Evolutionary divergence of developmental plasticity and learning of mating tactics in Trinidadian guppies

Abstract

1. Behavioral plasticity is a major driver in the early stages of adaptation, but its effects in mediating evolution remain elusive because behavioral plasticity itself can evolve.
2. In this study, we investigated how male Trinidadian guppies (*Poecilia reticulata*) adapted to different predation regimes diverged in behavioral plasticity of their mating tactic. We reared F2 juveniles of high- or low-predation population origins with different combinations of social and predator cues and assayed their mating behavior upon sexual maturity.
3. High-predation males learned their mating tactic from conspecific adults as juveniles, while low-predation males did not. High-predation males increased courtship when exposed to chemical predator cues during development; low-predation males decreased courtship in response to immediate chemical predator cues, but only when they were not exposed to such cues during development.
4. Behavioral changes induced by predator cues were associated with developmental plasticity in brain morphology, but changes acquired through social learning were not.
5. We thus show that guppy populations diverged in their response to social and ecological cues during development, and correlational evidence suggests that different cues can shape the same behavior via different neural mechanisms. Our study demonstrates that behavioral plasticity, both environmentally induced and socially learnt, evolves rapidly when organisms colonize ecologically divergent habitats to shape adaptation.

KEYWORDS: behavioral plasticity, social learning, alternative mating tactic, brain morphology, predation, guppy, G x E interaction

Introduction

Adaptation occurs through evolution over multiple generations, but environments can change within an individual's lifetime. Phenotypic plasticity, the ability of organisms to alter their phenotype in response to the environment, allows individuals to incorporate information and optimize their fitness to fine-scale environmental change (Schlichting & Pigliucci, 1998). Because plasticity can respond to selective pressures faster than evolution, it has been hypothesized to facilitate adaptation and promote diversification (Pfennig, 2021; West-Eberhard, 2003). In particular, behavioral traits are considered the "pacemaker" of evolution because they determine how animals interact with their environment, and thus shape the selective landscape they experience (Mayr, 1963). Behavioral traits exhibit remarkable plasticity and are often the first to respond to a novel environment (West-Eberhard, 2003). This plasticity ranges from developmental plasticity (shaped by ontogenetic experience and fixed after development) to behavioral flexibility (or "activational plasticity", a reversible response to the immediate environment ; Snell-Rood, 2013). Furthermore, behaviors can be shaped by learning among social group members (i.e. social learning), which allows an advantageous behavioral tactic to rapidly spread in the population without new genetic mutations (Feldman & Laland, 1996; Wright et al., 2022). Notable examples include tool use in primates (Whiten et al., 1999), song dialects in birds (Marler & Tamura, 1964), and oviposition site choice in fruit flies (Battesti et al., 2012). These characteristics of behavioral traits lead to the hypothesis that learning, or more broadly behavioral plasticity, facilitates adaptation (Axelrod et al., 2023; Baldwin, 1896; Simpson, 1953). However, in some scenarios, behavioral plasticity may also hinder adaptation because it can buffer deleterious genotypes from selection (Axelrod et al., 2023; Bogert, 1949; Ghalambor et al., 2007; Muñoz, 2022).

High levels of behavioral plasticity come at a cost, most notably the time and energy required to sample environmental cues and metabolically expensive investment in neural tissue (Snell-Rood, 2013).

As a result, low behavioral plasticity may also be adaptive if such plasticity does not confer a sufficient selective advantage. Therefore, the degree of behavioral plasticity itself may be under selection and can evolve. The evolution of behavioral plasticity and learning has been linked to adaptation to environmental variability in a variety of factors like temperature (e.g. rodents; Sassi et al., 2015), predation (e.g. frogs; Van Buskirk, 2002), and mate availability (e.g. insects; Carroll & Corneli, 1995). Furthermore, animals need to integrate cues from different sources using different modalities that provide information of varying reliability, and their combined effects are often non-additive (Amo et al., 2004; Westneat et al., 2019). For example, social learning of an anti-predator behavior is often facilitated by the presence of predator, and more efficient when both visual and chemical cues are present (Griffin, 2004; Kelley et al., 2003). Because of this, studies that examine how animals respond behaviorally to multiple ecological and social cues simultaneously, and how such plasticity patterns diverge among populations, are essential to evaluating the role of behavioral plasticity in adaptation and diversification.

In addition to documenting how behaviors change in response to environmental cues, disentangling the neural substrates that underlie these changes is essential for understanding the developmental and evolutionary constraints of behavioral plasticity (Duckworth, 2009; Snell-Rood, 2013). Variation in brain morphology, or the size and proportion of functionally specialized brain regions, has been proposed as a major mechanistic basis of behavioral variation (Gonda et al., 2013; Hofman & Falk, 2012; Lefebvre & Sol, 2008). Inter- and intraspecific variation in brain morphology correlates with a wide variety of behaviors including foraging (Axelrod et al., 2022), communication (Schumacher & Carlson, 2022), locomotion (Bertrand et al., 2021), and anti-predator behaviors (Jaatinen et al., 2019). Brain morphology can also show considerable plasticity in response to various environmental cues, and this change can further shape behavioral patterns and performance (Gonda et al., 2013). For example, white footed mice exposed to shorter photoperiods developed a smaller brain and hippocampus, resulting in decreased performance in spatial learning and memory (Pyter et al., 2005). Sticklebacks reared with visual or chemical stimulation developed larger optic tectum and larger olfactory bulbs respectively, and consequently alter their reliance on the two modalities when performing a cognitive task (Pike et al., 2018). Mechanistically, larger brains or brain regions can sustain more neurons and more complex connections, providing a better capacity for cognition, sensory integration or motor coordination (Dicke & Roth, 2016; Herculano-Houzel et al., 2006). However, changes in behavior can also occur via rewiring or biochemical switches in neural networks, change in neural density, or changes in neural activities (Harris & Hofmann, 2014), all of which may not reflect on (or be constrained by) brain morphology. To gain a comprehensive understanding of how behaviors and behavioral plasticity evolve, we should consider not only the pattern of behavioral plasticity across divergent populations, but also the architecture of its underlying neural substrates.

In this study, we examine the evolutionary and developmental mechanisms, as well as brain morphology correlates of behavioral plasticity in mating behavior using Trinidadian guppies (*Poecilia reticulata*). The Trinidadian guppy is a model system for behavioral evolution and rapid adaptation in the wild. Male guppies have bright color ornaments, and exhibit two alternative mating tactics: they achieve mating by either performing courtship displays that attract females ("sigmoid displays"), or by forced insemination that bypasses courtship and female choice ("sneak mating"; Liley, 1966). Courtship displays increase mating success (Evans & Magurran, 2001; Kodric-Brown & Nicoletto, 2001b), but may be more energetically costly than sneak behaviors (Cattelan et al., 2016). Guppy populations in Trinidad can be broadly categorized into two habitat types: downstream, high-predation (HP) sites, where guppies co-exist with large predatory fishes (e.g. *Crenicichla alta*), and upstream, low-predation (LP) sites, where these predators are absent (Endler, 1978, 1995; Haskins et al., 1961). In addition to predation risks, the HP and LP sites also differ in several ecological factors including population density, productivity, and competition regimes (reviewed in Travis et al., 2014), and there is considerable gene flow from upstream (LP) to downstream (HP) populations within the same drainage (Crispo et al., 2006). Males are generally less colorful, court less, and sneak more at HP sites than at LP sites (Endler, 1978; Luyten & Liley, 1985; Magurran & Seghers, 1990; Magurran, 2001; but see Yong et al., 2022). This is because conspicuous color and courtship displays incur higher predation costs (Endler, 1980; Godin & McDonough, 2003). Thus, male mating tactic, or the relative proportion of courtship *versus* sneak tactics used when encountering a female, is a critical behavioral trait that is under both natural and sexual selection. There is evidence of a genetic basis for variation in male mating tactic in guppies (Evans, 2010; Luyten & Liley, 1985; Yang et al., 2023; but see Evans et al., 2015). However, males also exhibit remarkable developmental plasticity in mating tactic in response to a variety of factors including diet quality (Devigili et al., 2013) and social experiences (Guevara-Fiore, 2012; Rodd & Sokolowski, 1995). They also adjust their mating tactic in response to the immediate environment (i.e. behavioral flexibility); for example, decreasing courtship displays in the presence of a predator (Godin, 1995; Magurran & Nowak, 1991). While many studies have investigated individual factors influencing male mating tactic in guppies, no study, to our knowledge, has investigated how males integrate socially learned information with environmental cues, and whether this pattern of behavioral plasticity can evolve to adapt to different environments.

In this study, we reared second generation (F_2) descendants of guppies collected from HP and LP populations under different combinations of social and predation environments to examine their integrated effects on male mating tactic. We used a 2 x 3 x 2 factorial design, rearing HP and LP juveniles (hereafter *population origin; HP/LP*) with either HP adults, LP adults, or in social isolation (hereafter *social treatment; HP tutor/LP tutor/solo*), and in water with or without chemical cues from a cichlid predator (hereafter *predator treatment; pred+/pred-*). This design allowed us to test how male guppies integrate social learning with environmental cues to optimize their mating tactic, and whether guppies adapted to high- and low-predation habitats differ in their reliance on the two types of information. We also assayed each male twice, in random order, in water with or without the same predator cues (here after, *assay water; pred+/pred-*) to measure behavioral flexibility. Finally, to test whether developmental plasticity in brain morphology is associated with the behavioral changes observed, we quantified brain size and the sizes of five brain regions (telencephalon, cerebellum, optic tectum, olfactory bulb, and hypothalamus) in a subset of individuals in each treatment.

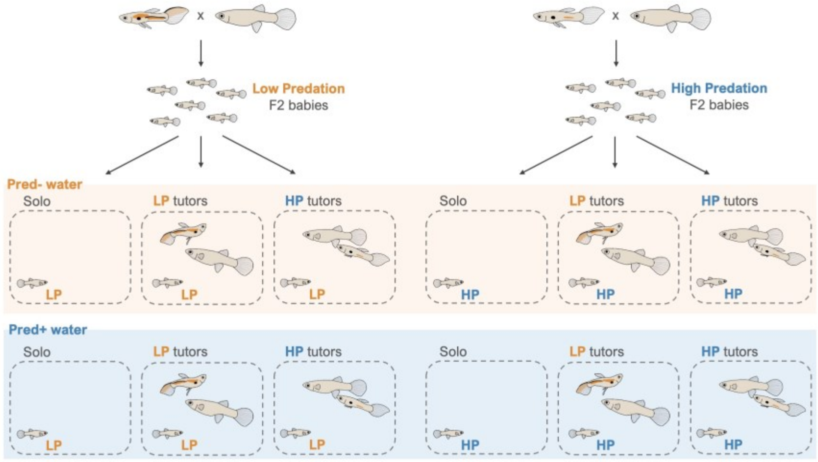
Methods

EXPERIMENTAL SETUP

We collected guppies from HP and LP localities at the Aripo drainage in Trinidad (HP site: 10.665681N; 61.228006W; LP site: 10.686783N; 61.232843W). Collected fish were then transported to Washington University in St. Louis, where they were quarantined and treated prophylactically for a variety of parasites. We kept the wild guppies (approximately 20 males and 20 females from each population) in population group tanks and collected all their resulting offspring. F1 offspring were raised in 9.5-liter tanks in flow-through aquatic housing systems (Aquaneering Inc., USA), and males and females were separated before sexual maturity. All fish in the lab were maintained at 25°C under a 12L:12D light cycle. Juvenile guppies were fed brine shrimp or liver paste, and adults were fed crushed Tetramin Tropical Flakes (Tetra Co., USA).

The first-generation lab-bred virgins were randomly paired to create 32 family lines (17 HP pairs, 15 LP pairs). We reared the F2 siblings in isolated tanks for 2 weeks, then randomly split them into 12 different treatments: 2 population origin x 3 social treatments x 2 predator treatments (**Fig. 1**). Total sample size was 236 males, with ~20 males per treatment combination. In the social treatments, we reared juveniles with either adults from HP or LP populations or on their own (HP tutor/LP tutor/solo, hereafter). In the two treatments with tutors, we added two adult males and one adult female from either HP or LP population into the juvenile tank at week two when juveniles were large enough to prevent adult cannibalism. We removed the tutor fish from the tanks on day 45 before the males were fully mature (except for one male that matured on day 42). In the predator treatments, we manipulated whether guppies experienced a high-predation or low-predation environment by raising them in water with or without predator chemical cues (pred+/ pred-, hereafter). In the pred+ treatment, we connected a tank containing a pike cichlid (*Crenicichla alta*) to the flow-through water system of the guppies. The guppies could not see the predator, but they received chemical cues through the water. Each day we fed two guppies to the cichlid, so the circulating water contains both cues from the cichlid and alarm cues from the predated guppies (Ghalambor et al., 2015). Experimental fish were kept in 1.8 liter tanks in flow-through aquatic housing systems (Aquaneering Inc., USA) and fed a quantified amount of brine shrimp or liver paste (bi-weekly increase as they age) throughout the experiment.

Figure 1 Experimental design. 2 population origin (HP/LP) x 3 social treatments (solo/HP tutor/LP tutor) x 2 predator treatments (pred-/pred+).



TUTOR BEHAVIOR OBSERVATION

On day 45, prior to isolating the focal males from their tutors, we transferred the three tutors and the focal males to a 31 x 16 x 21 cm glass tank filled with the same water they were reared in and allowed to acclimate overnight (~15h). The experimental tank was lit by two diffused light panels on either side, and the entire apparatus was covered by black cloth to minimize external disruption. In the morning, we waited until the first mating attempt (either a sigmoid display or a sneak mating attempt) or for 5 minutes, whichever was earlier to begin behavior observation. We quantified the behavior of the tutors by counting the total numbers of sigmoid displays and sneak attempts the two tutor males performed in 5 minutes. Tutor males directed most of their sigmoid displays toward the tutor females; however, there were rare instances in which the males may have been displaying toward each other or toward the juvenile focal male. To avoid introducing bias from different observers, we indiscriminately tallied all observed sigmoid displays. We calculated the proportion of courtship display, defined as # sigmoids / (#sneaks + #sigmoids), as an indicator of mating tactic of the two tutor males.

MATING ASSAY

We assayed the males for their mating tactic after they reached sexual maturity, defined as when the hood extended beyond the distal tip of the gonopodium. Each male was tested twice for each type of behavioral assay, once in pred- water and once in pred+ water. We randomly assigned approximately half of the males in each treatment combination with pred- water as the first assay, and the other half with pred+ water first (see **Table S1** for exact sample sizes). We assayed male mating tactic by quantifying behavioral interactions between a male and an unrelated, virgin female from the same population and treatment category. While we cannot rule out the indirect effect of female behavior on male behavior, we paired the individuals in this manner so that the males were interacting with the female phenotype that matched the specific environment simulated.

Prior to the assay, the male and the female were transferred to a 31 x 16 x 21 cm glass tank filled with either pred+ or pred- water and allowed to acclimate overnight (~15h). Pred+ and pred- water were acquired from the sump reservoirs of the flow-through water system of the pred+ and pred- predator treatments, respectively. The two fish were separated by an opaque divider that obstructed visual but not olfactory contact (small amount of water can pass through the bottom of the divider) during acclimation. Tank lighting setup was the same as the tutor assays. In the morning, we started the assay by removing the divider, waited until the first mating attempt (either a sigmoid display or a sneak mating attempt) or for 5 minutes, whichever was earlier, and then timed 10 minutes for behavioral recording. We recorded the following male behaviors: the number of sigmoid displays, the duration of each sigmoid display, the number of sneak mating attempts, the number of gonopodial swings, the number of aggressive physical contacts, and the number of successful matings (i.e. followed by male postcopulatory jerks that signal sperm transfer). We transferred the pair to a new experimental tank with the second assay water type and allowed them to acclimate for at least 3h before we repeated the same assay procedure in the afternoon. We calculated the proportion of courtship display, defined as # sigmoids / (#sneaks + #sigmoids), as an indicator of male mating tactic.

MORPHOLOGICAL MEASUREMENTS

After behavioral assays, males were euthanized, weighed, and photographed. We euthanized the males using overdosed MS-222 (tricaine methanesulfonate) and weighed the males to the nearest 1 mg using an Ohaus STR123 balance. We placed the males right-side-up on a white background, and carefully used a paintbrush to straighten their body position and spread the gonopodia away from body. We then took a photograph of the fish with a metric ruler and used it as a reference to measure their standard body length (snout to insertion point of the caudal fin rays) using ImageJ (Abràmoff et al., 2004). Bodies were then stored in 10% buffered formalin for brain dissection later.

BRAIN MEASUREMENTS

For a subset of males (10-12) in each treatment, we extracted the brains and measured brain mass and estimated volume of five superficial brain regions (cerebellum, optic tectum, telencephalon, olfactory bulb, and hypothalamus). Brains were extracted using dorsal dissection and photographed from dorsal, ventral, and lateral angles using a Leica MC190 HD microscope camera. Brains were then trimmed of excess nerves and their spinal cords were cut at the level of the obex. Blotted wet mass of the brains was then measured with a Mettler Toledo XPR2 microbalance to the nearest 0.01mg. The length, width, and depth of each brain region were measured using Image J, and the volumes were estimated using the ellipsoid formula ($V = L \times W \times H/\pi 6$; White & Brown, 2015). Only one side of the brain was photographed, and the depths of bilaterally symmetrical lobes were assumed to be the same. Detailed analyses and interpretation of brain morphology plasticity is reported in another paper (Axelrod *et al. in review*) and these results are summarized in the discussion. Here, we focused on testing whether plasticity in relative brain size (brain mass residuals against body mass) and relative brain region sizes (brain region volume residuals against brain mass) predict variation in male mating tactic (see ***confirmatory path analysis*** below).

STATISTICAL ANALYSIS

All Statistical analyses were performed in R version 3.3.2 (R Core Team, 2022).

Relative importance analysis

To analyze the relative contributions of each factor on male mating behavior, we used a linear model to estimate the influence of population origin, predator treatment, social treatment, assay water, and the interactions between population origin and the other three predictors on logit transformed sigmoid proportion. We quantified the relative importance of each main effect and interaction using the *calc.relimp* function in the *relaimpo* R package (Groemping & Matthias, 2021).

Mating tactic among treatments

Because a full model that includes higher order interaction terms (e.g. population x predator treatment x social treatment) often lead to model convergence failure, we subset the HP and LP males in subsequent analyses to more reliably explore how HP and LP males differ in their response to predator and social treatments. We tested whether male mating tactic of HP and LP males differed among the social and predator treatments, and whether assay water had an effect. We used binomial generalized linear mixed models (GLMMs) to test the effects of social treatment (HP tutor/LP tutor/no tutor), predator treatment (pred+/pred-), assay water (pred+/pred-), and their interaction terms on sigmoid proportions in HP and LP males, respectively. Individual ID and family ID were included as random effects. Assay order (first/second), whether females mated in the previous assay (Y/N), and their two-way interactions with social treatment, predator treatment, and assay water were included as covariates to control for potential confounding effects of time of day, different acclimation periods, and sexual experiences. Interactions that were not statistically significant were sequentially dropped from the models. We also included an observation level random effect (OLRE; (Harrison, 2015) to mitigate overdispersion. In 8.7% of the assays, the male exhibited neither sneak mating attempts nor courtship displays; these assays were excluded from the analyses. The GLMMs were performed using the *glmer* function in the R package *lme4* (Bates et al., 2015). Post-hoc comparisons (Tukey) were performed using the *emmeans* function in the R package *emmeans* (Lenth, 2022).

In addition to sigmoid proportion, we also modeled the number of sigmoid displays, total sigmoid time, average duration of each sigmoid display, the number of sneak mating attempts, the total number of mating behaviors, whether the pair successful mated, and the number of gonopodium swings, using the same predictor variables, covariates, and random effects as the above models. We applied negative binomial GLMMs, binomial GLMMs, and LMMs for count, yes/no, and continuous response variables, respectively. Full statistical results as well as the grouped summary of the behavioral metrics are reported in the **Supplemental Materials**.

Correlation with tutor behavior

To test whether male mating tactic was correlated with the behavior of the specific set of tutors the individual was reared with, we modeled the effect of the sigmoid proportion of the tutors (as a continuous predictor variable) on sigmoid proportion of the focal male using LMMs. We first ran a model with population, tutor sigmoid proportion, and their interaction, then conducted a post hoc analysis to test the estimated marginal means of linear trends of HP and LP males respectively for their significance against 0 (i.e. no correlation between tutor and focal behavior). We included individual ID as a random effect to account for the repeated assays in pred+ and pred- water. Data points were excluded when we did not observe any mating behavior in either the focal male mating assay or the tutor assay (19.0%). The LMMs were performed using the *lmer* function in the R package *lme4* (Bates et al., 2015). Post-hoc analyses were performed using the *emtrends* function in the R package *emmeans* (Lenth, 2022).

Confirmatory path analyses

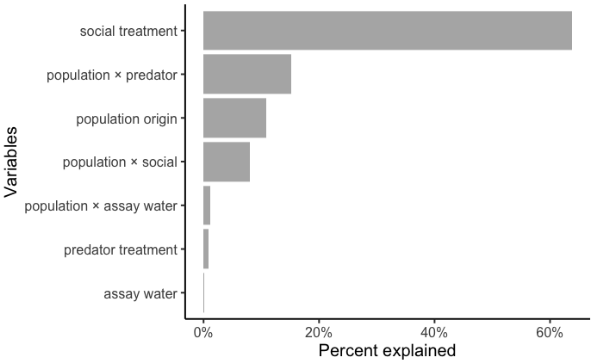
We conducted confirmatory path analyses using structural equation models (SEMs) to test the hypothesis that developmental plasticity in brain morphology is associated with the behavioral variations we observed in the experiment. We specified a pre-determined mediation pathway (**Fig. 4A**), where logit transformed sigmoid proportion is the dependent variable; social treatment, predator treatment, and assay water were the predictor variables; and brain size and brain architecture were the mediators. Brain size was measured as brain mass residuals against body mass. Brain architecture is a latent variable, with its indicators being the relative volumes (residuals against brain mass) of the five brain regions we measured (Tel: telencephalon; Cb: cerebellum; OT: optic tectum; OB: olfactory bulb; Hyp: hypothalamus). A latent variable is not directly measured, but its influence can be summarized through several indicator variables (Rosseel et al., 2023). We treated sigmoid proportion as a continuous variable because the execution and interpretation of non-Gaussian path analyses are more complex and have limited software support. Significant indirect effects (**Fig. 4A**, orange arrows) suggest that the treatments were influencing mating tactic through shaping brain morphology. Statistically significant direct effects (**Fig. 4A**, black arrows) indicate that other neurohormonal mechanisms that we did not measure underlie the mating behavior plasticity observed. Path analyses were performed using the *sem* function in the R package *lavaan* (Rosseel et al., 2023).

Results

Relative importance analysis

Among the main effects, social treatment accounted for the highest percentage of the explained variance (63.8%); population origin accounted for 10.8%, predator treatment accounted for 0.9%, and assay water accounted for 0.1% (**Fig. 2**). Interactions of population origin with social treatment, predator treatment, and assay water accounted for 8.1%, 15.2%, and 1.2%, respectively (**Fig. 2**).

Figure 2 Relative importance of population origin, social treatment, predator treatment, assay water, and interactions of population origin with the three other variables in a linear model predicting male mating tactic (courtship proportion, logit transformed). The metrics of the main effects and interactions were scaled to sum to 100%.



Mating tactic among treatments

Social treatment, predator treatment, and assay water influenced mating tactic in male guppies adapted to both HP and LP habitats but in different ways. Among the social treatments, HP males reared with LP tutors or in isolation performed proportionally more courtship displays than those reared with HP tutors (**Tables 1 & S2; Fig. 3A**). In contrast, LP males reared in isolation performed proportionally more courtship displays than those reared with either HP or LP tutors (**Tables 1 & S2; Fig. 3B**). For both HP and LP males, changes in sigmoid proportion in response to social treatment were likely a result of changes in both the number of sigmoid displays and the number of sneak mating attempts (**Table S3**, **Fig. S1**). The average duration of each sigmoid display was also influenced by social treatment. HP males reared with HP tutors exhibited marginally longer courtship displays in pred- water compared to pred+ water, while those reared with LP tutors or in isolation did not show an effect of assay water (**Table S3&S4**, **Fig. S1E**). In contrast, LP males reared in isolation performed longer displays than those reared with either HP or LP tutors (**Table S3&S5**, **Fig. S1F**).

Between the predator treatments, HP males reared in pred+ treatment performed proportionally more sigmoid displays compared to those in pred- treatment (**Table 1; Fig. 3A**). However, they did not change their mating behavior in response to assay water (**Table 1; Fig. 3A**). LP males reared in pred- and pred+ treatments did not overall differ in sigmoid proportions (β ± SE = -0.03 ± 0.18, z =-0.18, p = 0.861), but those in pred- treatment altered their mating tactic in response to assay water, performing proportionally less sigmoid displays when assayed in pred+ water compared to pred- water (β ± SE = 0.43 ± 0.16, z =2.65, p = 0.008**; Fig. 3B**). Changes in sigmoid proportion in response to predator treatment is primarily driven by changes in number of sigmoid displays in both HP and LP males (**Table S3**, **Fig. S1**). In addition, the average duration of each sigmoid display was influenced by predator treatment in LP males, but not in HP males (**Table S3; Fig. S1E-F**). LP males reared in pred- water exhibited longer displays compared to those reared in pred+ water, but regardless of assay water, this difference only appeared in the second behavioral assay (**Table S6**).

Table 1 Two GLMMs modeling proportion of sigmoid displays in mating assays on the two population origins. Dropped interaction terms are not shown or are denoted with "-"s.

		HP males				**LP males**			
Variables	df	β	SE	LR χ2	*p*	β	SE	LR χ2	*p*
tutor treatment	2			10.32	**0.006**			17.73	**<.001**
HP tutor		-1.21	0.47			-1.47	0.35		
LP tutor		0.25	0.47			-0.89	0.35		
predator treatment	1	1.02	0.38	7.32	**0.007**	-0.55	0.32	2.95	0.086
assay water	1	0.10	0.15	0.46	0.496	-0.15	0.12	6.83	**0.009**
predator × assay water	1	-	-	-	-	0.42	0.24	3.15	0.076
assay order	1	-0.39	0.16	6.14	**0.013**	-0.15	0.12	1.60	0.206
prior mating	1	0.63	0.35	3.26	0.071	-0.75	0.49	2.34	0.126

Baselines: tutor treatment = solo; predator treatment = pred-; assay water = pred-; assay order = first; prior mating = N

Figure 3 Marginal means and standard errors estimated from the GLMMs comparing mating tactic among treatments in HP and LP males (**Table 1**). Letters denote post hoc comparisons among the social treatments (**Tables S2**). See **Tables 1** and main text for effects of predator treatment and assay water.

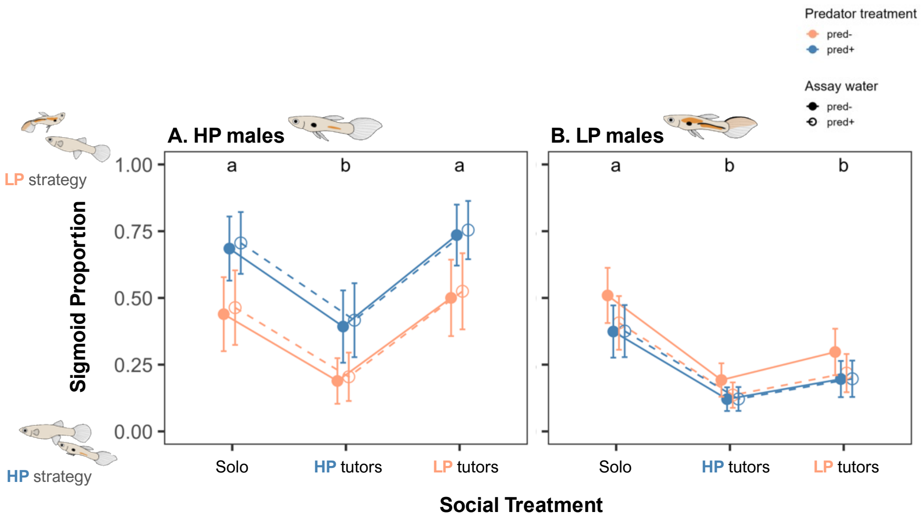
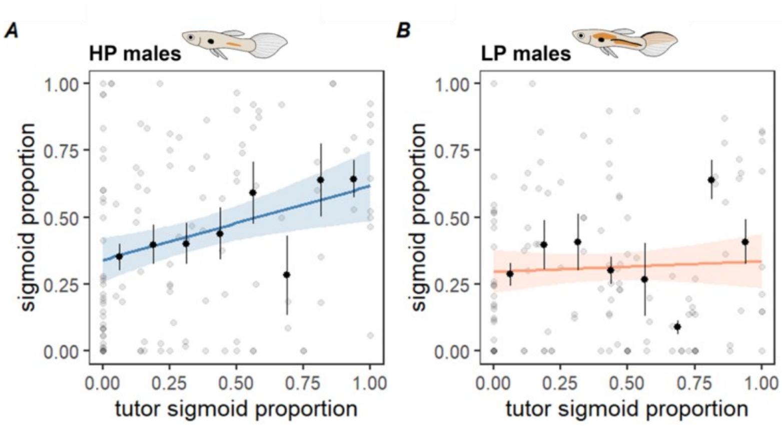


Figure 4 Correlation between mating tactic (y axis) of the HP males (**A**) and LP males (**B**) and the behaviors of the tutors they were reared with (x axis). Smaller grey dots are raw data points. Black dots and error bars are mean and standard error of the y variable calculated from the data points that fall within each of the eight x axis bins. The fitted lines and 95% confidence intervals (shaded area) were estimated from linear models on the raw data points.



Correlation with tutor behavior

We found a non-significant trend in the interaction between population and tutor sigmoid proportion (LR χ2 = 3.06, df = 1, p = 0.080). Post hoc analyses also showed that male mating tactic was positively correlated with the mating tactic of the specific set of tutors it was reared with in HP males (β ± SE = 0.27 ± 0.11, z =2.51, p = 0.013, **Fig. 4A**), but not in LP males (β ± SE = 0.01 ± 0.11, z =0.05, = 0.958; **Fig. 4B**).

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Commented [CA2R1]: This one is tricky because without an interaction effect there isn't a reason to continue to the post-hoc test which shows the main result. I think we need to include it even though it could be criticized. Maybe we could put in a sentence to get ahead of that like "we moved forward with the post-hoc test due to the apparent trend despite it's lack of significance". Or something better than that lol.

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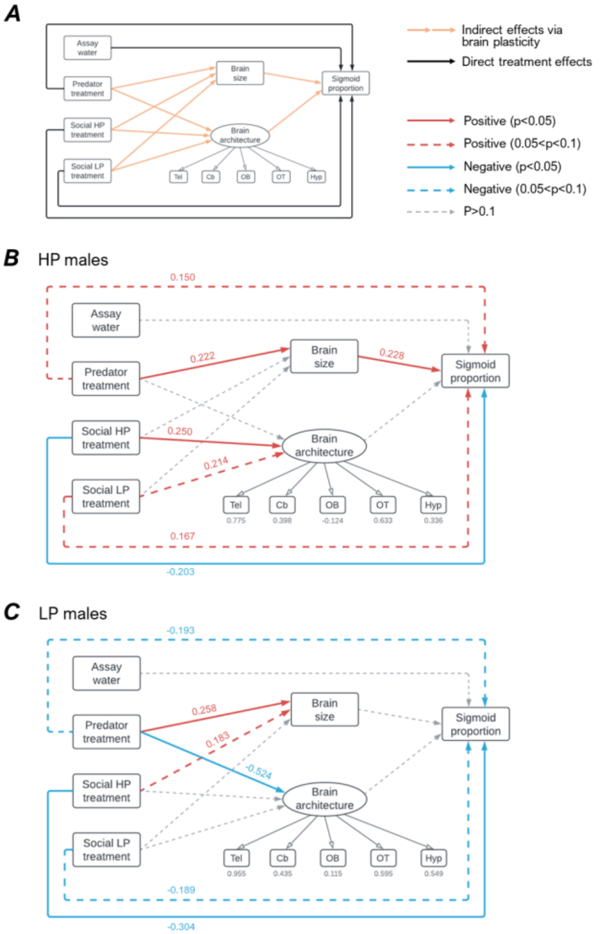
Confirmatory path analyses

In HP males, we found an indirect effect of predator treatment on mating tactic through brain size. Predator cues during development increased brain size, which increased courtship proportion in mating tactic (**Fig. 5B**). In LP males, predator cues increased brain size and altered brain architecture (i.e. the relative sizes of brain regions), but these changes in brain morphology did not translate to predictable changes in mating tactic (**Fig. 5C**). In contrast, the effects of social treatment on both HP and LP male mating tactic were both primarily direct. Exposure to conspecifics (either HP or LP tutors) during development altered brain architecture in HP males, and exposure to HP tutors marginally increased brain size in LP males. However, neither of these changes in brain morphology translated to changes in mating tactic (**Fig. 5B-C**). Detailed analyses on the effects of social and predator treatments on brain morphology is reported in another paper (Axelrod *et al. in review*) and summarized in the discussion.

Figure 5 Path analyses of the direct and indirect effects of predator and social treatments on male sigmoid proportion in mating assays. The arrow style and color indicate directions of effect and statistical significance (see key in figure), and the numbers next to the arrows show standardized coefficients. Squares indicate measured variables, and circles indicate latent variables. **A**: Conceptual diagram. **B:** HP male model estimates. **C:** LP male model estimates. (Baselines: tutor treatment = solo; predator treatment = pred-; assay water = pred-)



Discussion

Relative importance of genetic divergence and behavioral plasticity

Male mating tactic in HP and LP habitats was shaped by interactions among genetic divergence, social experience, and predator-induced plasticity. Population origin only accounted for 10% of the explained variance in proportion of courtship display. In contrast, social treatment accounted for more than 60% of the explained variance, indicating that social learning is an important factor shaping mating tactics in guppies. Interactions of population origin with social and predator treatments also accounted for a substantial amount of variation, suggesting that HP and LP males responded differently to the social and predator treatments. Notably, individuals were very consistent in their mating tactic. Despite being tested in pred+ and pred- water, repeatability of the two assays was very high (repeatability = 0.718), indicating that males persist in their tendency to court or sneak more across high- and low-immediate predation risk contexts. This matches with a previous study finding that while guppies show behavioral flexibility to different sex ratios, they persist in their mating tactic tendency across contexts (Magellan & Magurran, 2007).

Juvenile social learning shapes male mating tactic

The effect of social treatment was not dependent on predator treatment (i.e. no detectable social x predator treatment interaction; **Table 1**), suggesting that social learning tendency was not influenced by predation risk experienced during development. Comparisons between the two social treatments with tutors suggested the hypothesis that HP males socially learn their mating tactics whereas LP males do not. HP males reared with HP tutors adopted a characteristic HP mating tactic (more sneak dominant) whereas HP males reared with LP tutors adopted an LP mating tactic (more courtship dominant; **Fig.2A**). In contrast, LP males reared with HP tutors and LP tutors exhibited similar mating tactics (**Fig. 2B**). In concordance with this learning hypothesis, we found that a male's mating tactic was positively correlated with the mating tactic of the specific set of tutors it was reared with in HP males, but not in LP males (**Fig. 3**). In guppies, both adult males and females copy the mate choice of other individuals (Dugatkin & Godin, 1992; Pusiak et al., 2020). Our finding that HP, but not LP males copied their mating tactic from the social group they grew up with adds to the evidence that social learning is a key factor shaping sexual selection dynamics.

Predation risk is a strong evolutionary driver of social group formation (Krause et al., 2002) and social learning (Brown & Chivers, 2005; Coolen et al., 2005). Guppies under high predation risk shoal more (Seghers & Magurran, 1991) and form tighter social networks (Heathcote et al., 2017). HP habitats are also temporally and spatially more variable in predation risk. Similar to previous studies (Kotrschal et al., 2017; Reddon et al., 2018), we found that HP males overall had larger brains compared to LP males in this study ($\beta \pm SE = 0.36 \pm 0.16$, $t = 2.26$, $p = 0.037$). This is likely because of the higher cognitive demands of avoiding predators and forming a tighter social structure in HP environments (van der Bijl & Kolm, 2016). Combined, males in HP habitats likely have more opportunity, cognitive capacity, and evolutionary pressure to learn their mating tactic from their social group. Interestingly, a previous study found that LP males show greater plasticity in mating behaviors in response to the demographic composition of their social group compared to HP males (Rodd & Sokolowski, 1995). This suggests HP and LP guppies may respond differently to components of their social environment (e.g. sex ratio, density, or social knowledge) to adjust their mating tactics.

Previous studies have shown that HP and LP males reared in groups with other developing juveniles of the same population develop mating tactics that reflects their wild counterpart (Luyten & Liley, 1985; Yang et al., 2023), suggesting that at least part of the mating tactic divergence is genetic. However, instead of reflecting a genetic baseline of HP and LP mating tactic, we found that both HP and LP males reared without other conspecifics performed a comparatively high proportion of courtship and

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low proportion of sneak mating (**Fig. 2**). Social deprivation often leads to decreased or abnormal courtship or mating behaviors (e.g. rat: Duffy & Hendricks, 1973; fish: (Hesse et al., 2016). This suggests that while LP guppies don't learn the specific behavioral tactics of the adults they are reared with, social experiences still have an important effect on the development of their mating tactic.

Developmental plasticity and behavioral flexibility to varying predation risks

Ontogenetic exposure to elevated predation risk often induces phenotypic plasticity that increases defense against predators (Tollrian & Harvell, 1999). For mating tactic, an adaptive defense to elevated predation risk may be to reduce courtship displays and increase sneak mating attempts. Alternatively, an increasing courtship display in response to background predation risks may also be adaptive: the increased activity when no predators are immediately present can compensate for the lost time during actual antipredator responses (e.g. hiding). For example, both guppies and sticklebacks that have ontogenetic experiences with predators tend to resume feeding faster or feed at a higher rate after a predation stimulus (Álvarez & Bell, 2007; Elvidge et al., 2014). In this study, we found that HP males increased courtship proportion when reared with predator cue but did not change their mating tactic in response to predator cues in the immediate environment (**Fig. 2A**). In LP males, predator cue during development led to reduced behavioral flexibility to immediate predator cues: the behavioral flexibility of decreasing courtship proportion in response to immediate predator cue was only expressed when they were reared without predator cues (**Fig. 2B)**.

A factor that may influence our results is that we did not capture the response to predator cues in other modalities (e.g. visual cues). In many animals, predator cues from different modalities are often used simultaneously for assessing predation risks (Endler et al., 1997), and whether the cues provide aligning or conflicting information can influence an animal's behavioral decision (Ward & Mehner, 2010). For aquatic species, chemical cues can be detected from a further distance than visual cues but are less precise temporally and spatially. It is possible that for HP males, chemical predator cues on its own only signals higher background predation risk. HP males may increase allocation in reproduction by elevating their baseline courtship proportion when background mortality is high to compensate for the reduced courtship activity when they visually detect a predator. LP males reduced courtship in the presence of chemical predator cues, but only when they have not been developmentally exposed to the same cues. It is possible that the constant exposure to chemical predator cues without visual cues or observed predation events may lead to the chemical cues becoming a less reliable indicator of predator presence for the LP males. While these hypotheses require further experimental evidence, our results that HP and LP males responded differently to chemical predator cues in different temporal scales (ontogenetic *versus* immediate) suggests that behavioral plasticity has diverged between the two populations.

Several past studies provided some support that visual and chemical predator cues elicit different patterns of behavioral plasticity. Male guppies either show no response or an increased courtship proportion when they are developmentally or contextually exposed to predator cues (Broder, 2016; Chuard et al., 2020; Fischer et al., 2016). In contrast, studies that used live predators or visual cues found that males reduce courtship in the immediate presence of predators (Dill et al., 1999; Evans et al., 2002; Glavaschi et al., 2020; Godin, 1995; Magurran & Seghers, 1990). Furthermore, exposure to chemical cues may also alter how guppies respond to visual cues (Stephenson, 2016). It will be interesting for future studies to specifically tease apart how visual and chemical cues in the developmental and immediate environments interact to influence male mating tactic plasticity, and how such plasticity evolves to adapt to habitats with varying predation pressure.

Female-mediated behavioral change

We interpret the effects of rearing treatments and assay water as male behavioral plasticity. However, male guppies are sensitive to female receptiveness and often adjust their mating tactics accordingly. Studies have shown that males adjust mating behaviors even when only the female is exposed to predator visual cues (Dill et al., 1999; Evans et al., 2002). In this study, males were assayed using a female from the same population and treatment category. It is possible that our observed differences among populations and treatments may, at least partly, be mediated by female behavior. However, we also found that these same males showed similar tendencies of sigmoid display versus gonopodium thrust (similar to sneak behavior but toward a male) without females in a male-male aggression context (Yang et al. *unpublished data*). This suggests that differences in male behaviors observed in mating assays were not entirely dependent on female behavior. It would be interesting for future studies to tease apart the degree to which changes in male mating tactic is induced directly by environmental cues or indirectly by female behavior.

Mated and virgin females differ in receptiveness (Kodric-Brown & Nicoletto, 2001a), and prior mating experiences of a male can also have an effect on his mating tactic (Guevara-Fiore & Endler, 2018). We observed successful matings in ~10% of both first and second assays, and the probability of mating is not significantly different across treatments (**Table S3**). Whether the pair mated in the first assay did not influence male mating tactic in the second assay. We also re-ran the models excluding all assays with successful matings and received qualitatively similar results (**Table S8**). This suggest that our findings in male mating tactic plasticity in response to social and predator cues are robust to female receptiveness difference across treatments.

Brain morphology partly underlies plasticity in mating tactic

In this experiment, brain morphology was influenced by population, predator treatment, and social treatment (a more detailed analysis is presented in Axelrod et al. *in review.*). Our analyses revealed that HP males overall have larger brains than LP males regardless of the treatments. Predator cues and social interactions (regardless of tutor type) during development also led to larger brains in both HP and LP males. As for brain architecture, HP males overall have relatively smaller optic tectum and telencephalon than LP males. When reared with predator cues, both HP and LP males developed larger olfactory bulbs, but smaller cerebellum and telencephalon regions. LP males also developed smaller optic tectum and telencephalon regions when reared with predator cues, but HP males did not show this plasticity. In comparison, the effect of social treatment on brain architecture was limited.

We found that changes in mating tactic induced by predator cues were associated with developmental plasticity in brain morphology in HP males (**Fig. 4**). This suggests that shifts in mating tactic in response to developmental exposure to high predation risk were partly due to developmental plasticity in brain morphology. Interestingly, predator cues modified LP male brains in similar ways, but these changes in brain morphology did not translate to predictable changes in mating tactic. In contrast, changes in brain morphology due to social treatments were not associated with the mating behavior variations in the treatments (**Fig. 4**). This suggests that behavioral changes acquired through social learning stem from rewiring or biochemical switches of neural networks, change in neural density, or changes in neural activities at a level that does not change gross brain morphology (Zupanc & Lamprecht, 2000). We did not estimate neuron numbers in this experiment, so variation in brain mass and region volumes may relate to differences in neuronal density or non-neural cells such as glia. However, it is important to note that brain mass in guppies has been shown to correlate with neuron number, reflecting similar neuronal densities for large- and small-brained guppies (Marhounová et al., 2019). Further, while the scaling of neuron number with brain mass or region volume has been shown to differ between clades (Herculano-Houzel, 2011, 2017), it is not known to vary within species.

While the link we found between brain morphology and mating tactic is correlational, these results provide support that even for the same behavior, the neural mechanisms that underlie plastic changes may be different when induced by different cues. A species level comparison in African cichlids showed that ecological factors in general exert a broader influence on brain morphology compared to social factors (Pollen et al., 2007; Shumway, 2010). It will be interesting for future studies to test whether this pattern is broadly applicable to fish and other taxa. Developmental, seasonal, and immediate shifts in various behaviors have also been shown to be associated with changes in neurogenomic expression profiles in both vertebrates and invertebrates (Cardoso et al., 2015; Harris & Hofmann, 2014). For example, swordtail females learn olfactory mate preferences from early exposure to conspecifics or hybridizing sister species, and this behavioral plasticity is associated with gene expression changes in olfactory receptors, neurogenesis and synaptic transmission (Cui et al., 2017). In guppies, developmental exposure to predator chemical cues alters brain gene expression profile, but primarily in the opposite direction of evolved differences between HP and LP habitats (Ghalambor et al., 2015). There is also substantial brain gene expression plasticity in response to predator cue, as well as the evolution of expression plasticity itself, across populations (Fischer et al., 2021). Evolutionary history with and developmental exposure to predators are also both associated with lowered cortisol release (Fischer et al., 2014). Future investigation on gene expression profiles and hormonal responses associated with socially learned or predator cue induced behaviors, along with our findings for brain morphology as a potential driver of behavioral differences, will paint a more complete picture of the various mechanisms underlying behavioral plasticity in mating tactic.

Conclusion

Behavioral plasticity allows animals to respond rapidly to changes in the social and ecological environment with an optimal behavioral tactic. Understanding how behavioral plasticity evolves is key to elucidating its role in adaptation. Here, we show that male guppies adapted to high- and low-predation environments differ in their response to social and ecological information in adjusting their mating tactic, demonstrating that the capacity for behavioral plasticity has evolved and diverged between the two predation regimes. This suggests that the classic mating tactic pattern in wild populations, that LP males court more and sneak less than HP males, is likely due to the combined effects of genetically inherited and developmentally plastic responses to predators and the social environment. Changes in mating tactic induced by predator cue were associated with developmental plasticity in brain morphology, but changes acquired through social learning were not. This suggests that even for the same behavior, plasticity induced by different cues may be achieved by different neural mechanisms. Our study demonstrates that behavioral plasticity evolves upon colonizing new environments and can strongly influence the direction of adaptation.

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DATA ACCESSIBILITY STATEMENT

Data and analysis code will be archived on Dryad or Figshare upon acceptance.

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