore, 1999). Quantifying the interaction between female choice and male-male competition is especially crucial in speciation research because it dictates not only sexual signal evolution but also non-random mating patterns, and consequently, gene flow among the divergent phenotypes.

Recent research and syntheses have identified two potential roles of male-male competition in speciation by sexual selection. First, theoretical studies suggest that speciation via divergent female preference can only occur under a limited set of conditions, and these conditions are

especially limited when there is gene flow between the incipient species. A key challenge to speciation by sexual selection is maintaining mating trait and preference variation during the speciation process (Arnegard & Kondrashov, 2004; van Doorn *et al.*, 2004). Recent studies suggest that male-male competition can maintain this variation if males bias their aggression towards phenotypically similar rivals (through negative-frequency dependent selection; Mikami *et al.*, 2004; Seehausen & Schluter, 2004; van Doorn *et al.*, 2004). However, whether this promotes speciation depends critically on the assumption that the advantage gained in male-male competition (e.g. holding a territory) translates to higher reproductive success despite assortative female preferences (Dijkstra *et al.*, 2008b). Secondly, divergent male types (or closely related species) are often asymmetric in competitive ability (Pryke & Griffith, 2006; Sefc *et al.*, 2015; Martin *et al.*, 2017). Females may not be able to choose their preferred phenotype when non-preferred males are better competitors (Reichard *et al.*, 2005; Dijkstra *et al.*, 2008b), thus limiting mate preferences from translating to assortative mating. Such asymmetric male dominance could lead to competitive exclusion of the weaker phenotype or directional introgression of the stronger phenotype (Pearson & Rohwer, 2000; Teeter *et al.*, 2008; Sefc *et al.*, 2015; While *et al.*, 2015), breaking down the potential for sexual isolation due to divergent female preference. Testing the hypothesis that the outcome of male-male competition can limit or prevent the expression of divergent female preferences is critical to understanding the role of male-male competition in speciation.

The strawberry poison frog (*Oophaga pumilio*) exhibits extreme, heritable color polytypism in the Bocas del Toro region of Panama (**Figure 1-1**; Summers *et al.*, 2003). Most described color variants occur among isolated island populations, but there are a few populations that show sympatric color polymorphism (summarized in Yang *et al.*, 2019b). Coloration in males

and females is qualitatively similar in this species (Summers *et al.*, 2003; Maan & Cummings, 2009) and functions both as an aposematic signal (Saporito *et al.*, 2007) and in intraspecific communication (Maan & Cummings, 2008; Crothers & Cummings, 2015; Yang *et al.*, 2018). Females generally prefer males of the local color morph over an unfamiliar color morph (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008; Richards-Zawacki & Cummings, 2011; but see Yang *et al.*, 2016), and this assortative preference pattern has been interpreted as evidence that sexual isolation is evolving among divergent color morphs. However, this inference does not account for male-male territorial competition, which is also mediated by color and has a substantial effect on male courtship success (Meuche & Pröhl, 2011). Males respond more aggressively toward rivals of the local (familiar) color compared to an unfamiliar color in territorial contests (Yang *et al.*, 2018, 2019b). Males of conspicuous color morphs are also generally more aggressive than duller morphs (Rudh *et al.*, 2013), suggesting the potential for asymmetry in competitive ability to evolve in association with divergent coloration. Both patterns (aggression biases toward different-colored rivals and asymmetric aggressiveness among color morphs), as discussed above, have the potential to influence the evolution of reproductive isolation among *O. pumilio* color morphs.

Here, we conducted a breeding experiment with two territorial treatment groups in which male-male competition and female mate choice act either i) in the *same* direction, or ii) in *opposing* directions. We allowed two size-matched males to compete for dominance, and subsequently introduced a female with a preference for the *winner's* or the *loser's* coloration. By comparing the mating patterns between the two territoriality treatments, we explicitly tested whether male-male territorial contests limited the expression of assortative color preferences in *O. pumilio* females. Testing this hypothesis is relevant to the role of male-male competition in both i) maintaining

sexual trait and preference polymorphism in the face of gene flow, and ii) limiting the expression of female assortative preference when non-preferred phenotype is the superior competitor.

4.3 Methods

4.3.1 Study Species

Oophaga pumilio is a small terrestrial poison frog native and restricted to the Neotropics (Savage, 1968). This frog is diurnal and has trichromatic color vision that enables it to distinguish variation in conspecific color signals (Siddiqi *et al.*, 2004). Male *O. pumilio* defend territories year round through vocalization, visual displays, and physical combat (Bunnell, 1973; Pröhl, 1997; Pröhl & Berke, 2001; Gardner & Graves, 2005). Females have larger home ranges that overlap several males' territories, and they visit calling males within their home ranges when searching for potential mates (Pröhl & Berke, 2001). Unlike most frogs, males of this species do not clasp females during mating; females can therefore terminate courtship at any time prior to mating (Yang *et al.*, 2019a). When courtship is successful, females lay a clutch of ~5 eggs in the male's territory (Limerick, 1980; Haase & Pröhl, 2002). Although non-territorial males often attempt to court females, there are no documented cases of successful mating resulting from this satellite strategy in the wild (Meuche & Pröhl, 2011; Meuche *et al.*, 2012). After oviposition, males guard and hydrate the terrestrial eggs. Upon hatching, females return to transport the tadpoles to individual nurseries (e.g. water-filled leaf axil; Dugas, 2018). Throughout larval development, tadpoles rely on the mother providing unfertilized eggs as their only food source (~45 d, Weygoldt, 1980; Dugas *et al.*, 2016).

4.3.2 Experiment Design

Our breeding experiment had two territorial treatment groups in which male-male competition and female mate choice acted either i) in the *same* direction, or ii) in *opposing* directions. Females were housed with two size-matched males, one of the same color morph as her own (her preferred color), and one of an unfamiliar color morph. We designed the trials so that the female was either the same color morph as the holder of the territory (terrarium) in which the three frogs resided (treatment AW, *Attractive Winner*) or the same color morph as the male who had lost the territorial contest (treatment AL, *Attractive Loser*; **Figure 4-1A**). We did not assay the females for color preference before introducing them into a trial. However, females of the three color morphs that we used (see below) have been shown to exhibit assortative color preferences in the wild (Maan & Cummings, 2008; Richards-Zawacki & Cummings, 2011), and these preferences persist in pure-bred captive individuals in our breeding colony (preferences are shaped by maternal imprinting; Yang *et al.*, 2019b). We therefore assumed that the pure-bred females in our study displayed assortative preferences in our experiment (i.e. prefer males with their, and their mother's color compared to a contrasting, unfamiliar color). These trios were kept together until one pair produced tadpoles, which we then genotyped to reveal paternity as direct evidence of reproductive success.

4.3.3 Animals

We conducted our experiment using three color morphs in a breeding colony that was established from wild-caught individuals collected from three allopatric populations from Bocas del Toro, Panama: a bright orange-red morph from the southern tip of Isla Bastimentos (hereafter,

orange; 9°15.080' N, 82°80.433' W), a red morph with coarse black patterning from the northwestern tip of Isla Bastimentos (hereafter, *red*; 9°20.808' N, 82°12.384' W), and a green morph with a bright yellow belly from Isla Popa (hereafter, *green*; 9°80.260' N, 82°70.391' W; exemplars of each morph are shown on the x axis of **Figure 4-1B**). Color is heritable in this species and all individuals used in the experiment were pure-bred (i.e. produced by individuals caught from the same wild population or their descendants). All animal enclosures were housed in the same walk-in environmental chamber (Darwin Chambers Company, St. Louis) at the University of Pittsburgh. The chamber was maintained at 25 °C and 70% relative humidity, under a 12L/12D light cycle. Frogs were fed with vitamin dusted fruit flies (*D. melanogaster*). Other animal care and maintenance details were as described in Dugas & Richards-Zawacki (2015).

For both territoriality treatments, we used four different color morph combinations: i) green female with green and red males, ii) green female with green and orange males, iii) orange female with orange and green males, and iv) red female with red and green males. We did not include the two combinations that asked the female to choose between orange and red males, because a previous study demonstrated that dorsal color hue (and not patterning) is the main target of female preference in *O. pumilio*, and that the red and orange morphs may not be different enough for the females to distinguish (Siddiqi *et al.*, 2004; Maan & Cummings, 2008). Color combinations were included as a categorical covariate of 4 levels in statistical analyses. Using multiple color morph combinations permitted us to ask whether the patterns we found were likely to be universal versus unique to certain phenotype combinations.

4.3.4 Determining Male Territoriality

For each trial, we first introduced two differently-colored, size-matched males into a 30 x 20 x 20cm terrarium. Each terrarium floor was lined with moist sheet moss and each terrarium contained a live *Peperomia Scandens* vine for egg deposition, and 4 water-filled PVC tubes for tadpole rearing. These terraria were kept in the same environmental chamber and condition as the rest of the breeding colony and were misted with RO filtered tap water several times a day. We observed the male pairs 2-3 times each week until the males established a stable hierarchy, operationally defined as the winner exhibiting behavioral dominance over the loser for three consecutive observations. Dominant behaviors include calling, chasing, wrestling, and pinning; and submissive behaviors include escaping and freezing (Baugh & Forester, 1994; Yang *et al.*, 2018). During each hour-long observation, we observed up to 16 terraria via scan sampling, and recorded the dominant and subordinate males in each tank based on the tallied behaviors at the end of the observation. We marked a male as the territory holder (winner) when he was recorded as the dominant male in three consecutive observations.

4.3.5 Mating Trials

After the male pair had resolved their dominance hierarchy, we randomly introduced a female into the terrarium that was either i) the same color morph as the winner (treatment AW), or ii) the same color morph as the loser (treatment AL). Females were assumed to display assortative preference (i.e. prefer males of the same color morph, see **Experiment Design** above). The tanks were censused every week for new tadpoles. Trials were terminated when the trio produced its first set of tadpoles, or when the trio failed to produce tadpoles in 200 days.

During the time that the trio were housed together, we continued to observe the behavior of the two males 2-3 times each week. Winner males were often observed courting females or acting aggressively toward the loser males (e.g. chasing, wrestling, calling). We pooled these courtship and agonistic behaviors together because it is often difficult tell whether the winner male was calling to the female or the loser male. Most loser males were socially inactive, but some displayed variable degrees of agonistic or courtship behaviors. To test whether the difference in loser behavior influenced the reproductive dynamics, we further categorized the losers as "submissive" or "aggressive" according to their behavior after the females were introduced to the terrarium. Loser males were classified as "submissive" when we did not observe any agonistic or courtship behavior during any of our observations, and classified as "aggressive" when agonistic and/or courtship behaviors was observed at least once.

We collected toe-clips of adults and tail-clips of tadpoles, extracted genomic DNA, and genotyped individuals at 6 polymorphic microsatellite loci (Hauswaldt *et al.*, 2009) to determine paternity. With a known mother and only two candidate fathers in each trial, paternity could be assigned unambiguously by eye in all cases. We used tadpoles as our indicator of reproductive success because egg production is difficult to reliably monitor in the breeding colony (Dugas & Richards-Zawacki, 2015). Tadpole production requires successful courtship, fertilization, egg development, male care (egg tending) and female care (tadpole transport), so our measurement likely underestimated the number of mating events. Previous studies in the same colony revealed that intra- and inter-morph breeding is similarly successful, suggesting no intrinsic reproductive barriers under captive conditions (Dugas & Richards-Zawacki, 2015).

4.3.6 Statistical Analyses

To test the hypothesis that the outcome of male-male territorial contests limits a female from choosing her preferred color morph, we compared the mating pattern between the two territoriality treatments, using only the trials that produced tadpoles within 200 days. We tested the effect of territoriality treatment (AW/AL) on whether (yes/no) the female mated assortatively based on coloration (i.e. whether mate choice is in concordance with presumed mate preference) using a binomial generalized linear mixed model (GLMM). In this model, we included color morph combination (4 total combinations, see methods) and loser behavior (submissive/aggressive) as covariates, and male and female IDs as random effects. We then tested whether bearing an attractive color trait can increase reproductive success on top of being territorial, with the prediction that the females are more likely to mate with a winner in the AW treatment compared to the AW treatment. We tested the effect of territoriality treatment (AW/AL) on whether (yes/no) the female chose to mate with the winner, including color morphs and the loser behavior as covariates.

We then analyzed the factors that influenced the timing of reproduction (latency to produce tadpoles) using all data points (inclusive of trios that produced no tadpoles). We first compared the reproductive timing of the winners in the two territory treatments, modeling the effect of territoriality treatment (AW/AL) on the latency for winners to produce tadpoles using a Cox proportional hazards regression. We then compared the reproductive timing of the losers, similarly modeling the territoriality treatment's effect (AW/AL) on the latency for losers to produce tadpoles. Cox proportional hazards regressions test for effects of variables of interest on both the timing and the probability of occurrence of an event. Note that because there were trials in which neither the winner nor the loser produced any tadpoles in 200 days, the two Cox regression

analyses were not identical. Color morph combination and loser behavior were included in both models as covariates.

All analyses were performed in R 3.6.2 (R Core Team, 2019). We used the "glmmPQL" function in the *MASS* package (Venables & Ripley, 2013) to fit the GLMMs. We used the "coxph" function in the *survival* package (Therneau & Grambsch, 2000) to fit the Cox proportional hazards regressions. We tested the significance of the main effects (territoriality treatment and the covariates) using a likelihood ratio test with the "Anova" function in the *car* package (Fox & Weisberg, 2018), which compares overall model fit with and without a particular effect. *Post hoc* Tukey pairwise comparisons of the significant main effects with more than two levels were made using the 'glht' function in the *multcomp* package (Hothorn *et al.*, 2008).

4.4 Results

We conducted a total of 88 trials, 71 of which successfully produced tadpoles within 200 days (mean ± sd = 50 ± 39 days, excluding trials that ended without tadpoles). Proportion of reproductive successes (number of trials resulting in tadpoles/total number of trials) in each treatment group were: *Attractive Winner*: 36/43 (84%), *Attractive Loser*: 35/45(78%); sample sizes for each color morph combination are listed in **Table 4-1**. In 66% of the trials, we did not observe any agonistic or courtship behavior from the loser male during any of our observations ("submissive losers"), while in the remaining 34% of the trials, the losers displayed some degree of agonistic and/or courtship behavior ("aggressive losers"). We were able to unambiguously assign parentage genetically for all tadpoles, and tadpoles in the same clutch (clutch size range: 1-

7 tadpoles) were always sired by the same male. We used the 71 trials which successfully produced tadpoles in *Analysis of mating pattern* analyses, and all 88 trials in *Reproductive timing* analyses.

Table 4-1 Sample size breakdown for the breeding experiment

Number of total trials and number of trials that successfully produced tadpoles within 200 days (in parenthesis) of the four color morph combinations in the two territoriality treatments.

		Territoriality Treatment	
Female Morph	**Male morphs**	Attractive Winner	Attractive Loser
green	green & red	9 (6)	9 (8)
green	green & orange	15 (14)	9 (8)
red	red & green	10 (10)	10 (8)
orange	orange & green	9 (6)	17 (11)

4.4.1 Analysis of Mating Patterns

Territoriality treatment (AW/AL) had a significant effect on female mate choice (binomial GLMM, LRχ2 = 26.45, df = 1, $p < 0.0001$): 75% of the females in the AW treatment mated assortatively by color, while only 23% of the females in the AL treatment did so (**Figure 4-1B**). Neither color morph combination nor loser behavior had a significant effect on female mate choice (color morph combination: LRχ2 = 1.62, df = 3, p =0.654; loser behavior: LRχ2 = 0.139, df = 1, p = 0.709). Females were equally likely to mate with the winners of the same color morph (AW treatment) as winners of an unfamiliar color morph (AL treatment; binomial GLMM, LRχ2 = 0.005, df = 1, p = 0.945). Because this analysis used only trials that produced tadpole(s), the result also means that females were equally likely to mate with losers of either color. Color morph combination had no effect on the paternal identity of the tadpole(s) (LRχ2 = 6.57, df = 3, p =

0.087); however, aggressive losers were more likely to sire tadpole(s) compared to submissive losers (LRχ2 = 7.21, df = 1, p = 0.007).

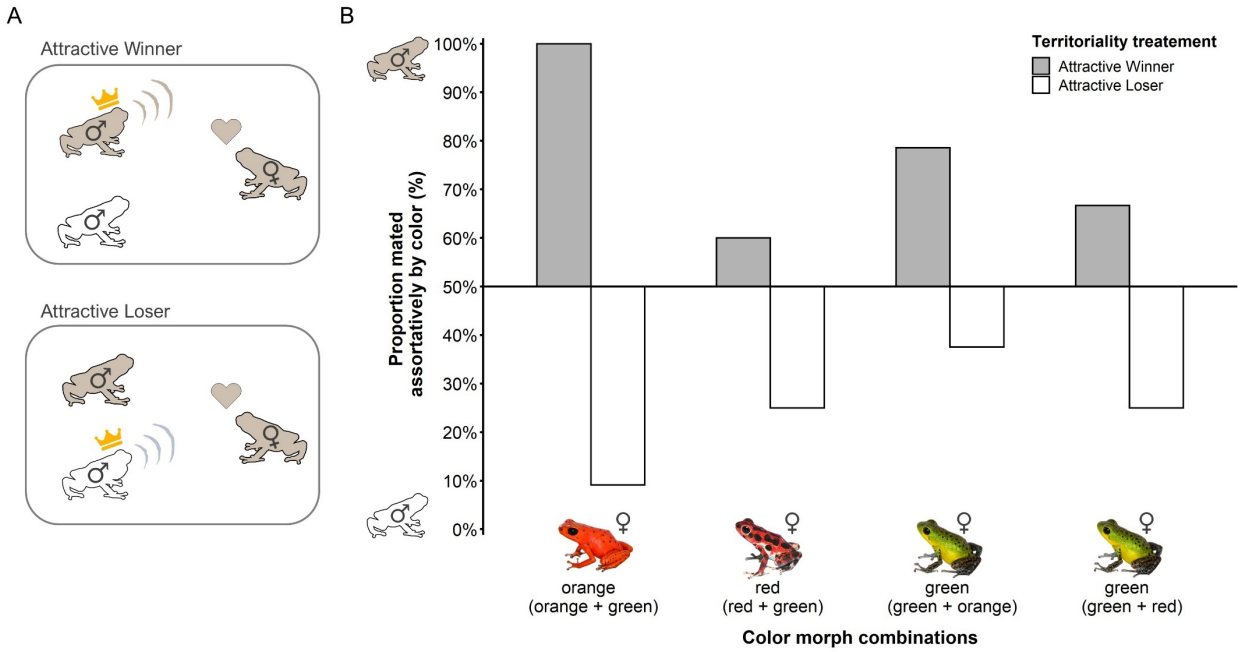

Figure 4-1 Breeding experiment design and results

A: Experimental design. In both treatments, the female was housed with a male of her own color morph and a male of a contrasting, unfamiliarcolor morph. In the *Attractive Winner* treatment, the territorial male was the same color morph (presumed to be her preferred morph) as the female; in the *Attractive Loser* treatment, the territorial male was the contrasting color morph (presumed to be less attractive). **B**: The proportion of trials in which the female mated assortatively in the breeding experiment. Bars above the x axis (y = 50%) indicate an assortative mating pattern, and bars below the x axis indicate disassortative mating. Images on the x axis shows an exemplar of each color morph. Colors in parentheses indicate the color morphs of the two males with which the female was housed. Sample sizes are reported in **Table 4-1**.

4.4.2 Reproductive Timing

The reproductive timing of winners was not significantly different between the two territoriality treatments (Cox regression, LRχ2 = 0.057, df = 1, p = 0.811). Color morph combination had no effect on the winner's reproductive timing (LRχ2 = 1.71, df = 3, p = 0.635); however, winners housed with an aggressive loser produced tadpoles significantly later compared

to those housed with an submissive loser (LRχ2 = 16.56, df = 1, p < 0.0001, **Figure 4-2A**). In comparison, the reproductive timing of a loser was not predicted by territoriality treatment (Cox regression, LRχ2 = 0.064, df = 1, p = 0.801) or by whether he behaved aggressively or submissively (LRχ2 = 1.71, df = 3, p = 0.635, **Figure 4-2B**), but was significantly different among color morph combinations (LRχ2 = 8.03, df = 3, p = 0.045). However, none of the pairwise comparisons among the color morph combinations was significant in the Tukey *post hoc* test (all p > 0.145).

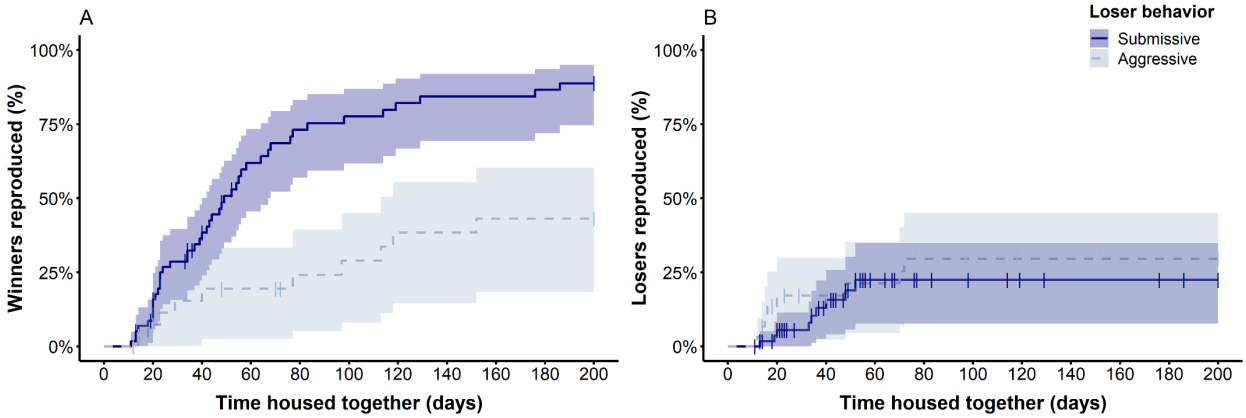

Figure 4-2 Reproductive timing results

Time-to-event curves showing the reproductive timing of territory winners (**A**) and losers (**B**) in trials with an aggressive (dashed line) or a submissive (solid line) loser. Shaded areas represent 95% confidence intervals, and vertical lines on the curve indicate censor points (i.e. trials terminated because the other male sired a tadpole(s), or the 200 day limit had been reached). The two territoriality treatments were pooled together because the treatment effect was not significant in the Cox regression analysis (see **4.4.2** for details).

4.5 Discussion

We provide empirical evidence that supports the hypothesis that the outcome of male-male territorial contests can limit the expression of divergent color preference in the strawberry poison frog (*O. pumilio*), a species with highly divergent mating trait and mate preferences. We found

that females mated primarily with the territorial winner, regardless of his coloration. Furthermore, the territorial losers of the female's preferred color were no more reproductively successful than losers of an unfamiliar, less preferred color, suggesting that bearing an attractive color trait was not sufficient to rescue the reproductive success of a non-territorial male. Together with previous studies showing that that both male competitive ability and aggression biases have diverged in concert with coloration (Rudh *et al.*, 2013; Yang *et al.*, 2018), our study adds to the weight of evidence that male-male competition is an important driver in trait divergence and evolution of reproductive isolation in *O. pumilio.*

Although female color preferences had no effect on mating patterns in our experiment, we do not mean to suggest that color preferences have no effect on color evolution or reproductive patterns in nature. In the *Attractive Loser* treatment, the female could choose a mate bearing her preferred color or a mate with a territory but not both. In the wild, females may be able to sample among multiple male territories before mating, increasing the chance that she would encounter a male that is both territorial and bears her preferred color. In other words, color may not be important when a female is choosing between a territorial male and a non-territorial male, but may become relevant when females are choosing between two territorial males. The number of potential mates a female can sample is often variable and restricted by social or ecological context, which dictates the sampling costs (Jennions & Petrie, 1997; Rosenthal, 2017). For example, population density is a strong determinant of how females sample potential mates in *O. pumilio* (as in many other animals; Kokko & Rankin, 2006). Females in a low-density population mate with the closest calling male without comparing them to other potential mates (Meuche *et al.*, 2013), but appear to be choosier in populations with higher densities (Gade *et al.*, 2016; Yang *et al.*, 2019a). The degree to which male dominance restricts the expression of female preference

may therefore be variable among *O. pumilio* populations, potentially increasing as density decreases. However, even in populations where females sample multiple males, the outcome of male-male territorial competition can still determine the encounter rate of territorial males of her preferred versus less preferred color, indirectly restricting the expression of mate preferences.

In the wild, *O. pumilio* males that have lost a territorial contest may leave the location in search of another territory. However, it is also common for these non-territorial males to stay and attempt to intercept and court females attracted by the winner's advertisement call (Meuche & Pröhl, 2011). These satellite males court females by emitting quiet courtship calls to avoid detection by territorial males (Meuche & Pröhl, 2011). We observed such behaviors in our experiment: 34% of the loser males in our trials attempted to court the females or exhibited some degree of agonistic behavior toward the territory winner. In the analysis using only the 71 trials that successfully produced tadpoles, females were more likely to mate with an aggressive loser compared to a submissive loser. However, trials with an aggressive loser were also less likely to produce tadpoles within 200 days compared to trials with a submissive loser (60% vs 91%). When we considered these failed trials in the reproductive timing analyses, the reproductive success and timing of aggressive losers were not significantly different from submissive losers. On the other hand, the reproductive timing of the territorial winners was significantly delayed in the presence of an aggressive loser. This suggests that satellite males that are actively courting or fighting can inflict costs on the territorial owner even when territories do not change hands. This matches anecdotal observations in the wild, that females often lose interest in courtship when the territorial male engages in agonistic interaction with another male (Y. Yang, personal observation; H. Pröhl, personal communication).

A recent study revealed that both color-based female preference and male-male aggression biases in *O. pumilio* are formed through maternal imprinting instead of genetically inherited (Yang *et al.*, 2019b). Based on this finding, Yang et al. (2019b) proposed a mechanism whereby this combination of learned behaviors may facilitate speciation by sexual selection. In this model, imprinted aggression biases generate negative frequency dependent selection, which can maintain a stable polymorphism and allow sexually imprinted female mate preferences to reduce gene flow in sympatry. Our findings in this study lend support to two important assumptions of the Yang et al. (2019) model: that winning a territory confers major reproductive advantages to a male, and that aggression or challenges a territorial male receives decrease his reproductive success, even if he is able to maintain the territory.

The Yang et al. (2019) model incorporated individual male aggression biases toward rivals of different color morphs (Yang *et al.*, 2018), but not asymmetric behavioral dominance among color morphs (Rudh *et al.*, 2013). Females may not be able to choose males of their preferred color morph if competing morphs are superior in acquiring and defending territories. How this additional factor would impact the evolutionary trajectories described in the Yang et al. (2019) model is unclear. Intuitively, asymmetric dominance should disrupt reproductive isolation and make it more difficult for a stable polymorphism to arise. However, the effect likely also depends on the degree of asymmetry in competitive ability and the relative strength of female choice and male-male competition. A more comprehensive analysis is required to test this verbal argument and explore the range of evolutionary outcomes that result from different scenarios.

Research on speciation by sexual selection has traditionally focused on divergent female preferences, and studies elucidating the role of male-male competition are just starting to gain momentum (Tinghitella *et al.*, 2017). Recent studies have proposed two main roles of male-male

competition in speciation: i) maintaining sexual trait and preference polymorphism in the face of gene flow, and ii) limiting assortative mating when males of the non-preferred phenotype are superior competitors. Our study tested and confirmed a shared, critical assumption of both scenarios. Male-male competition does indeed limit the expression of divergent female mate preferences in *O. pumilio*, lending support to inferences made in previous studies. That sexual traits can mediate both female mate preference and male territorial competition (Andersson, 1994; Berglund *et al.*, 1996; McCullough *et al.*, 2016), and that females pay attention to both male traits and territory status/quality (Jennions & Petrie, 1997; Dijkstra *et al.*, 2008b) have been demonstrate across a range of animal taxa. Exploring whether the degree to which male territorial competition limits divergent female preference varies among species, and whether this variation correlates with these lineages' progression toward full reproductive isolation would be an exciting avenue for future research.

4.6 Acknowledgements

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5.0 Chapter 5: Imprinting Sets the Stage for Speciation

The contents in this chapter are adapted from the following publication:

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5.1 Chapter Summary

Sexual imprinting, the phenomenon where offspring learn parental traits and later use them as a model for their own mate preferences, can generate reproductive barriers between species. When the target of imprinting is a mating trait that differs among young lineages, imprinted preferences may contribute to behavioral isolation and facilitate speciation. However, in most models of speciation by sexual selection, divergent natural selection is also required; it acts to generate and maintain variation in sexually-selected trait(s) and the mating preferences that act upon them. Here we demonstrate that imprinting, in addition to mediating female mate preferences, can also shape male-male aggression biases; these aggression biases can act similarly to natural selection in maintaining trait and preference variation, facilitating reproductive isolation driven entirely by sexual selection. Using a cross-fostering study, we show that both male and female strawberry poison frogs (*Oophaga pumilio*) imprint on coloration, a mating trait that has diverged recently and rapidly in this species. Cross-fostered females prefer to court mates of their foster mother's color, and cross-fostered males are more aggressive toward rivals of their foster mother's color. We also demonstrate with a simple population genetic model that when both male

aggression biases and female mate preferences are formed through parental imprinting, sexual selection alone can (i) stabilize a sympatric polymorphism and (ii) strengthen the trait-preference association leading to behavioral reproductive isolation. Our work constitutes the first evidence of imprinting in an amphibian, and together with our model, suggests that this rarely considered combination of rival and sexual imprinting can reduce gene flow between individuals bearing divergent mating traits, setting the stage for speciation by sexual selection.

5.2 Introduction

Sexual selection can drive rapid divergence in mating signals and preferences, which may then lead to behavioral isolation among phenotypic variants, thereby facilitating speciation (Ritchie, 2007). Although the potential for speciation-by-sexual-selection has long been acknowledged, theoretical work has identified two major challenges for this mechanism to occur when there is gene flow between incipient species: (i) the association between a genetic mating trait and a genetic preference can easily be broken down by recombination (Felsenstein, 1981), and (ii) assortative mating often degrades genetic variation in mating traits and preferences, eliminating the polymorphisms that provide the basis for future divergence (Arnegard & Kondrashov, 2004; van Doorn *et al.*, 2004). Sexual imprinting, the phenomenon whereby offspring learn parental phenotypes as the basis of their mate preference, presents a solution to the problem of recombination (Irwin & Price, 1999; Verzijden *et al.*, 2012). Because offspring inherit their mating trait from the parent(s) they imprint on, the trait-preference association reforms anew in each generation. The second challenge, achieving stable polymorphisms, can be resolved by incorporating divergent ecological selection that acts directly on mating traits (i.e., “magic traits”;

Servedio *et al.*, 2011; Servedio & Boughman, 2017) or mating preferences (e.g., sensory drive; Boughman, 2002). In these scenarios, however, natural selection is arguably a more important driver of speciation than sexual selection because of the former's contribution to the origin and maintenance of trait and preference variation.

Assortative male-male competition mediated by the same mating signal can also generate divergent selection through negative-frequency dependent selection (i.e., rare male advantage; Seehausen & Schluter, 2004), and may provide an alternative mechanism by which sexual selection, on its own, can stabilize a polymorphism (van Doorn *et al.*, 2004; Dijkstra & Border, 2018). In this scenario, the mating trait inherently becomes a magic trait, affecting both divergence and reproductive isolation via solely sexual selection, and without the need for a pleiotropic ecological effect. This mechanism may be widespread because sexually selected traits are often used for both mate choice and intrasexual aggression (Berglund *et al.*, 1996). Furthermore, "species recognition" (stronger behavioral responses toward conspecifics) often involves behavioral biases in both sexes (Grether *et al.*, 2017). Mathematical models (Mikami *et al.*, 2004; van Doorn *et al.*, 2004) that have incorporated male-male competition as the source of balancing selection have assumed that individuals are more competitive/aggressive toward their own phenotype. However, as has been demonstrated for mating biases (Verzijden *et al.*, 2012), the mechanisms that shape aggressive behavioral biases are diverse (e.g., genetic vs plastic; Hansen & Slagsvold, 2003; Verzijden *et al.*, 2008; Dijkstra & Border, 2018), and the evolutionary trajectories resulting from biases generated through these various mechanisms remain largely unexplored.

Here, we tested for imprinted behaviors in a species that shows evidence of recent, rapid divergence in a sexually selected trait on which both female mate preferences and male aggressive

biases act. The strawberry poison frog, *Oophaga pumilio,* exhibits extreme, heritable color polymorphism in and around the Bocas del Toro archipelago of Panama (Summers *et al.*, 2003; Dugas & Richards-Zawacki, 2015). While most color variation occurs among isolated island populations, there are a few areas of sympatric polymorphism (**Figure 1-1**; Summers *et al.*, 2003; Richards-Zawacki *et al.*, 2012; Yang *et al.*, 2016). The *O. pumilio* populations in this region have likely experienced periods of vicariance and reconnection due to the rise and fall of sea-levels; however, comparisons of neutral genotypic with phenotypic variation suggest a major role of selection in the rapid color divergence (Brown *et al.*, 2010). Despite evidence that color can be aposematic in this species (Saporito *et al.*, 2007), color variants appear to incur similar predation risk (Hegna *et al.*, 2013; Richards-Zawacki *et al.*, 2013; Yeager, 2015). This suggests that differential predation, the most obvious candidate for natural selection, may have played a relatively minor role in shaping coloration in *O. pumilio* compared to other poison frog species (Rojas, 2017). In contrast, sexual selection seems to be a strong driver of *O. pumilio* color evolution. Females, in general, prefer to court with their own color morph over novel color morphs (Maan & Cummings, 2008; Yang *et al.*, 2016). Males, although not studied as extensively, are also more aggressive toward their own color morph (Yang *et al.*, 2018). Because no post-zygotic incompatibilities appear to exist (Dugas & Richards-Zawacki, 2015), these divergent, color-biased sexual behaviors likely represent the most salient reproductive barrier among the color morphs.

In this study, we tested the hypothesis that imprinting shapes both female mate preferences ("sexual imprinting"; Irwin & Price, 1999; Verzijden *et al.*, 2012) and male aggression biases ("rival imprinting"; Hansen & Slagsvold, 2003) among three color morphs of *O. pumilio* using a rearing experiment. We tested for color biases in female mate preference and male-male aggression of lab-reared, socially naïve frogs that were either pure-bred (both parents of the same

color), cross-bred (each parent a different color), or cross-fostered (raised by foster parents of a different color than the biological parents). Upon finding empirical evidence for both sexual and rival imprinting, we further explored the evolutionary implications of these imprinted behaviors using a simple population genetic model in which imprinting shapes both female mate preferences and male aggression biases.

5.3 Methods

5.3.1 Rearing Experiment

5.3.1.1 Experiment Design

We reared socially naïve *O. pumilio* individuals from frogs in a breeding colony (Dugas & Richards-Zawacki, 2015), haphazardly selecting from three allopatric, differently colored O. pumilio lineages from Bocas del Toro, Panama: red (Tranquilo Bay, Isla Bastimentos: 9°15.080' N, 82°80.433' W), green (Punta Laurel, Isla Popa: 9°80.260' N, 82°70.391' W) and blue (Shark Hole, Tierra Oscura: 9°12.047' N, 82°12.049' W; **Figure 1-1**). The three lineages show divergence in neutral genetic markers (Wang & Shaffer, 2008; Wang & Summers, 2010), but no evidence of intrinsic post-zygotic isolation (Dugas & Richards-Zawacki, 2015).

The rearing experiment included three treatments: (i) pure-bred: offspring raised by their biological parents, both of whom were of the same color morph, (ii) cross-bred: offspring raised by their biological parents, each of whom were a different color morph, and (iii) cross-fostered: offspring raised by foster parents that were of a different color from the biological parents (**Figure 5-1A**). The biparental care exhibited by this species provides ample opportunity for tadpoles to

observe adult colors. Males tend their eggs while females transport tadpoles to waterholes on their backs and later feed their begging tadpoles with their own unfertilized eggs throughout larval development (Dugas, 2018). Offspring were removed from rearing enclosures within 24 h of reaching Gosner stage 46 (complete metamorphosis; Gosner, 1960) and transferred to a separate enclosure, physically and visually isolated from other frogs in the colony until the behavioral assay, which was conducted after the individual had reached sexual maturity (10 – 12 months after metamorphosis). We tested for color-based behavioral biases in the male and female offspring using a two-way choice test (**Figure 5-1B**; see below) among males of contrasting colors (pure-breeding: own vs another color; cross-breeding: mother's vs father's color; cross-fostering: biological vs foster color).

We used a total of 42 males and 40 females, out of which we matched up a total of 49 unique pairs during the course of the rearing experiment. Each pair produced 1-3 of the focal individuals used in our behavioral assays (not including individuals that died during the larval period or before sexual maturity). The csv file containing parent IDs of each individual is archived at https://doi.org/10.6084/m9.figshare.9628406. Sample sizes were: pure-bred male = 11 (blue = 3; green = 7; red = 1); pure-bred female = 12 (blue = 5; green = 6; red = 1); cross-bred male = 16 (blue mother x green father = 2; green mother x red father = 14); cross-bred female = 19 (blue mother x green father = 9; green mother x red father = 10); cross-fostered male = 7 (biologically blue, green fostered = 4; biologically blue, red fostered = 2; biologically green, blue fostered = 1); cross-fostered female = 7 (biologically blue, green fostered = 1; biologically blue, red fostered = 2; biologically green, blue fostered = 2; biologically green, red fostered = 1; biologically red, blue fostered = 1).

5.3.1.2 Cross-fostering Protocol

Following successful courtship in nature, female *O. pumilio* lay terrestrial eggs in the male's territory (Pröhl & Hödl, 1999). Males tend and moisten the clutches; upon hatching, the female transports each tadpole to a separate water-filled leaf axil (Weygoldt, 1980; Dugas, 2018). Throughout larval development, tadpoles are fully dependent upon trophic eggs provided by their mother. Females visit their tadpoles every 1-8 days for ~6 weeks (Brust, 1993; Dugas, 2018). During these visits, the mother submerges her body in the water inside the leaf axil and her tadpole vibrates its body against her body to beg for egg provisioning (Stynoski & Noble, 2012; Dugas, 2018). In the colony, the breeding pairs were housed in plastic enclosures (37 x 22 x 25cm) that each contained four artificial leaf axils (water-filled PVC tubes) for tadpole rearing. Other animal housing and care details follow Dugas & Richards-Zawacki (2015). Breeding enclosures were checked daily for newly deposited tadpoles. Cross-fostering was accomplished by swapping PVC tubes, and the newly-hatched tadpoles they contained, among enclosures within 24h post-deposit. Cross-fostering was possible since females recognize tadpole rearing sites using location cues, but not tadpole identity (Stynoski, 2009).

While we acknowledge that a treatment group where tadpoles were swapped among breeding pairs of the same color morph would have made a more effective control than our pure-bred treatment, the low odds of rearing a swapped tadpole to adulthood dissuaded us from including this type of control in our experiment. To minimize the potential for imprinting on the biological parents, we swapped tadpoles among tanks within 24h of them being deposited in their water holes. Therefore, we could only swap tadpoles when two breeding pairs had newly hatched tadpoles on the same day. Knowing that the mortality rate between hatching and metamorphosis was near 80% (Dugas & Richards-Zawacki, 2016) and given that we had a limited number of

breeding pairs to work with, we prioritized swapping tadpoles between different color morphs when the opportunity for cross-fostering arose.

5.3.1.3 Behavioral Assay

Two-way choice experiments were carried out to assess the behavior of offspring toward males of different phenotypes: the focal individual could move freely in an arena, and the two stimulus males were confined under transparent, plastic domes (**Figure 5-1B**). Unrelated adult males were used as stimulus males, and stimulus male pairs were matched for size and mass but differed in color. The experimental arena was a plastic container (30 x 40 x 20 cm) that was opaque (white) on the sides and bottom, and covered on top with plastic mesh to allow behavioral observations from above. All observations were conducted in a dark room, with illumination that mimics forest floor lighting condition (*sensu* Maan & Cummings, 2008). The specific experimental setup and protocol fully followed Yang *et al.* (2016) except (i) the arena size was different, and (ii) the focal individuals were given a choice between two instead of three stimulus males.

For both female preference and male aggression assays, we quantified (i) association time, defined as the cumulative time the focal frog spent in each of the 4 cm (~2 body lengths) interaction zones surrounding each male's dome, and (ii) approaches, defined as the number of times the focal frog oriented toward and entered each interaction zone. In the wild, aggressive male *O. pumilio* perform the following behaviors, typically in this order, as contest escalates (Yang et al., 2018): i) 'track', orienting toward and facing an intruder; ii) 'approach', moving toward the intruder; iii) 'call', typically accompanied an approach, but could occur any time; iv) 'challenge' if a male continued an approach onto the intruder's perch; and v) 'attack', physically wrestling with the intruder. For male aggression assays, because the focal individual and the stimulus males were

separated by the transparent plastic dome, the most aggressive behavior the focal males could perform was to approach the confined stimulus males as close as possible. Therefore, we similarly used association time (in the 4 cm interaction zone) and number of approaches to assess male aggression. We did not use calling as a metric because it was often difficult to distinguish which stimulus male the focal male was calling to when the focal male was not in the interaction zone.

5.3.1.4 Statistical Analyses

To test for female mate preference and male aggression biases, we calculated the proportions of the total association time male and female offspring spent with one of the two male phenotypes presented to them (pure-bred: own/(own + other); cross-bred: mother/(mother + father); cross-foster: foster/(foster + biological)). We tested the hypothesis that these association time proportions were > 0.5 for the three rearing treatments and two sexes separately with one-tailed, one-sample permutational t-tests. We also ran the same analyses with ratios of the number of approaches to each stimulus phenotype as a second, supplementary confirmation of the pattern. All analyses mentioned in the main text were performed in R 3.6.2 (R Core Team, 2019). We used the 'perm.test' function in the jmuOutlier package (Garren, 2018). Effect sizes (Cohens' D) were calculated using the 'cohensD' function in the lsr package (Navarro, 2015).

5.3.2 Population Genetic Model

5.3.2.1 Design

We built a simple population genetic model to explore the possible evolutionary trajectories driven by imprinted female mate preferences and male aggression biases. In nature, the genetic architecture of polymorphic mating traits appears highly variable, ranging from simple

Mendelian to highly polygenic, but a commonly observed pattern is one in which a single mutated allele leads to a drastic phenotypic change (e.g. MC1R and animal melanic coloration; Hoekstra *et al.*, 2006; Uy *et al.*, 2009). While studies of the genetic architecture of coloration in *O. pumilio* are still in their infancy, there are examples suggesting both polygenic (Dugas & Richards-Zawacki, 2015; Yang *et al.*, 2018) and simple Mendelian inheritance of color (Richards-Zawacki *et al.*, 2012) (**Figure 1-1**). As an initial step, we used a diploid model that incorporates a mating trait governed by a diallelic Mendelian locus (with dominant and recessive alleles) on which both female mate preferences and male aggression biases act. We defined preference strength α and aggression bias strength β such that upon encounter, females are $1+\alpha$ times as likely to mate with the imprinted phenotype than with the alternative phenotype, and males are $1+\beta$ times as aggressive toward the imprinted phenotype in male-male competition. In our model, both female preferences and male aggression biases are learned by imprinting on a parent's mating trait, as we found evidence for in *O. pumilio*.

5.3.2.2 Model

We developed a diploid model with discrete, non-overlapping generations. The mating trait is governed by a single diallelic locus T with a dominant allele (T_1) and a recessive allele (T_2). We also used a behavior "locus" P (actually a phenotype) that denotes which trait the individuals have imprinted on. P is inherited via maternal or paternal imprinting, and governs both female mate preference and male aggression biases, being either P_1 (biased toward trait 1) or P_2 (biased toward trait 2). The population is therefore described by "phenogenotypes" that contain a diploid mating trait locus T and a haploid behavior "locus" P. The frequencies of the six phenogenotypes $T_{11}P_1$, $T_{12}P_1$, $T_{22}P_1$, $T_{11}P_2$, $T_{12}P_2$ and $T_{22}P_2$ are designated x_1, x_2, x_3, x_4, x_5 and x_6, respectively.

The life cycle consists of male-male competition, female mate choice, reproduction and imprinting. During male-male competition, males are $1+\beta_k$ times as aggressive toward the imprinted mating trait phenotype *k*. The total aggression received by mating phenotype 1, for example, is $A_1 = (1 + \beta_1)\, p_1 + p_2$, where p_1 and p_2 represent the frequencies of trait 1- and trait 2-biased males, respectively ($p_1 = x_1 + x_2 + x_3$, $p_2 = x_4 + x_5 + x_6$). We assumed that males that receive more aggression from other males are less likely to establish a territory and thereby have reduced reproductive success (Grether *et al.*, 2017), and calculated the effective male genotype frequencies that enter the mating pool, from which the females choose their mates. The fitness (ω) of males with mating phenotype *k* decreases as the total aggression received increases, such that $\omega_k = 1 - s_k$, where $s_k = A_k / \sum_k A_k$. Because the behavioral bias of males is not relevant in the portion of the life cycle that comes after male-male competition, we pool P_1 and P_2 males and calculate only the three male genotype frequencies: T_{11}, T_{12} and T_{22} males are designated $x_{1,m}$, $x_{2,m}$, $x_{3,m}$, where subscript *m* denotes males. The effective frequency of males with mating genotype *i* that enter the mating pool after male competition is therefore:

$$x^*_{i,m} = \frac{\omega_k x_{i,m}}{\sum_i \omega_k x_{i,m}},$$

where $k = 1$ when $i = 1$ or 2, and $k = 2$ when $i = 3$. There is no competition between females (denoted by subscript *f*) in this model, so the female phenogenotype frequency that enters the mating pool is $x^*_{j,f} = x_{j,f}$, where the six female phenogenotypes $T_{11}P_1$, $T_{12}P_1$, $T_{22}P_1$, $T_{11}P_2$, $T_{12}P_2$ and $T_{22}P_2$ are designated $x_{1,f}$, $x_{2,f}$, $x_{3,f}$, $x_{4,f}$, $x_{5,f}$ and $x_{6,f}$, respectively.

After male competition, the females choose their mates according to the behavior "locus" P, such that upon encounter, females are $1+\alpha_k$ times as likely to mate with males possessing the

imprinted mating phenotype k (following ref (Kirkpatrick, 1982)). Thus, the frequency of mating between each combination of male genotype i and female phenogenotype j is:

$$F_{i,j} = \frac{x^*_{i,m} x^*_{j,f} (1+d_{i,j}\alpha_k)}{\sum_i x^*_{i,m} (1+d_{i,j}\alpha_k)},$$

where $d_{i,j} = 1$ if the female behavior locus P matches the male trait phenotype T ($i = 1$ or 2 with $j = 1$, 2 or 3, and $i = 3$ with $j = 4$, 5 or 6) and $d_{i,j} = 0$ otherwise, and where $k = 1$ when $j = 1$, 2 or 3, and $k = 2$ when $j = 4$, 5, or 6. The denominator normalizes the frequencies to ensure females have equal mating success (i.e. strict polygyny).

Reproduction and imprinting happen after mating, where the phenogenotype frequencies of the resulting zygotes are calculated. The mating trait locus T is genetically inherited with Mendelian segregation. The phenotypic "locus" P is obtained by either maternal or paternal imprinting. All offspring with a trait 1 (T_{11} or T_{12}) parent (mother for maternal printing; father for paternal printing) are P_1 individuals, and all offspring with a trait 2 (T_{22}) parent are P_2 individuals.

5.3.2.3 Analysis

Details of the recursion equations and numerical analyses are described in **Appendix B**. The recursion equations were not solvable analytically, and were analyzed by estimating numerical solutions using Mathematica (Wolfram Research, 2018) and using deterministic simulations. We considered two conditions important for assessing progress toward the evolution of reproductive isolation (Verzijden *et al.*, 2005): (i) whether the polymorphic equilibrium of mating trait T is stable (**Figure 5-2A**), and (ii) whether the mating traits (T) and the behaviors (P) were associated (i.e., in phenogenotypic linkage disequilibrium) at the polymorphic equilibrium (**Figure 5-2B**). We also assessed the extent to which the potential for achieving a stable polymorphic state

depended upon starting allele frequencies (**Appendix Figure 5**). Our basic model (presented in the main text) assumed symmetrical selective strengths on both mating phenotypes (i.e. $\alpha_1 = \alpha_2$ and $\beta_1 = \beta_2$). We also analyzed scenarios in which the strength of the behavioral biases differs between the P_1 and P_2 individuals (i.e. $\alpha_1 \neq \alpha_2$ and $\beta_1 \neq \beta_2$; presented in supporting information). The asymmetries allow the polymorphic equilibrium to stabilize at a wide range of phenotype frequencies (from 0 to 1), but the qualitative conclusions reported above regarding polymorphism stability and the evolution of linkage disequilibrium between the mating trait and the behaviors were robust **(Appendix B.2.2, Appendix Figure 3 & Appendix Figure 4)**. All analyses were performed using Mathematica (Wolfram Research, 2018).

5.4 Results and Discussion

5.4.1 Rearing Experiment

Overall, males and females showed similar behavioral patterns (courtship in females and rival aggression in males) in all three treatments (**Figure 5-1C & D**, **Table 5-1 & Table 5-2**). Pure-bred offspring biased their interactions toward males of their own color over another color, establishing that lab-reared frogs exhibit similar color-biased behaviors to wild *O. pumilio*. Cross-fostered offspring showed a bias toward the foster parents' color over their biological parents' color, suggesting that imprinting is more influential than genetics in shaping these color-mediated behaviors. Additionally, Cross-bred offspring biased their interactions toward males of their mother's color over their father's, suggesting that interaction with the mother is more influential than interaction with the father. We hypothesize that *O. pumilio* learn their mother's coloration

during the tadpole stage, and use it as a template for mate preference and rival aggression biases in adulthood. Our results suggest that maternal imprinting may be the key mechanism mediating the color-assortative behaviors seen among recently diverged *O. pumilio* color morphs.

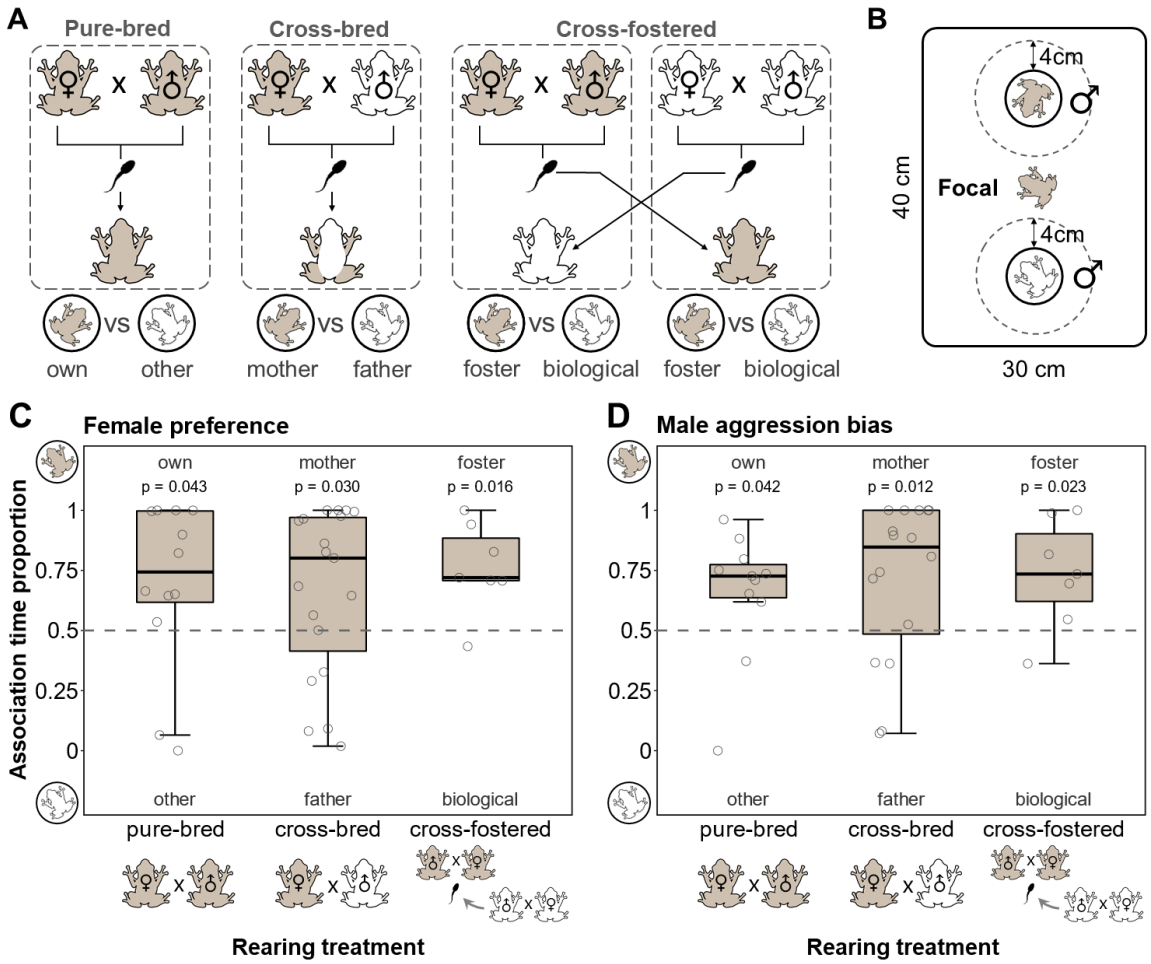


Figure 5-1 *O. pumilio* rearing experiment

A: Experimental design. Pure-bred = offspring raised by their biological parents, both of whom were of the same color morph. Cross-bred = offspring raised by their biological parents, each of whom were a different color morph. Cross-fostered = offspring raised by foster parents (a different color morph than the biological parents). **B**: Experimental apparatus used in behavioral assays. During behavioral observations, the two stimulus males were confined under clear plastic domes, and the focal individual could move freely in the arena. **C-D**: The proportions of the total time female (**C**) and male (**D**) offspring spent with one of the two stimulus male phenotypes during the behavioral assay (pure-bred: own/(own + other); cross-bred: mother/(mother + father); cross-foster: foster/(foster + biological)). Values above the dashed line (y = 0.5) indicate a preference/aggression bias consistent with maternal imprinting. P-values were based on one-sided, one-sample permutational t-tests (see **Table 5-1**). Bold lines indicate medians. Boxes enclose 25th – 75th percentiles. Error bars enclose data range (excluding outliers). Dots are data points; those vertically outside the error bars are outliers (below Q1 – 1.5 × IQR or above Q3 + 1.5 × IQR). Sample sizes (number of focal animals) were: pure-bred male = 11; pure-bred female = 12; cross-bred male = 16; cross-bred female = 19; cross-fostered male = 7; cross-fostered female = 7.

Table 5-1 Proportional association time for the three groups of rearing treatment

One-sided one-sample permutational t-tests testing the association time ratios against 1 separately in the three rearing treatments and two sexes. A summarized version that shows only p values is incorporated into **Figure 5-1C & D**.

		Female				Male			
	Ratio of time spent with	N	Median ± IQR	ES*	*p*	N	Median ± IQR	ES	*p*
Pure-bred	Biological's color	12	3.28 ± 240.9	0.60	0.011	11	2.65 ± 1.66	0.53	0.008
Cross-bred	Mother's color	19	4.01 ± 31.67	0.41	0.002	16	5.94 ± 297.0	0.58	0.002
Cross-fostered	Foster's color	7	2.57 ± 7.79	0.40	0.031	7	2.75 ± 34.17	0.41	0.047

*ES: effect size, Cohens' D

Table 5-2 Proportional approaches for the three groups of rearing treatment

One-sided one-sample permutational t-tests testing the proportions of approaches against 0.5 separately, in the three rearing treatments and two sexes

		Female				Male			
	Ratio of approaches toward	N	Median ± IQR	ES*	*p*	N	Median ± IQR	ES	*p*
Pure-bred	Biological's color	12	2.48 ± 52.2	0.54	0.020	11	1.99 ±0.50	0.94	0.031
Cross-bred	Mother's color	19	1.00 ± 0.99	0.37	0.026	16	2.49 ± 150.1	0.54	0.004
Cross-fostered	Foster's color	7	2.98 ± 2.72	0.39	0.063	7	1.99 ± 2.72	0.39	0.047

*ES: effect size, Cohens' D

5.4.2 Population Genetic Model

We further explored the evolutionary implications of these imprinted behaviors using a simple population genetic model in which imprinting shapes both female mate preferences and male aggression biases. We compared results from two versions of the model that differ only in whether imprinting was on the mother's or the father's mating trait. The two yield qualitatively similar conclusions. We were interested in identifying the conditions under which both mating trait phenotypes coexist stably in our model. We found that the stability of such a polymorphism

is dependent upon the relative strengths of the female mate preference (α) and male aggression bias (β) (**Figure 5-2A**). While initial allele frequencies affect the maintenance of trait variation, an initially rare trait allele (frequency = 0.0001) can increase in frequency to reach stable polymorphism across a wide range of preference and aggression strengths (**Appendix Figure 5**). Incomplete imprinting (i.e., when not every individual imprints successfully on the parent's phenotype), on the other hand, will linearly decrease the realized strength of both α and β, resulting in a diagonal shift toward the origin in **Figure 5-2A** (See **Appendix B.4**). Mechanistically, mating trait frequencies, through sexual imprinting, determine the behavior frequencies (both female mate preferences and male aggression biases) of the offspring, which then serve as the source of sexual selection acting on the mating trait in the next generation. Therefore, selection generated by both female preference and male aggression is frequency-dependent, delayed by one generation. Consistent with previous studies (Verzijden *et al.*, 2005; Servedio & Boughman, 2017), imprinted female mate preference exerts positive-frequency dependent selection that favors the more common mating trait allele and would, on its own, drive that allele to fixation. Male aggression bias, in contrast, generates negative-frequency dependent selection that counters the pull toward fixation(van Doorn *et al.*, 2004), generating a stable polymorphism in the mating trait when the aggression bias is sufficiently strong (i.e., tan area of **Figure 5-2A**). In this basic model, the mating trait phenotype frequency always stabilizes at 0.5; however, when we extend the model to allow asymmetry in the strengths of selection (i.e., when the value of α and β varies with the phenotype that the individual imprinted on), the trait polymorphism can stabilize at broad range of frequencies (see **Appendix B.2.2**, **Appendix Figure 3 & Appendix Figure 4**).

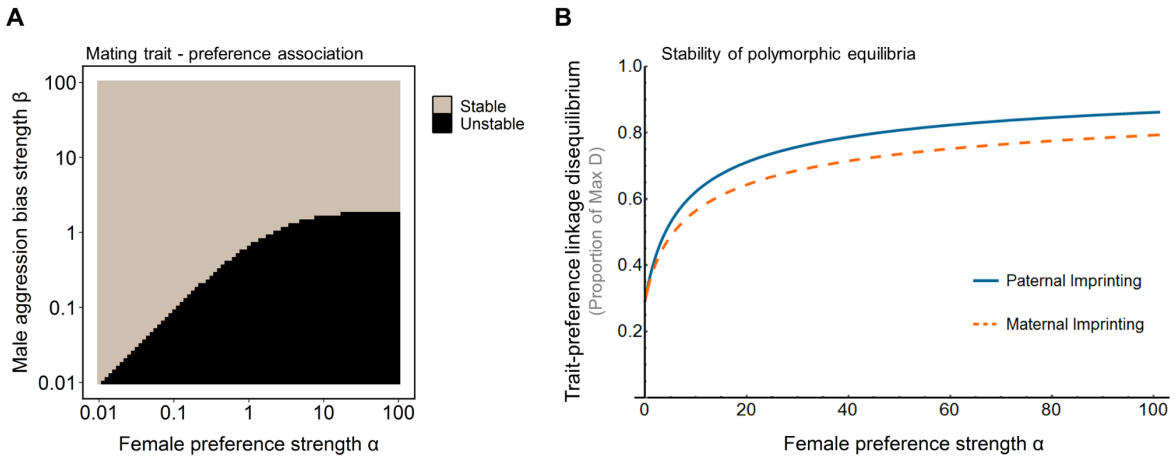


Figure 5-2 Polymorphism stability and trait-preference association

A: Stability of polymorphic equilibria for combinations of female preference strength α and male aggression bias strength β from 0.01 to 100, with a step size of 100.05. Axes are on log scales. Maternal and paternal imprinting models yield visually identical results. Across the range of parameters tested, the strength of the male aggression β bias required to maintain a stable polymorphism increases nonlinearly with female preference strength α. **B**: Association between mating trait locus T and female mate preference "locus" P (measured as trait-preference phenogenotypic linkage disequilibrium, presented as proportion of maximum D) at the polymorphic equilibrium (trait 1 phenotype frequency = 0.5) increases with female preference strength α. Note that the stability of such a polymorphic equilibrium depends on the relative strength of α and β (see **A**). Trait-preference linkage disequilibrium, expressed in terms of α (it is independent of β), are described in **Appendix B.2.1**.

We also evaluated the association between the mating trait and mate preference at the polymorphic equilibrium. Divergent mating and aggressive behaviors will not generate two completely isolated mating groups unless female mate preference is absolute (i.e., females never mate with the non-preferred phenotype, $\alpha = \infty$). Instead, we looked for parameter space where a positive trait-behavior association was formed, as this indicates reduced gene flow between the two trait variants, which could set the stage for speciation(Felsenstein, 1981). We found that formation of this positive trait-behavior association (calculated as phenogenotypic linkage disequilibrium) is independent of aggression bias strength β and increases with preference strength α (**Figure 5-2B**). However, as the preference strength α increases, the minimum aggression bias strength β required for a stable polymorphism also increases (**Figure 5-2A**). Consequently, pre-

mating behavioral isolation is most likely to evolve via this mechanism when female preference is strong, but cannot do so without being accompanied by male aggression bias that is strong enough to maintain a stable polymorphism during the process. Along with simulations that explore the robustness of these findings to other initial trait frequencies, our model suggests that in the rapidly diverging *O. pumilio*, maternally imprinted sexual behaviors may begin to generate reproductive isolation when allopatric color morphs come into secondary contact (as has likely occurred many times due to sea-level change), when parapatric populations contribute new trait variants by one-time migration, or even among color morphs that arise in sympatry as long as sexual selection is strong.

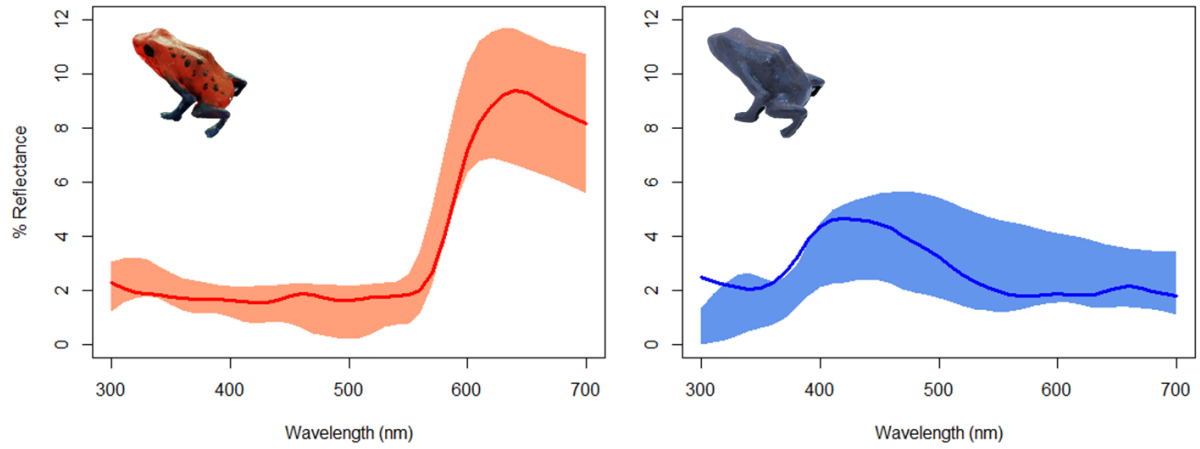
5.5 Conclusion

Sexual selection is likely to facilitate speciation when divergent traits are accompanied by divergent sexual behaviors. We provide empirical evidence that suggests that maternal sexual imprinting on a heritable, polymorphic mating trait mediates both female mate choice and male-male competition in a poison frog. We also demonstrate using a mathematical model that imprinted aggression biases toward rival males can act in concert with sexually imprinted female mate preferences to maintain a stable polymorphism and reduce gene flow between divergent mating phenotypes in sympatry. Thus, parental imprinting provides a plausible and effective mechanism by which a sexually-selected trait and the behaviors that act on it may co-diverge as a result of sexual selection alone, reducing gene flow between sympatric lineages and setting the stage for speciation.

Appendix A Supplemental Information for Chapter 3

Appendix A.1 Visual and Acoustic Properties of the Simulated Intrusion

Appendix A.1.1 Intruder Models



Appendix Figure 1 Visual properties of the intruder models

Photographs and the reflectance spectra of the hand-painted red and blue plastic models used as simulated intruders. Solid lines show the reflectance of models, and shaded areas that enclose them show the range between the brightest and dullest males from a sample of 10 males of each morph in the contact zone.

Appendix A.1.2 Acoustic Playback

The spectral and temporal parameters of male advertisement calls do not differ significantly across the red-blue contact zone (Pröhl *et al.*, 2007; M. L. Dye, *unpublished data*), so we used the same call playback for trials in all four populations. We used a recorded call from a Dolphin Bay red male that had parameters within the ranges for all focal populations (dominant

frequency 5038.5 Hz, call rate: 0.009, call duration: 40 seconds, between-calls interval: 12 seconds). The sound pressure level of playbacks was adjusted to fit the value of a calling male (~ 61 dB at 60 cm from the source, measured by Velleman AVM2050; Crothers & Cummings, 2015).

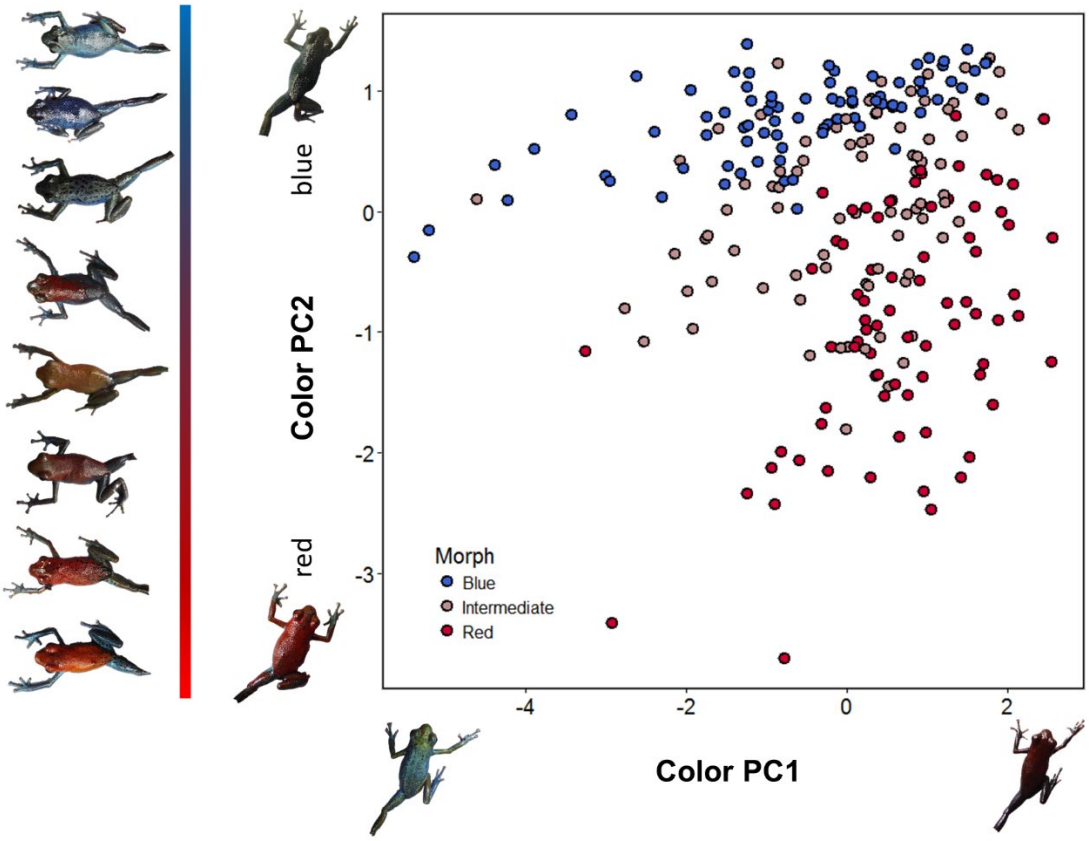
Appendix A.2 Quantitative Photograph Color Scores

PC1 captures the brightness (but much higher green and blue loadings) of the male dorsum; PC2 captures hue, or how blue the male was along the red-blue spectrum (**Appendix Table 1**). When grouped by eye, red, intermediate and blue morphs differ significantly in both PC1 (ANOVA, $F_{2, 241} = 20.95$, $p < .001$) and PC2 (ANOVA, $F_{2, 241} = 114.33$, $p < .001$). PC1 was highest in red males, lower in intermediate males, and lowest in blue males (Tukey *post hoc*, red – intermediate: $p = 0.005$, red-blue: $p < .001$, intermediate – blue: $p = 0.002$). PC2 was highest in blue males, lower in intermediate males, and lowest in red males (Tukey *post hoc*, red – intermediate: $p < .001$, red-blue: $p < .001$, intermediate – blue: $p < .001$).

Appendix Table 1 PCA for quantantative color scores

Results of a principal components analysis of mean red, green, and blue color scores (RGB values) from the dorsum of *O. pumilio* from polymorphic populations in Bocas del Toro, Panama. Color was measured in and averaged between five 20 × 20 pixel areas on the dorsum using imageJ (Dugas *et al.*, 2015).

	Eigenvalue	Variance Explained	PC loadings		
			Red	*Green*	*Blue*
PC1	1.98	65.9%	-0.254	-0.704	-0.664
PC2	0.98	32.7%	-0.941	0.022	0.336



Appendix Figure 2 By-eye color categories plotted on quantitative color space

Appendix A.3 Sample Size Breakdown

Appendix Table 2 Sample size breakdown for the simulated intrusion experiment

Responsive males of each male color/model intruder color combination in the four populations.

	red monomorphic	**high-red polymorphic**			**high-blue polymorphic**			**blue monomorphic**
intruder color	Red	red	int	blue	red	int	blue	blue
red	21	25	24	23	21	23	24	22
blue	27	20	25	23	20	25	26	23

Appendix A.4 Analyses Excluding Samples with Conspecific Interactions

In **3.4**, we included conspecific interactions (y/n) as a fixed effect in the statistical models that included our observations of all territorial males, as these activities lowered the potential time a male could interact with the simulated intruder. Here, we provide an alternative approach to addressing focal male interaction with conspecifics during trials: we re-ran all models presented in **3.4** with a dataset restricted to observations in which the focal male did not interact with conspecifics during the trial (original n = 372, in these analyses n = 251).

Appendix A.4.1 Does Male Aggression Pattern Vary with Population Morph Frequency?

As was the case in the full dataset, there was no significant population × intruder color interaction in the likelihood that a focal territorial male (i) tracked, (ii) approached, (iii) called, or (iv) challenged the model intruder (**Appendix Table 3**). Also consistent with patterns in the full dataset, we found a significant population × intruder color interaction with respect to the likelihood that a focal male attacked the simulated intruder (**Appendix Table 3**).

Appendix Table 3 GLMs: male agonistic behaviors

Generalized linear models estimating the likelihood of a territorial male to (i) track, (ii) approach, (iii) call at, (iv) challenge and (v) attack the intruder model. Re-run from **Table 3-1** using a dataset that excluded all trials with conspecific interactions.

		Track		**Approach**		**Call**		**Challenge**		**Attack**	
Parameters	df	LR $\chi2$	*p*	LR $\chi2$	*p*	LR $\chi2$	*p*	LR $\chi2$	*p*	LR $\chi2$	*p*
Population	3	3.80	0.284	1.03	0.792	1.53	0.675	6.62	0.085	9.98	0.019
intruder color	1	0.04	0.849	0.48	0.489	1.50	0.221	0.47	0.494	4.51	0.034
population × intruder color	3	0.53	0.911	1.50	0.681	1.17	0.759	5.37	0.146	14.62	**0.002**
perch height	1	0.12	0.912	0.09	0.771	0.02	0.895	3.26	0.071	0.10	0.749

Bonferroni correction: alpha level is set to 0.01

In the two monomorphic populations, the reduced data set with only observations in which conspecific interactions did not occur revealed similar patterns as the full dataset: Focal males in the monomorphic red population were more likely to attack a red (10/15) than a blue (3/20) model intruder (GLM, $LR\chi^2 = 7.60$, df = 1, $p = 0.006$), and males in the monomorphic blue population were more likely to attack a blue (11/16) than a red (5/15) model intruder (GLM, $LR\chi^2 = 3.99$, df = 1, $p = 0.046$). In the full dataset presented in Results, we found that males in the two polymorphic populations were equally likely to attack red or blue simulated intruders. However, in this reduced dataset, males in the high-red polymorphic population were more likely to attack a blue (21/37) than a red (16/48) model intruder ($LR\chi^2 = 4.81$, df = 1, $p = 0.028$), and in the high-blue polymorphic population, this pattern was similar but non-significant (red model: 27/50; blue model: 17/50, $LR\chi^2 = 3.60$, df = 1, $p = 0.058$). In none of these four follow up models did perch height have a significant effect (GLM, all $LR\chi^2 < 1.48$, all $p > 0.223$).

Similar to the full dataset results, there was no effect of population, intruder color or the interaction term on the number of attacks (GLM, population: $LR\chi^2 = 1.15$, df = 3, $p = 0.764$; intruder color: $LR\chi^2 = 1.17$, df = 1, $p = 0.279$; interaction term: $LR\chi^2 = 1.33$, df = 3, $p = 0.722$). Perch height was non-significant ($LR\chi^2 = 0.23$, df = 1, $p = 0.635$) in this model.

Appendix A.4.2 Do Males Bias Aggression toward Intruders of Their Own Color in the Polymorphic Populations?

In the high-red polymorphic population, patterns in the reduced dataset were similar to those in the full dataset. Neither the main effects of male color and intruder color nor their interaction were a significant predictor of the probability of attack, regardless of whether we treated color as categorical or used PC scores (**Appendix Table 4 & Appendix Table 5**). In the

high-blue population, we found no significant effects when using all observations, and similarly found that there was no significant interaction effect between focal male and simulated intruder color (the key prediction of the "own-type" hypothesis) in this reduced data set. However, in the reduced data set, we found that males in the high-blue polymorphic population were more likely to attack a blue model intruder (**Appendix Table 4**). The intruder color effect was non-significant when we re-ran the model with color PC scores, but as was the case with the full dataset, the likelihood of attack was positively associated with focal male PC2 (**Appendix Table 5**).

Appendix Table 4 GLM: likelihood of attacking in the two polymorphic populations (excluding trials with conspecific interactions)

Generalized linear models of the likelihood of attack in the two polymorphic populations, re-ran from **Table 3-2** using a dataset that excluded all trials with conspecific interactions.

Attack (y/n)		**High-red polymorphic**		**High-blue polymorphic**	
Parameters	df	LR $\chi 2$	*p*	LR $\chi 2$	*p*
male color	2	3.67	0.159	1.16	0.560
intruder color	1	0.21	0.650	4.98	**0.026**
male × intruder color	2	1.66	0.436	2.03	0.362
perch height	1	1.02	0.312	1.41	0.235

Appendix Table 5 GLMs: likelihood of attacking in the two polymorphic populations (color scores, excluding trials with conspecific interactions)

Generalized linear models of the likelihood of attack in the two polymorphic populations, re-run from **Appendix Table 6** using a dataset that excluded all trials with conspecific interactions.

Attack (y/n)		**High-red polymorphic**		**High-blue polymorphic**	
Parameters	df	LR $\chi 2$	*p*	LR $\chi 2$	*p*
PC1	1	0.32	0.571	1.50	0.221
PC2	1	0.87	0.352	8.85	**0.003**
intruder color	1	3.62	0.057	2.29	0.065
PC1 × intruder color	1	0.52	0.471	0.18	0.668
PC2 × intruder color	1	0.45	0.504	0.30	0.581
perch height	1	2.07	0.150	3.96	**0.047**

Appendix A.5 Supplemental Results

Appendix Table 6 GLM: likelihood of attacking in the two polymorphic populations (color scores)

Generalized linear models of the likelihood of attack in the two polymorphic populations, re-run from **Table 3-2**, replacing by-eye color groups (red, intermediate and blue) with color score PC1 and PC2.

Attack (y/n)		**High-red polymorphic**		**High-blue polymorphic**	
Parameters	df	LR $\chi 2$	*p*	LR $\chi 2$	*p*
PC1	1	0.11	0.739	0.45	0.505
PC2	1	0.35	0.554	6.49	**0.011**
intruder color	1	0.94	0.332	0.81	0.369
PC1 × intruder color	1	0.32	0.571	0.10	0.752
PC2 × intruder color	1	0.00	0.995	0.87	0.351
conspecific interaction	1	6.90	**0.009**	0.76	0.383
perch height	1	1.33	0.249	7.37	**0.007**

The four tables below are generalized linear models evaluating the influence of male color (red, intermediate and blue), model intruder color (red, blue) and their interaction term on the likelihood of a territorial male to track (**Appendix Table 7**), approach (**Appendix Table 8**), call (**Appendix Table 9**) and challenge (**Appendix Table 10**) in the two polymorphic populations. Perch height and conspecific interaction (y/n) were included as covariates.

Appendix Table 7 GLMs: likelihood of tracking

Track (y/n)		**High-red polymorphic**		**High-blue polymorphic**	
Parameters	df	LR $\chi 2$	*p*	LR $\chi 2$	*p*
male color	2	2.10	0.351	4.25	0.119
intruder color	1	1.87	0.171	0.60	0.436
male × intruder color	2	4.17	0.124	0.05	0.977
conspecific interactions	1	1.55	0.213	3.58	0.058
perch height	1	1.89	0.169	8.60	**0.003**

Bonferroni correction: alpha level is set to 0.01

Appendix Table 8 GLMs: likelihood of approaching

Appraoch (y/n)		**High-red polymorphic**		**High-blue polymorphic**	
Parameters	df	LR $\chi 2$	*p*	LR $\chi 2$	*p*
male color	2	2.77	0.250	1.77	0.412
model color	1	0.01	0.929	0.004	0.952
male × intruder color	2	1.71	0.424	0.26	0.878
conspecific interactions	1	2.52	0.113	0.87	0.351
perch height	1	0.47	0.491	4.92	0.027

Bonferroni correction: alpha level is set to 0.01

Appendix Table 9 GLMs: likelihood of calling

Call (y/n)		**High-red polymorphic**		**High-blue polymorphic**	
Parameters	df	LR $\chi 2$	*p*	LR $\chi 2$	*p*
male color	2	4.16	0.125	0.61	0.736
intruder color	1	0.16	0.687	0.26	0.610
male × intruder color	2	2.80	0.247	2.84	0.242
conspecific interactions	1	0.92	0.338	1.43	0.231
perch height	1	4.12	0.042	11.55	**<.001**

Bonferroni correction: alpha level is set to 0.01

Appendix Table 10 GLMs: likelihood of challenging

Challenge (y/n)		**High-red polymorphic**		**High-blue polymorphic**	
Parameters	df	LR $\chi 2$	*p*	LR $\chi 2$	*p*
male color	2	2.33	0.312	3.16	0.205
intruder color	1	0.09	0.758	0.03	0.858
male × intruder color	2	1.67	0.432	1.10	0.578
conspecific interactions	1	5.95	0.015	5.71	0.017
perch height	1	0.72	0.395	0.33	0.565

Bonferroni correction: alpha level is set to 0.01

Appendix B Supplemental Information for Chapter 5

Appendix B.1 Recursion Equations

The following recursion equations are based on the mating frequency table F described in **5.3.2.2**. Note that the recursion equation of x_4 ($T_{11}P_2$) always equals zero because a T_{11} offspring can only have phenotype 1 parents (T_{11} or T_{12}) and therefore it is impossible for these offspring to acquire P_2 via imprinting.

Maternal Imprinting

$x_1(t+1) = F_{1,1} + ½ F_{2,1} + F_{4,1} + ½ F_{5,1} + ½ F_{1,2} + ¼ F_{2,2} + ½ F_{4,2} + ¼ F_{5,2}$

$x_2(t+1) = ½ F_{2,1} + ½ F_{5,1} + ½ F_{1,2} + ½ F_{2,2} + ½ F_{4,2} + ½ F_{5,2} + F_{1,3} + ½ F_{2,3} + F_{4,3} + ½ F_{5,3}$

$x_3(t+1) = ¼ F_{2,2} + ¼ F_{5,2} + ½ F_{2,3} + ½ F_{5,3}$

$x_4(t+1) = 0$

$x_5(t+1) = F_{3,1} + F_{6,1} + ½ F_{3,2} + ½ F_{6,2}$

$x_6(t+1) = ½ F_{3,2} + ½ F_{6,2} + F_{3,3} + F_{6,3}$

Paternal Imprinting

$x_1(t+1) = F_{1,1} + ½ F_{2,1} + F_{4,1} + ½ F_{5,1} + ½ F_{1,2} + ¼ F_{2,2} + ½ F_{4,2} + ¼ F_{5,2}$

$x_2(t+1) = ½ F_{2,1} + F_{3,1} + ½ F_{5,1} + F_{6,1} + ½ F_{1,2} + ½ F_{2,2} + ½ F_{3,2} + ½ F_{4,2} + ½ F_{5,2} + ½ F_{6,2}$

$x_3(t+1) = ¼ F_{2,2} + ½ F_{3,2} + ¼ F_{5,2} + ½ F_{6,2}$

$x_4(t+1) = 0$

$x_5(t+1) = F_{1,3} + ½ F_{2,3} + F_{4,3} + ½ F_{5,3}$

$x_6(t+1) = ½ F_{2,3} + F_{3,3} + ½ F_{5,3} + F_{6,3}$

Appendix B.2 Numerical Analyses

Variables

The frequencies of the six phenogenotypes $T_{11}P_1$, $T_{12}P_1$, $T_{22}P_1$, $T_{11}P_2$, $T_{12}P_2$ and $T_{22}P_2$, are denoted as x_1, x_2, x_3, x_4, x_5 and x_6, respectively (see **5.3.2.2**).

Parameters

Female mate preference strengths are α_1 and α_2 and male aggression bias strengths are β_1 and β_2. Females are $1 + \alpha_k$ times as likely to mate with the imprinted phenotype k upon encounter, and males are $1 + \beta_k$ times as aggressive to the imprinted phenotype in male-male competition.

Appendix B.2.1 Basic Model: Symmetrical Selection

In the basic model, we assumed symmetrical selective strength on both mating phenotypes (i.e. $\alpha_1 = \alpha_2$ and $\beta_1 = \beta_2$). The recursion equations were not solvable analytically with unknown α and β, so we solved numerically with every combination of α and β from the set {0.01, 0.1, 0.2, 0.5, 1, 2, 3, 5, 10, 20, 50, 100}, using Mathematica (Wolfram 2018). We applied the constraint $x_6 = 1- x_1 - x_2 - x_3 - x_4 - x_5$ and $x_4 = 0$ (based on the x_4 recursion equation, see previous section). For each combination, we found three to five biologically relevant equilibria: fixation to trait 1 (x_1=1), fixation to trait 2 (x_6=1), and between one and three polymorphic equilibria. We used x_1, x_2, x_3, x_5 to form the Jacobian matrix in each model and performed a linear stability analysis on these equilibria. The only possible stable polymorphic equilibrium found occurred when the trait 1 phenotype frequency = 0.5 (including T_{11} and T_{12}, calculated as $x_1 + x_2 + x_4 + x_5$). The condition for instability of the polymorphic equilibrium (whether the absolute value of the leading

eigenvalue > 1) was determined numerically to be dependent on both female mate preference (α) and male aggression bias (β). Maternal and paternal imprinting models yield similar results in terms of the α and β parameter space that allows for a stable polymorphism (Mathematica code and R code presenting these analyses, as well as the output csv files, are archived at https://doi.org/10.6084/m9.figshare.9628406).

We then proceed to determine each of the phenogenotype frequencies (x_1 to x_6) at the stable polymorphic equilibria of the trait 1 phenotype frequency = 0.5. For these analyses, we therefore added two additional constraints: i) $x_1 + x_2 + x_4 + x_5 = 0.5$, and ii) $x_1 + x_2 + x_3 = 0.5$. The second constraint was added because at these equilibria, trait 1 phenotype ($x_1 + x_2 + x_4 + x_5$) will equal the frequency of P_1 ($x_1 + x_2 + x_3$) in the next generation. With these additional constraints, we can analytically solve the recursion equations with unknown α and β. At equilibrium, the x_i (expressed in terms of α and β) are independent of β and can be expressed in terms of α only. Note that the stability of these equilibria depends on the relative strength of α and β. We used these solutions to generate **Figure 5-2A** with a total of 6,561 simulations, including every combination of α and β from 0.01 to 100, modeled every $10^{0.05}$. We also used the x_i expressions to calculate the phenogenotypic linkage disequilibrium(Goodisman *et al.*, 1998) between T and P:

$$D = freq\,(T_1P_1) - freq\,(T_1) \cdot freq\,(P_1) = (x_1 + \tfrac{1}{2} x_2) - (x_1 + \tfrac{1}{2} x_2 + x_4 + \tfrac{1}{2} x_5)\,(x_1 + x_2 + x_3).$$

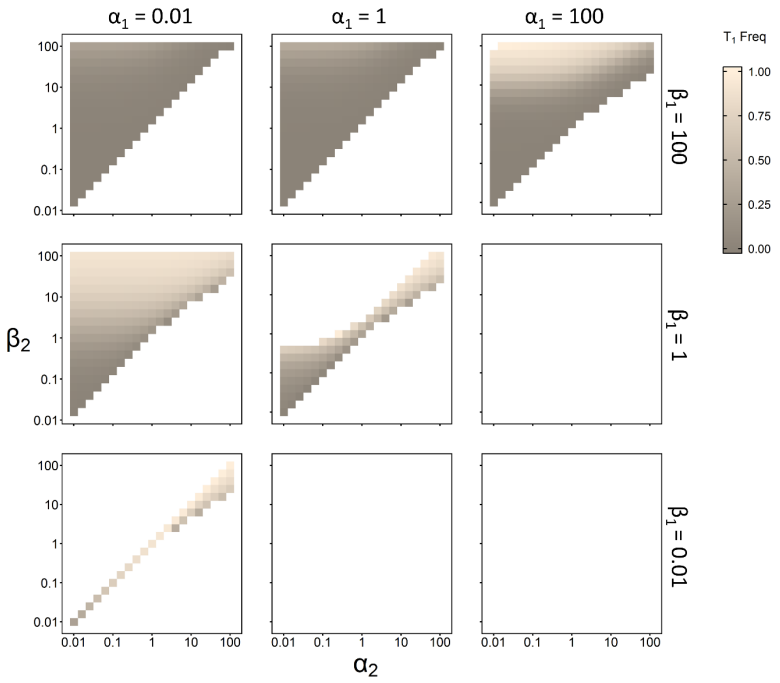
We then calculated the proportion of maximum LD (calculated as $\frac{D}{\sqrt{p_1 p_2 t_1 t_2}}$, hereafter D_{cor}). The resulting D_{cor} by α graph is presented in **Figure 5-2B**. The paternal imprinting model has a slightly higher phenogenotype linkage disequilibrium compared to the maternal imprinting model.

Appendix B.2.2 Model Extension: Asymmetrical Selection

Our basic model (presented in the main text) assumed symmetrical selective strength on both mating phenotypes (i.e. $\alpha_1 = \alpha_2$ and $\beta_1 = \beta_2$). We also analyzed scenarios in which the strength of behavioral bias differs between the P_1 and P_2 individuals (i.e. $\alpha_1 \neq \alpha_2$ and $\beta_1 \neq \beta_2$). The recursion equations were not solvable analytically with unknown α_k and β_k; we therefore first held α_1 and β_1 constant (starting with $\alpha_1 = \beta_1 = 0.01$), and solved numerically with every combination of α_2 and β_2 from the set $\{0.01, 0.1, 0.2, 0.5, 1, 2, 3, 5, 10, 20, 50, 100\}$. We then altered α_1 and β_1 and repeated the process, so that we had 9 sets of 144 simulations, including every combination of α_1 and β_1 from the set $\{0.01, 1, 100\}$. Similar to the basic model, we used x_1, x_2, x_3, x_5 to form the Jacobian matrix in each parameter set, and performed a numerical linear stability analysis on these equilibria. When we found any stable polymorphic solution in a certain α_1 and β_1 combination, we re-ran that particular set of simulations with every combination of α_2 and β_2 from 0.01 to 100, modeled every $10^{0.2}$ for higher resolution graphs (**Appendix Figure 3**). We were unable to numerically solve the recursion equations with some parameter combinations; in these cases, we ran simulations consisting of iterations of the exact recursion equations with several initial phenogenotype frequencies to confirm whether there was a stable polymorphic equilibrium.

Deviation from symmetric selective strengths showed robust results regarding the previously described conditions for polymorphism stability: the two mating trait phenotypes can coexist stably in the population when negative frequency dependent selection generated by a male aggression bias counters the positive frequency dependent selection generated by female preference. However, the asymmetries allow polymorphisms to stabilize at a broader range of phenotype frequencies (**Appendix Figure 3**). If male aggression bias toward trait 1 is stronger

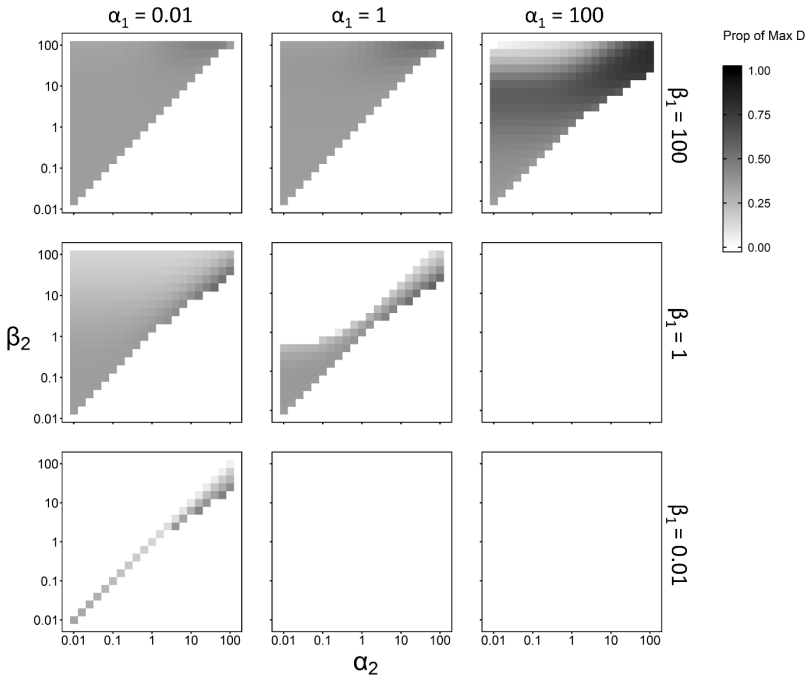
than that toward trait 2 (i.e. $\beta_1 > \beta_2$), trait 2 frequency at the stable equilibrium increases; if the female preference strength for trait 1 is greater than that for trait 2 (i.e. $\alpha_1 > \alpha_2$), trait 1 frequency at the stable equilibrium increases. The maternal imprinting and paternal imprinting models produced qualitatively similar results: there are slight differences (<0.1) in the equilibrium frequencies of phenogenotypes (x_1 to x_6) as well as the equilibrium frequency of the trait 1 phenotype ($x_1 + x_2 + x_4 + x_5$) for a given α_k and β_k combination (**Appendix Figure 3**). Mathematica code and the output csv files are archived at https://doi.org/10.6084/m9.figshare.9628406.



Appendix Figure 3 Trait 1 phenotype frequency at stable polymorphic equilibrium

T_1 phenotype frequency (including T_{11} and T_{12} individuals, $x_1 + x_2 + x_4 + x_5$) at the stable polymorphic equilibrium of the extended models (asymmetrical selection). Each panel represents a particular α_1 and β_1 combination from the set {0.01, 1, 100}, labeled on the top and the right. Within each panel, we ran every combination of α_2 and β_2 from 0.01 to 100, with a step size of $10^{0.2}$. Axes are on log scales. The white area in each panel is the parameter space where no stable polymorphism can be found. T_1 phenotype frequency at polymorphic equilibrium for a given α_k and β_k combination is slightly different in maternal and paternal imprinting models (< 0.1, not shown).

We also calculated the proportion of maximum LD (D_{cor}) between T and P, as we had in the basic model analysis (**Appendix Figure 4**). The trait-behavior linkage is strongest when the trait 1 phenotype frequency ($x_1 + x_2 + x_4 + x_5$) is close to 0.5. Similar to the symmetric models, the trait-behavior linkage roughly increases with α_k, but decreases when the equilibrium phenotype frequency is too close to T_1 or T_2 fixation. Overall, the paternal imprinting models produced higher D_{cor} than maternal imprinting models, but the differences were very small (< 0.1, not shown).



Appendix Figure 4 Proportion of maximum linkage disequalibrium (D_{cor}) at stable polymorphic equilibrium

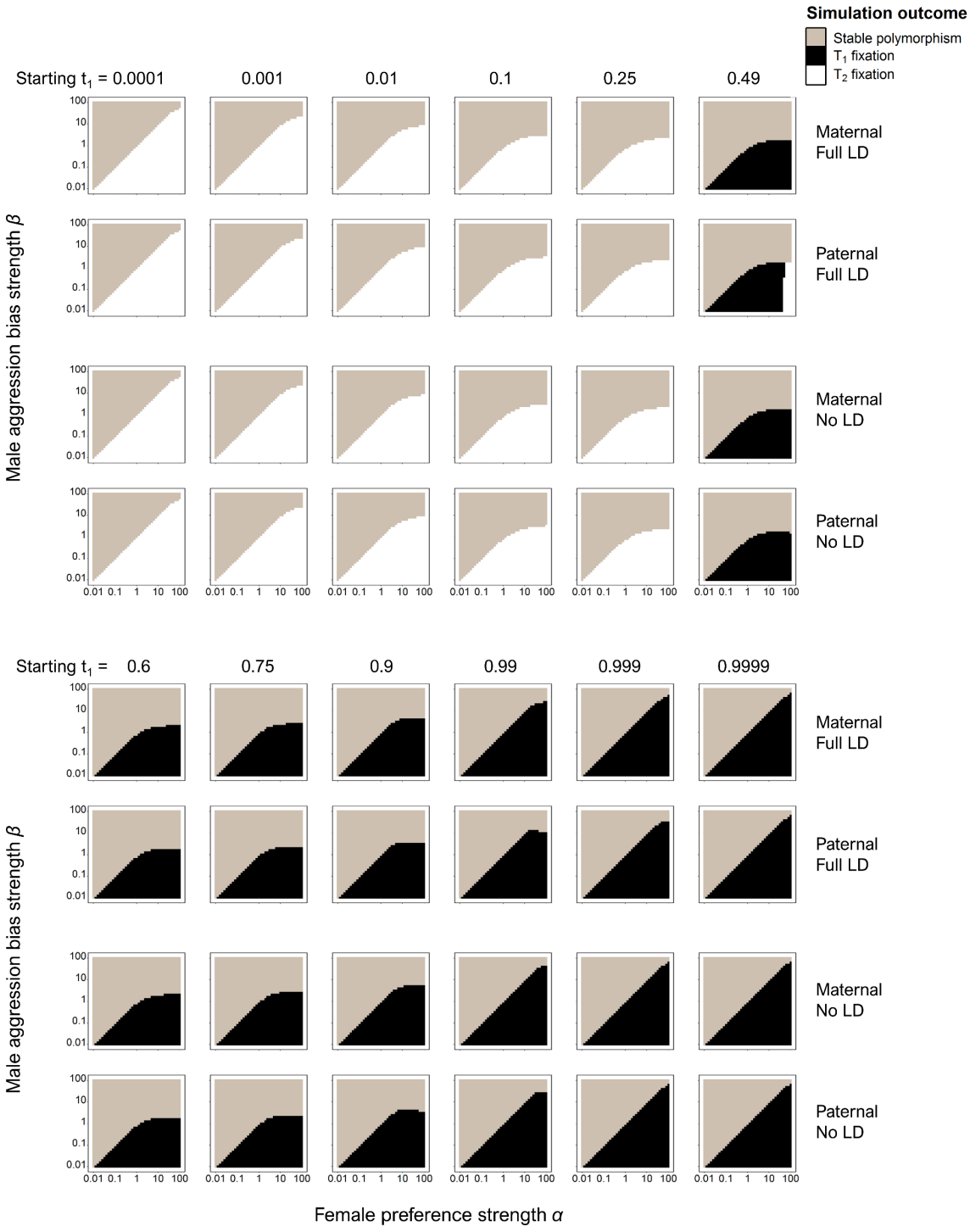
In this extended model, the value of α and β varies with the phenotype that the individual imprinted on. Trait – behavior linkage disequilibrium between the trait genotype and the behavioral phenotype (D_{cor}, calculated as $D/\sqrt{(p_1p_2t_1t_2)}$) at the stable polymorphic equilibrium. Each panel represents a particular combination of α_1 and β_1 from the set {0.01, 1, 100}, labelled on the top and the right. Within each panel, we ran combinations of α_2 and β_2 from 0.01 to 100, with a step size of 100.2. Axes are on logarithmic scales. The figure presents results from maternal imprinting models. Overall, the paternal imprinting models produced higher D_{cor} at polymorphic equilibrium than did the maternal imprinting models, but the differences were very small (<0.1, not shown).

Appendix B.3 Effects of Starting Frequency

To assess the extent to which the ability of the population to reach stable polymorphism depends upon the starting mating trait frequencies in the basic model (symmetrical selection), we ran simulations consisting of iterations of the exact recursion equations described above with a range of initial phenogenotype frequencies. We ran simulations of 2000 generations with T_1 initial frequency from the set {0.0001, 0.001, 0.01, 0.1, 0.25, 0.49, 0.6, 0.75, 0.9, 0.99, 0.999, 0.9999}. For certain parameter combinations, 2000 generations are not sufficient for the population to reach equilibrium; therefore, we also compared the final frequency with the initial frequency to determine if the population is moving toward T_1 fixation, T_2 fixation, or the stable polymorphism. To simplify the analysis, we only started with homozygotes (i.e. $x_2 = x_5 = 0$). For each T_1 initial frequency t_1, we set the initial phenogenotype frequencies in two ways, so that the population started either with i) maximized LD: only $T_{11}P_1$ and $T_{22}P_2$ individuals; $x_1 = t_1$, $x_6 = 1 - t_1$, $x_2 = x_3 = x_4 = x_5 = 0$, or ii) no LD: mating trait and behavior combinations as expected from random assortment; $x_1 = t_1^{\,2}$, $x_3 = x_4 = t_1 (1 - t_1)$, $x_6 = (1 - t_1)^2$, $x_2 = x_5 = 0$. For each t_1 – LD combination, we ran every combination of α and β from 0.01 to 100, with a step size of $10^{0.1}$. We therefore had 24 sets (12 initial frequencies, 2 LD criteria) of 1,681 simulations for both maternal and paternal imprinting models (**Appendix Figure 5**). Mathematica code and the output csv files are archived at https://doi.org/10.6084/m9.figshare.9628406.

The simulations show that it is possible for a rare allele (frequency = 0.0001) to increase in frequency and reach a stable polymorphism over a wide range of parameter space (tan area in **Appendix Figure 5**). Overall, the rarer an allele initially is in the population, the more restricted the parameter space that allows a stable polymorphism to evolve. In the area where there is no stable polymorphism, the threshold that leads to dominant allele (T_1) fixation over a

polymorphism, versus recessive allele (T_2) fixation over a polymorphism, is asymmetric: the recessive allele requires a greater starting allele frequency (as evidenced by the predominant fixation of T_1 in the t_1 = 0.49 column, see **Appendix Figure 5**). However, the exact starting frequency threshold depends on the mechanism of imprinting (maternal vs paternal) as well as the starting LD condition (e.g. **Appendix Figure 5**, t_1 = 0.49). Paternal imprinting has a larger parameter space that allows stable polymorphism to evolve compared to maternal imprinting, but the difference is very small. The starting LD condition also influences the parameter space that leads to stable polymorphism and which allele becomes fixed when there is no stable polymorphism, but again the difference is small.



Appendix Figure 5 Effects of starting frequency on polymorphism maintenance

Simulations of 2000 generations demonstrating the effects of starting allele frequencies on polymorphism maintenance. Each panel represents a combination of a particular starting T_1 frequency (t_1, labeled on top), mating trait-behavior association (phenogenotypic linkage disequilibrium, labeled on right), and types of imprinting (maternal or paternal; labeled on right). Within each panel, we ran every combination of α and β from 0.01 to 100, with a step size of $10^{0.1}$. Axes are on log scales.

Appendix B.4 Incomplete Imprinting

Appendix B.4.1 Behavioral Pattern of the Individuals that Fail to Imprint

We considered a scenario in which not every individual imprint successfully on the parent's phenotype. Conceptually, we considered three alternative hypotheses: individuals that fail to imprint i) have no behavioral bias, ii) display a genetically determined behavioral bias, and iii) display behavioral bias toward their own phenotype (self-referent phenotype matching). Results suggest that ii) and iii) are unlikely. If the individuals that failed to imprint develop a behavioral pattern according to their genes or their mating phenotype, we would expect the behavioral bias (toward own color) in the pure-bred treatment to be stronger than the behavioral bias (toward the foster color) in the cross-fostered treatment (as individuals who failed to imprint would be expected to show a bias in the opposite direction). However, we did not detect any significant difference between the strength of the behavioral biases of these two groups (unpaired two-sided, two-sample permutational t test; all $P > 0.33$, for both male and female offspring, based on proportions of both association time and approaches). Therefore, it seems most likely for the individuals that fail to imprint to display no preference/aggression bias. We therefore made the assumption that the individuals that fail to imprint on the parent show no aggression bias/mate preference in the next section.

Appendix B.4.2 Modeling Incomplete Imprinting

We added an imprinting success parameter S, which represents the proportion of offspring that succeed in imprinting on the parent's phenotype (equivalent to an offspring imprinting on the

parent phenotype with the probability *S*). For the remaining 1 - *S* percent that fail to imprint, they display no mate preference (females) or aggression bias (males; see below for justification of this assumption). We incorporated imprinting success *S* into the male-male competition and female mate choice stages in the life cycle described in **5.3.2**:

Male-male Competition

In our main model males are $1+\beta_k$ times as aggressive toward the imprinted mating trait phenotype *k*. The total aggression received by mating phenotype 1, for example, is $A_1 = (1 + \beta_k)p_1 + p_2$, where p_1 and p_2 represent the frequencies of trait 1- and trait 2-biased males, respectively ($p_1 = x_1 + x_2 + x_3$, $p_1 = x_4 + x_5 + x_6$) Incorporating imprinting success *S*, the total aggression received by mating phenotype 1 becomes

$$A_1 = (1 + \beta_1) \cdot S \cdot p_1 + (1 - S)p_1 + p_2 = (1 - S \cdot \beta_1)p_1 + p_2,$$

which is equivalent having a male aggression bias strength of $S \cdot \beta_1$.

Female Mate Choice

In our main model, females choose their mates according to the behavior "locus" P, such that upon encounter, females are $1+ \alpha_k$ times as likely to mate with males possessing the imprinted mating phenotype k. Thus, the frequency of mating between each combination of male genotype i and female phenogenotype j is:

$$F_{i,j} = \frac{x^*_{i,m} x^*_{j,f} (1+d_{i,j}\alpha_k)}{\sum_i x^*_{i,m} (1+d_{i,j}\alpha_k)},$$

where $d_{i,j} = 1$ if the female behavior locus P matches the male trait phenotype T (i = 1 or 2 with j = 1, 2 or 3, and i = 3 with j = 4, 5 or 6) and $d_{i,j} = 0$ otherwise, and where $k = 1$ when j = 1, 2 or 3, and $k = 2$ when j = 4, 5, or 6. Incorporating incomplete imprinting success, the frequency of mating between $T_{11}P_1$ female and T_{11} male ($F_{1,1}$), for example, becomes

$$F_{1,1} = \frac{x^*_{1,m}x^*_{1,f}\left((1+\alpha_1)\cdot S+(1-S)\right)}{x^*_{1,m}\left((1+\alpha_1)\cdot S+(1-S)\right)+x^*_{2,m}+x^*_{3,m}} = \frac{x^*_{1,m}x^*_{1,f}(1+S\cdot\alpha_1)}{x^*_{1,m}(1+S\cdot\alpha_1)+x^*_{2,m}+x^*_{3,m}},$$

which is equivalent to having a female preference strength of $S \cdot \alpha_1$.

Thus, in both cases, our parameters α and β could include a linear scaling factor that describes the proportion of the population that successfully imprinted. The figures in the main text can therefore be interpreted as including in their parameters the effective strength of preference (for α) or aggression bias (for β) of the pool of individuals with that genotype, given that imprinting is only partly successful.

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