DISSERTATION

EVOLUTION AND PLASTICITY OF TRINIDADIAN GUPPIES IN THE FIELD,

THE LABORATORY, AND THE CLASSROOM

Submitted by

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Graduate Degree Program in Ecology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2016

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ABSTRACT

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A fundamental question in evolutionary biology is how organisms respond to new and changing environments. This question also has conservation implications in the face of human induced rapid environmental change, including invasive species, habitat loss, and climate change. In response to new or changing environments, populations may evolve genetic changes across generations, and individuals may also respond via phenotypic plasticity within a generation. We can use experimental methods and model systems to increase our understanding of the way that genes and the environment interact to shape phenotypes. The Trinidadian guppy is a small freshwater fish that exhibits phenotypic plasticity as well as rapid evolution in response to changes in the environment, namely changes in the predator community. We utilized experimental introductions and common garden experiments to investigate plasticity and evolution of cerebral laterality, genitalia, and mating behavior in guppies.

Predation pressure is thought to select for a higher degree of cerebral laterality, or consistency in the partitioning of tasks between hemispheres of the brain. However, we found no difference in laterality between populations that evolved with high versus low levels of predation in the wild (Chapter 1). Instead, brothers reared with chemical predator cues were more highly lateralized than their brothers reared without cues, which is likely adaptive plasticity since a higher degree of laterality is associated with enhanced antipredator behavior. This study revealed

the important but largely overlooked role of developmental plasticity in shaping cerebral laterality.

Next, we took advantage of an experimental introduction of guppies from an environment with many predators to four replicate streams that contained few predators. In only 4-8 guppy generations, males in the introduced populations evolved shorter gonopodia for a given body size compared to the source population with high predation risk (Chapter 2). This suggests that longer gonopodia are advantageous in environments with predators, consistent with the hypothesis that longer genitalia facilitate forced copulations and allow males to circumvent female choice. We also measured male mating behavior using the same experimental introduction. In approximately 8-12 generations, we documented evolutionary changes in several mating behaviors, but these patterns were not consistent across populations (Chapter 3). We also found that low food levels during development reduced mating effort, but we found no evidence of developmental plasticity in response to predator cues in the rearing environment. Instead, we found an important role for contextual plasticity, a reversible and rapid response to the current situation, evident in behavioral changes with acute chemical cues of predation. Contextual plasticity is though to be especially important for behavioral traits allowing flexibility in response to rapidly changing conditions. This represents one of the few empirical studies designed to explore evolution, developmental plasticity, and contextual plasticity in the same experiment.

The Trinidadian guppy is also a model system for science education, with locally adapted populations that provide an accessible example of evolution by natural selection. We created a hands-on authentic science program with live guppy experiments to teach evolution to middle school students (Chapter 4). Authentic science allows students to discover knowledge by conducting science as if they were practicing scientists, which should be particularly effective at

teaching evolution, yet few programs have been developed. Students who participated in our program exhibited significant increases in both knowledge and acceptance of evolution.

Our work with Trinidadian guppies documented patterns of evolution and plasticity in new environments for a series of traits using a powerful experimental framework. These experiments revealed a role for both genes and the environment in the way predation risk shapes cerebral laterality, genitalia, and mating behavior, suggesting that the relationship between plasticity and evolution is complex and likely depends on the trait being studied. We also demonstrated how the guppy system, and other organisms that exhibit local adaptation, can be used to develop engaging and effective authentic science programs to teach evolution to K-12 students.

DEDICATION

For my crushes Barak Obama and Stephen Colbert.

TABLE OF CONTENTS

1. PREDATOR-INDUCED PHENOTYPIC PLASTICITY OF LATERALITY

Summary

Lateralized brain function, or partitioning of tasks to separate hemispheres of the brain, directly impacts behaviour and fitness. Highly lateralized individuals tend to outperform nonlateralized individuals in many survival-related behaviours, such as the ability to learn, escape predators and multitask. Despite these benefits to lateralization, there is a great deal of observed variation in this trait within and between populations that is likely determined by the balance between costs and benefits of laterality in a given environment. Laterality appears to be heritable, but it can also vary with the environment experienced during development. We investigated the role of evolutionary history and developmental plasticity in determining the degree of laterality in the Trinidadian guppy, *Poecilia reticulata*, a freshwater fish that experiences variable predation pressure in its native range. We compared pairs of closely related populations that experience either high or low levels of predation in the wild, and manipulated the perceived predation risk in the rearing environment using a common garden split-brood design. We assayed laterality in mature male guppies using a detour test. Fish reared with exposure to chemical predator cues were more lateralized than their brothers reared without predator cues. This plastic response is in the direction we would predict if lateralization is favoured in environments with high predation risk. However, unlike findings in related species, we did not detect repeatable differences in degree of laterality associated with historical predation regime, suggesting that predation risk experienced over evolutionary history does not shape laterality patterns in guppies. Thus, although it is currently underemphasized in the literature, plasticity is likely an important contributor to variation in laterality.

Introduction

An animal's fitness is impacted by its degree of cerebral asymmetry, or laterality, which is the partitioning of tasks to separate hemispheres of the brain (Dadda, Bisazza, & Nepomnyashchikh, 2012; Rogers, 2000). Laterality is likely a very ancient adaptation in vertebrates, as it is found among birds, mammals, fish, reptiles and amphibians (Bisazza, Rogers, & Vollortigara, 1998; Vollortigara, Rogers, & Bisazza, 1999). Highly lateralized individuals outperform nonlateralized individuals in spatial orientation (Sovrano, Dadda, & Bizassa, 2005), shoaling in fish (Bisazza & Dadda, 2005), predator escape (Dadda, Koolhaas, & Domenici, 2010), learning (Bibost & Brown, 2014; Magat & Brown, 2009), foraging in the presence of predators (Dadda & Bisazza, 2006a) and foraging while avoiding harassment from conspecifics (Dadda & Bisazza, 2006b).

Because lateralized individuals tend to outperform nonlateralized individuals, it has been proposed that selection, particularly from predation (Rogers, Zucca, & Vallortigara, 2004), should consistently favour lateralization (Ghirlanda & Vallortigara, 2013; Rogers, 2000). Yet we observe incredible variation in the degree of laterality among species, populations and individuals (Bisazza, Pignatti, & Vallortigara, 1997a; Vallortigara et al. 1999), suggesting that there may be disadvantages and costs to being highly lateralized (Dadda, Zandona, Agrillo, & Bisazza, 2009). Lateralized individuals tend to respond differently to stimuli from the left and right side, which may create a disadvantage when stimuli appear at random in the environment (Valortigara & Rogers, 2005), and they may perform poorly when tasks require coordination between hemispheres (Dadda et al., 2009). Additionally, the link between laterality and personality traits may be disadvantageous; lateralized individuals are consistently bolder (Reddon & Hurd, 2009) and more aggressive (Reddon & Hurd, 2008) than nonlateralized

individuals, which may not be optimal in all situations and environments. The balance between costs and benefits of laterality likely determine the degree of asymmetry observed in any given species and environment (Corballis, 2006, 2008).

Laterality appears to be heritable (Bisazza, Facchin, & Vallortigara, 2000), allowing researchers to artificially select for lateralized and nonlateralized lines (Facchin, Argenton, & Bisazza, 2009). However, genetic differences cannot explain all of the variance in laterality (Bisazza et al., 2000), and furthermore, some lateralized functions cannot be artificially selected (Bisazza, Dadda, & Cantalupo, 2005), suggesting a potential role for the environment in shaping laterality (Rogers, 2006). Developmental plasticity, where the phenotype expressed by a given genotype can vary depending on the rearing environment, can influence laterality (Vallortagara & Rogers, 2005). For example, light exposure during development can affect the degree and direction of lateralization in pigeons (Güntürkün, 1993), chickens (Rogers, 1997), and a topminnow fish (Dadda & Bisazza, 2012). Hormones, particularly androgens like testosterone and corticosterone, have also been implicated, although this work is underemphasized (Rogers, 2006). Despite the recognized importance of genes, the environment, and their interaction in shaping phenotypes to a particular environment (West-Eberhard, 2003), theories about the roles of genes and the environment in shaping laterality have not been experimentally integrated (but see Rogers, 1995).

To investigate the roles of evolutionary history and the rearing environment in shaping laterality, we used the Trinidadian guppy, *Poecilia reticulata,* a small freshwater fish. Previous studies have demonstrated cerebral lateralization in guppies (i.e. the use of the left side of the brain when viewing potential mates; Bisazza et al., 1997a) and in other poeciliids (Bisazza & de Santi, 2003; Bisazza, Facchin, Pignatti, & Vallortigara, 1998; Bisazza et al., 1997a; Brown et al.,

2004), although the direction and degree of lateralization varies across species, populations and contexts. Trinidadian guppies naturally occur in streams that vary in predation pressure because barrier waterfalls typically exclude predators, but not guppies, from upstream migration (Houde, 1997; Magurran, 2005). Thus, river drainages often contain populations of guppies exposed to high levels of predation (hereafter referred to as HP) and closely related upstream populations that exist with only one other fish, a minor guppy predator *Rivulus hartii* (Carvalho, Shaw, Magurran, & Seghers, 1991; Crispo, Bentzen, Reznick, Kinnison, & Hendry, 2006; Fajen & Breden, 1992). These low-predation (hereafter referred to as LP) populations have evolved unique phenotypes that differ from nearby high-predation populations across multiple traits, including colour, behaviour, morphology and life history strategy (Houde, 1997; Magurran, 2005). Although laterality has not been compared between high- and low-predation populations of *P. reticulata*, environments with high levels of predation risk are expected to favour lateralization because of its associated predator escape, multitasking and shoaling benefits (Brown, Western, & Braithwaite, 2007; Dadda et al., 2012; Rogers et al., 2004).

To measure the role of evolutionary history in shaping laterality, we compared two pairs of natural high- and low-predation populations of *P. reticulata* reared in a common garden environment. We predicted that guppies from high-predation sites would show greater lateralization than those from low-predation sites if predation risk selects for laterality. To experimentally integrate the role of developmental plasticity in shaping laterality, we used a split-brood design to rear brothers in an environment with or without chemical predator cues. We predicted that males exposed to predator cues during development would show greater lateralization than their brothers reared without predator cues if the trait exhibits adaptive plasticity in response to perceived risk.

4

Methods

Collection and Husbandry

  We collected juvenile *P. reticulata* from two HP rivers and two LP rivers (Aripo HP_1, Guanapo HP_2, Naranjo LP_1 and Tumbason LP_2) on the southern slope of the Northern Range Mountains in Trinidad, West Indies, in May 2011. The Aripo is a high-predation river that is downstream from the low-predation Naranjo River in one drainage. Similarly, the high-predation Guanapo is downstream of the low-predation Tumbason River in a neighbouring drainage. We collected 50 fish from each location, transported them to Colorado State University, and reared them for two generations under common garden conditions to minimize maternal and other environmental effects. Wild-caught guppies were housed in mixed-sex pairs (approximately 25 pairs per population) until females produced first-generation broods. We separated each brood by sex at 5 weeks of age when females could be distinguished from males by a patch of melanophores near the gonopore (following Reznick, 1982). We randomly selected one male and one female from each family line to rear to adulthood and randomly crossed unrelated pairs to produce a second generation. Within 24 h of birth, we divided each second-generation brood into two treatments that differed in exposure to predator cues (following Handelsman et al., 2013). Half of each brood was placed in a 1.5-litre tank that received recirculating water from a sump that housed a common guppy predator, the pike cichlid, *Crenicichla frenata.* Cichlids were fed two guppies daily so that experimental guppies were exposed to predator kairomones as well as alarm cues released by the epidermal club cells of consumed conspecifics. The other half of each brood was reared in an identical 1.5-litre tank that received recirculating water from a sump that did not house a predator. We sexed the second-generation fish at 5 weeks of age and continued to rear one male per family in an individual 1.5-litre tank in its respective treatment until we

assayed its laterality; thus, at the time of the assays, males reared in the predator treatment were exposed to the chemical cues of predation their entire lives. We used males rather than females in this experiment because they were available as part of another study on male mating behaviour that utilized the same rearing design, and we lacked space to maintain females individually under these treatments. Fish were maintained at 24 °C on a 12:12 h light:dark cycle and were fed a specific quantity of food adjusted to their age twice daily (a paste made of TetraMin® Tropical Flakes (Spectrum Brands, Inc., Cincinnati, OH, U.S.A.) in the morning and brine shrimp nauplii, *Artemia* spp. (Brine Shrimp Direct, Inc., Ogden, UT, U.S.A.) in the evenings). Experimental fish were held in individual 1.5-litre clear plastic tanks that contained neutral gravel as well as a constant flow of water. Although fish had continuous visual access to guppies in neighbouring tanks, direct social experience was limited to 24 h of exposure to three females and a total of 2 h of experience with six other males from the same population and treatment. These controlled social interactions, which formed the basis of another study, were provided to each male systematically over the course of 4 weeks before the laterality assays.

Laterality Assays

We assessed laterality in sexually mature males (mean ±SE age = 130±2.4 days, $N = 133$) from all four populations and both treatments using a detour test. Detour tests, frequently used to assay laterality in fish (Bisazza, Pignatti, & Vallortidara, 1997b), force the individual to turn either right or left to view an object directly in front of them, thus using one eye and the corresponding opposite side of the brain to process information about the object. We designed and constructed a testing apparatus that was circular to avoid having either choice yield a dead end, which could influence choices in subsequent tests; instead, fish could choose to view an

6

object with either the left or right eye and then continue swimming indefinitely in that direction. The apparatus was made of acrylic and consisted of an acclimation chamber (8 × 12 cm) attached to a circular arena (30 cm diameter) that contained a semi-opaque circular barrier (15 cm diameter) with a target in its centre (Figure 1.1). We used a target (an object for the fish to view with one eye) because it has been found that laterality may be underestimated when assays are performed without one (Bisazza et al., 1997b; Brown et al., 2007). The target was a fish lure consisting of two identical generic fish models (3.5 cm long) facing opposite directions to avoid influencing the direction chosen by the test fish. Because the goal of this study was to measure the degree of lateralization and not to map the side of the brain that responds to a particular stimulus (e.g., a female or a predator), we used an arbitrary fish-shaped target that did not closely resemble either a guppy or a guppy predator.

Laterality assays were conducted during 21 May 2012–5 March 2013. Prior to assaying laterality, the testing apparatus was filled to a depth of 15 cm with water that did not contain predator cues. We used a net to transfer an individual fish to the acclimation chamber. After 2 min (following Reddon & Hurd, 2008, 2009), we slowly removed the barrier, allowing the fish to exit the acclimation chamber and enter the circular arena. If the fish remained in the acclimation chamber after 30 s and was not actively exploring (36% of tests), we directed the fish towards the arena with a small green dip net (following Bisazza et al., 1997b; Reddon & Hurd, 2008). For each laterality assay, we recorded whether the fish turned left or right after it swam past the walls of the acclimation chamber (double dashed lines in Figure 1.1). After each test, the fish was returned to its individual tank. Each fish completed the detour test 10 times (following Bisazza et al., 2000) in 7 days with no more than two assays per day (separated by a minimum of 1 h). Because some guppies can learn to avoid negatively reinforced stimuli after

fewer than 10 experiences in succession (Budaev & Zhuikov, 1998), we spaced the trials out over 1 week to minimize the possibility that handling after each trial might cause fish to turn a different direction in the subsequent trial. We also included three individuals that completed fewer than 10 trials: two that completed seven trials and one that completed nine trials.

Analysis

To calculate a laterality score for each individual based on its 10 laterality assays, we used the following equation (Bisazza et al., 1997b): ((right turns – left turns)/(right turns + left turns)) × 100. Positive values indicate an individual's propensity to turn right, use the left eye to view the target, and thus use the right side of the brain to process the target. Conversely, negative values indicate an individual's tendency to turn left, use the right eye to view the target, and thus use the left side of the brain. Because we were particularly interested in measuring the degree of laterality, more so than direction (left or right), we took the absolute value of the laterality scores. Degree of laterality is a better measure of individual consistency in laterality since it ignores direction, whereas mean laterality scores include positive and negative values that may cancel each other out, resulting in a more conservative mean value that may not differ from zero even though a large portion of individuals may be highly lateralized (Brown et al., 2007). We then estimated differences in both the laterality score and the degree of laterality between predation regimes and treatments in two separate mixed-effects ANOVAs (following Bisazza, Facchin, et al., 1998) with predation regime (HP or LP), treatment (reared with or without cues), and their interaction included as fixed effects. We included family identity as a random effect to account for brothers across treatments. The age at which males were assayed did not differ across treatments ($F_{1,131} = 0.77$, $P > 0.1$), although the HP guppies were 10 days younger (mean $\pm$SE =

8

125.90 ± 3.13, $N = 79$; $F_{1,131} = 4.09$, $P = 0.045$) than the LP guppies (mean ±SE = 135.92 ± 3.77, $N = 54$) because of their earlier maturation (consistent with Reznick, Shaw, Rodd, & Shaw, 1997). However, age was not predictive of laterality score or degree of laterality (both $F_{1,131}$ <1.67, P >0.1) and hence was not included in our analysis. Similarly, laterality score and degree of laterality did not differ across drainages (both $t_{131} < 0.56$, $P > 0.1$); thus, we present results from both drainages combined. These analyses were conducted using JMP® version 11 (SAS Institute, Cary, NC, U.S.A.).

Ethical Note

To incorporate both conspecific alarm cues and predator kairomones in the predator rearing treatment, we fed live guppies to *C. frenata* predators housed in the sumps of our recirculating systems. Although other methods are available for extracting alarm cues (i.e. lacerating the skin of euthanized guppies), we used live guppies because the wild-caught predators required a live fish diet, and direct predation provided a way to administer realistic quantities of alarm cues. These methods were approved by Colorado State University's Institutional Animal Care and Use Committee (protocol number 11-3072A).

Results

The laterality score of an individual can range from -100 (left side of brain dominant) to +100 (right side of brain dominant), and averages of these scores for a group of individuals can provide information about the consistency of directionality. We observed no difference between guppies from the HP and LP predation regimes in laterality score ($F_{1,129} = 0.99$, $P > 0.1$), in part because the average laterality scores were not strongly positive or negative for either of these

9

groups (mean ±SE: HP: -10.21±4.66, $N = 79$; LP: -2.94±5.62, $N = 54$). We did observe a treatment effect ($F_{1,129} = 5.14$, $P = 0.03$); fish reared with predator cues were more negatively lateralized (mean ±SE = -14.40±5.05, $N = 68$), tending to use the left side of the brain more than fish reared in the absence of predator cues (mean ±SE = 1.23±4.99, $N = 65$). There was no interaction between predation regime and treatment ($F_{1,129} = 0.31$, $P > 0.1$).

Degree of laterality is measured as the absolute value of the individual's laterality score and can range from 0 to +100, and averages of these scores indicate the degree of lateralization of individuals within a group, regardless of the consistency in directionality. We observed no differences between predation regimes, HP or LP, in degree of lateralization ($F_{1,129} = 1.11$, $P > 0.1$; Figure 1.2). We did observe a treatment effect ($F_{1,129} = 4.19$, $P = 0.04$); fish reared with predator cues were more highly lateralized than fish reared in the absence of predator cues (Figure 1.2). There was no interaction between predation regime and treatment ($F_{1,129} = 0.14$, $P > 0.1$).

Discussion

Much of the previous work on cerebral laterality has focused on its evolutionary origins and the selective pressures shaping the trait (e.g., Takahashi & Hori, 1994; Vallortigara et al., 1999), including the potential benefits for predator evasion (Dadda et al., 2012). However, studies investigating natural geographical variation in laterality across variable predation regimes have failed to evaluate environmental contributions to individual laterality. We used a common garden design to compare populations that evolved under low and high predation risk and we manipulated the perceived predation risk during development to simultaneously investigate the roles of evolutionary history and rearing environment on laterality in guppies. We did not find

consistent differences in either laterality score or degree of laterality between populations that evolved under high and low predation risk. However, we did find that males reared in the presence of predator cues were more negatively lateralized, indicating greater use of the right side of the brain and the left eye to view the stimulus. Similarly, degree of laterality, or individual consistency ignoring direction, was greater for guppies reared with predator cues, supporting predictions.

Previous work by Brown et al. (2004, 2007) comparing poeciliid fish (*Brachyraphis episcopi*) from high- and low-predation environments found that HP populations were more highly lateralized than LP populations. Furthermore, the similarity in degree of laterality between wild-caught fish and first-generation laboratory-reared fish suggested that the pattern was heritable (Brown et al., 2007). Our findings suggest that some of the established geographical variation in laterality may be explained by developmental plasticity rather than evolutionary history. In our study, second-generation laboratory-born fish did not differ among populations in laterality score or degree of laterality, but fish reared in the presence of predator cues showed a higher degree of laterality than fish reared in the absence of predator cues. Note, however, that our power of inference was limited to two pairs of high- and low-predation populations, and thus our findings may not be generally representative of laterality patterns across predation gradients.

Differences between our findings and those of Brown et al. (2004, 2007) could be attributed to a number of factors. Laterality patterns are known to vary depending on the species (Bisazza et al., 1997a; Bisazza, Facchin et al., 1998), the sex (Bisazza, Facchin et al., 1998; Brown et al., 2007) and the target used in laterality assays (Bisazza, Facchin et al., 1998; Brown et al., 2004, 2007), so our use of a different species, of males rather than females, and of a different target could explain some differences. Females are generally expected to show greater

11

laterality than males in order to more efficiently multitask as they avoid forced copulations and predation while foraging (Dadda & Bisazza, 2006b). However, male–female comparisons have yielded mixed results (e.g., Bisazza, Facchin, et al., 1998), finding sex differences only in certain populations and treatments (Brown et al., 2007). It would be interesting to repeat our study, testing both male and female responses to multiple targets, to estimate sex differences in the strength and direction of laterality. Another difference between our study and Brown et al. (2004, 2007) is that our fish were reared in individual tanks rather than in large group tanks. If laterality facilitates social interactions and shoaling (Bisazza & Dadda, 2005; Brown et al., 2007), it is possible that direct social contact promotes the development of laterality and that our simple rearing environment led to lower laterality scores. However, if this lack of social complexity affected all fish similarly, it would have been unlikely to influence our population and treatment comparisons. Finally, an important difference between our study and Brown et al. (2004, 2007) is that we used second-generation laboratory-reared fish, rather than wild-caught or first-generation fish, which likely allowed us to reduce maternal and other environmental effects more fully in our population comparisons.

The plastic response we observed was similar in direction and strength for guppies from both high- and low-predation environments, as indicated by the similarity in slope and intercept of the reaction norms (Figure 1.2). This plasticity is likely adaptive since a higher degree of laterality should increase fitness in environments with greater predation risk (Pitcher, 1986; Rogers et al., 2004). Laterality is thought to be a mechanism that facilitates social interactions, particularly shoaling (Bisazza & Dadda, 2005; Brown et al., 2007) and enhances cognitive ability (Megat & Brown, 2009), thus increasing survival in environments with high predation risk.

Here we suggest three possible explanations for the consistency of this adaptively plastic response across predation regimes. First, there could be strong selection for plasticity in environments with predators, and this plasticity persists even when guppies colonize locations without predators. Low-predation populations have been repeatedly founded from high-predation populations throughout Trinidad (Houde, 1997; Magurran, 2005), and plasticity in response to predator cues could persist in the absence of the selective force of predation if it is not costly to maintain. However, this may not be likely if plasticity is costly (DeWitt, Sih, & Wilson, 1998), or if lateralization is maladaptive in the absence of predation, for example because individuals respond inappropriately to stimuli from the right and left side (Valortigara & Rogers, 2005) or if they perform poorly at tasks requiring coordination between hemispheres (Dadda et al., 2009). Second, selection on correlated traits may maintain plasticity. If there is selection on traits, like aggression, that are tightly and positively correlated with laterality (Reddon & Hurd, 2008, 2009), we might expect plasticity to persist. This is perhaps unlikely since Irving and Brown (2013) found only weak links between laterality and several plastic personality traits like boldness, activity level and sociability in a feral population of guppies. A third explanation is that low-predation environments contain enough predation risk to maintain the plastic response. The multipredator hypothesis suggests that different antipredator responses are pleiotropically linked such that removal of some, but not all, predators would result in the persistence of antipredator behaviour (Blumstein, 2006). Guppies in low-predation environments still experience some predation through cannibalism and predation from *R. hartii,* which is known to prey on juvenile guppies.

In the future it will be interesting to explore the mechanisms during development that are responsible for the plastic response we observed. Hormones, specifically testosterone and

corticosterone, are known to affect laterality in birds (Rogers, 2006; Vallortigara et al., 1999) and may act similarly in fish. Predator cues experienced by male guppies during development reduces the amount of glucocorticoid steroid hormone (cortisol) that fish release into the water (Fisher, Harris, Hoffman, & Hoke, 2014).

Finally, this work is germane to the recent conversation about behavioural responses to human-induced rapid environmental change (Sih, Stamps, Yang, McElreath, & Ramenofsky, 2010). The plasticity of laterality in response to predation may have consequences for the ways that animals respond to rapid changes in predation regime, particularly given that laterality is correlated with many other behaviours and traits that are intimately linked with fitness (e.g., Bisazza & Dadda, 2005; Dadda & Bisazza, 2006a, 2006b; Dadda et al., 2010). Thus, animals may be able to respond via lateralization to the introduction or removal of a novel predator in just one generation. However, a better understanding of the mosaic nature of traits is needed to predict responses to environmental change. Although laterality is positively correlated with a variety of antipredator behaviours like shoaling (Bisazza & Dadda, 2005) and multitasking (Dadda & Bisazza, 2006a), we still lack a general understanding for how groups of plastic and nonplastic traits respond to changes in the environment, for example whether they respond independently or in an integrated manner (Ghalambor, McKay, Carroll, & Reznick, 2007). Investigating behavioural and personality traits in conjunction with laterality (e.g., Irving & Brown, 2003) will improve our understanding of the potential role of cerebral laterality as an overarching mechanism for correlations among traits.

This study is one of the first to assess the roles of both evolutionary history and rearing environment on laterality and illustrates the importance of developmental plasticity. We suggest

that developmental plasticity is currently underemphasized, and we encourage researchers investigating geographical variation of traits to consider its potential contribution.

Contributors

Figure 1.1. Schematic diagram of detour test apparatus. The square acclimation chamber was separated from the circular arena with a removable barrier (double solid line). The target was centered inside a semi-opaque barrier (dotted circle). Fish choice was recorded as left or right when the fish travelled past the bounds of the acclimation chamber (crossing one of the double dotted lines).
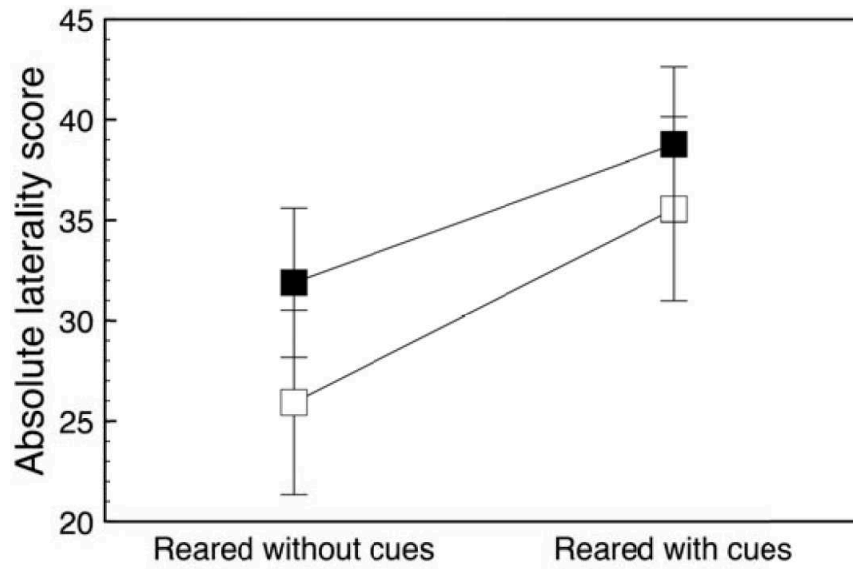
Figure 1.2. Reaction norms showing the degree of laterality, or the mean absolute laterality score ±SE for the high-predation (filed squares) and low-predation (open squares) populations across the two treatments, reared without and with predator cues.

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II. DEVELOPMENTAL PLASTICITY AND RAPID EVOLUTION OF GENITALIA

Summary

Male genital morphology exhibits a great deal of variation both among and within species and is intimately linked with mating success and fitness. Sexual selection, nonmating natural selection, and neutral forces have all been explored as potential evolutionary drivers of male genital divergence. Though less explored, genital diversity may also be influenced by plasticity if the rearing environment shapes the development of genitalia. In poeciliid fishes the length of the male intromittent organ, the gonopodium may be shaped by sexual selection, if longer gonopodia attract females or aid in forced copulation attempts, or by nonmating natural selection, if shorter gonopodia allow predator evasion. Males also tend to emphasize forced copulations over attracting females in environments with greater risk, complicating predictions for whether predation pressure selects for longer or shorter gonopodia. Further, it is unknown whether gonopodia are plastic in response to the rearing environment, potentially explaining some of the geographic trait variation observed. We tested whether a reduction in predation risk causes the evolution of genitalia, and whether predator cues and food levels in the rearing environment shape the development of genitalia. To do this, we took advantage of an experimental introduction of guppies from a high predation environment to four replicate streams in Trinidad that lacked major predators. After two and three years, we reared fish from the source and introduced populations in a common garden environment to detect evolutionary differences between populations, and we used a split-brood design to evaluate plasticity in response to different rearing environments (with and without predator cues, under high and low food levels). Compared to the source population, the introduced populations showed a decrease in

gonopodium length after accounting for body size suggesting that a reduction in predation risk selects for shorter gonopodia. Although gonopodium length seemed to be shorter in males reared with predator cues, we did not detect a significant decrease after accounting for the decrease in body size. Brothers reared on low food levels had longer gonopodia relative to body size than those on high food, reflecting a maintenance of gonopodium length despite a reduction in body size, which may be adaptive if gonopodium length is tied to reproductive success. This study demonstrates the importance of both evolution and phenotypic plasticity in explaining genital variation.

Introduction

Variation in genitalia within and among species tends to be greater than other morphological traits, so much so that genitalia are often used to distinguish among closely related species (Eberhard 1985; Hosken and Stockley 2004). In an effort to understand rapid genital divergence, much work has focused on the selective forces shaping intromittent organs (Eberhard 1985; Hosken and Stockley 2004). These selective forces include selection to avoid interspecific hybridization (lock-and-key hypothesis, Eberhard 1985), selection on other traits (the pleiotropy hypothesis, Mayr 1963), as well as several sexual selection mechanisms including female choice (the good genes hypothesis, Hamilton and Zuk 2016 and the sexy sons hypothesis, Weatherhead and Robertson 1979), sperm competition (Parker 1984), and sexual conflict (Eberhard 1985; Cameron et al. 2003; Hosken and Stockley 2004). Non-mating natural selection has also been implicated, as the size or shape of genitalia might affect the ability to avoid predation (Langerhans et al. 2005; Langerhans 2011).

Though there has been greater focus on the way selection shapes genetic differences in genitalia, it is also possible that developmental plasticity may play a role if the rearing environment influences genitalia. For example, male genitalia can be highly plastic in response to wave action (Neufeld and Palmer 2008, in barnacles), temperature (Andrade et al. 2005, in fruit flies) and food stress (Arnqvist and Thornhill 1998, in water striders). Genitalia may be canalized, exhibiting no plasticity, as a result of stabilizing selection for an optimal shape and size to match the average female size (Eberhard 1985; House and Simmons 2007). However, plasticity in response to food stress is expected if genitalia are condition-dependent traits (e.g., as predicted under sexual selection models where they indicate male quality; Arnqvist and Thornhill 1998). Additionally, nutrition-dependent developmental plasticity has been shown to alter the allometric relationship between sexual ornaments and body size in insects (Snell-Rood and Moczek 2012; Koyama et al. 2015).

Poeciliid fishes provide an excellent system in which to study the factors that influence the development and evolution of genitalia. Males have large, non-retractable intromittent organs called gonopodia that vary within and between species. Males also exhibit mating tactics that range from courting females with elaborate displays to forced mating attempts, and these behaviors correlate and may coevolve with genitalia (Wang et al. 2015). Males from species with shorter gonopodia have more complex precopulatory display behavior (Rosen and Tucker 1961), and individuals with relatively longer gonopodia perform more forced copulation attempts (Reynolds et al. 1993). Sexual conflict has been implicated to explain this pattern (Gasparini et al. 2011), since longer gonopodia are better able to make contact with females and more effectively circumvent female choice (Evans et al. 2011). However, the opposite pattern has also been found (Kolluru et al. 2014), with females preferring longer gonopodia, such that

26

gonopodium length reflects female choice rather than male-female conflict (Brooks and Caithness 1995; Kahn et al. 2009).

Nonmating selection on genitalia has also been studied in poecliids, specifically driven by the risk of predation. It is thought that large gonopodia provide drag, reducing swim performance and increasing predation risk (Langerhans et al. 2005; Langerhans 2011). In this system, sexual selection is overlaid with natural selection since mating behavior differs depending on the predation risk a population experiences. Presumably to minimize conspicuousness to predators, high predation males perform more forced copulation attempts and fewer displays than their low predation counterparts (Luyten and Liley 1985; Magurran and Seghers 1994), suggesting longer gonopodia may be selected in high predation environments to improve forced copulation success. However, when comparing gonopodia between high and low predation populations, findings have been inconclusive. High predation populations may have longer gonopodia (Kelly et al. 2000; Jennions and Kelly 2002, in *Poecilia reticulata*) or shorter gonopodia (Langerhans et al. 2005, in *Gambusia affinis* and *Gambusia hubbsi*) than their low predation counterparts, or there may be no difference (Evans et al. 2011, in *P. reticulata*). These inconsistencies may reflect the relative importance of proposed hypotheses in different systems, i.e., whether increased predation pressure leads to shorter gonopodia to minimize predation risk or because of reduced female preferences, or longer gonopodia to aid in forced copulations.

Despite extensive work on the evolution of gonopodia in poeciliid fishes, no work to date has used an experimental approach to investigate both the evolution and plasticity of gonopodia. We took advantage of experimental introductions of the Trinidadian guppy, *Poecilia reticulata*, to measure the direction of evolution of gonopodium length in four replicate populations of guppies that experienced a reduction in predation pressure. We used a common garden, split-

brood design to separate genetic evolution from environmentally induced plastic shifts in gonopodium length. We expected the four introduced populations to diverge from the source population if genital evolution can occur within 2-3 years, or 8-12 generations. If sexual selection plays an important role in genital evolution and long genitalia increase the success of forced copulations, we expected introduced males to evolve shorter gonopodia in the low predation environments where forced copulation is a less common strategy. We expected the opposite pattern if long gonopodia are selected through female choice, which should be stronger in the low predation environments, or if reduced predation risk allows for longer gonopodia because of relaxed selection on swim performance and predator evasion. To examine plasticity, we reared guppies with and without exposure to chemical cues of predation and under high and low food levels. If gonopodium length responds adaptively to perceived predation risk and is related to forced copulation success, we predicted males would develop longer gonopodia when reared with predator cues. Alternatively, males reared with predator cues could develop shorter gonopodia if the trait is primarily associated with precopulatory female choice or predator avoidance. We further expected gonopodium length to increase with food levels if it is a condition-dependent trait.

Methods

Experimental introduction

In a separate experiment to investigate eco-evolutionary dynamics, *P. reticulata* were moved from a large river in Trinidad containing predators to four headwater streams absent of guppies and major predators (for details see: Travis et al. 2014; Handelsman et al. 2013, 2014). The source site was a high predation location on the Guanapo River in Saint George, Trinidad

with many predators including cichlids (*Crenicichla frenata*, *Aequidens pulcher*) and characins (*Astayanax bimaculata*, *Hemibrycon dentatum*, *Hoplias malabaricus*). The four introduction sites previously contained only one fish species, *Rivulus hartii,* which is not a major guppy predator. Introductions into these low predation streams occurred in March 2008 (Lower Lalaja and Upper Lalaja; henceforth Intro1 and Intro2) and March 2009 (Caigual and Taylor; henceforth Intro3 and Intro4).

Collection and husbandry

In March 2011, three years post-introduction for the Intro1 and Intro2 populations and two years post-introduction for the Intro3 and Intro4 populations, 40 juvenile guppies were collected from each of the four introduction locations and the source population and brought to the laboratory at Colorado State University. Fish were reared in a common garden laboratory environment for two generations to minimize maternal and other environmental effects (rearing methods described in Handelsman et al. 2013, 2014, modified from Reznick 1982). Guppies were held in a flow through aquarium system fitted with 1.5-liter individual tanks on a 12:12 light:dark schedule at 27 ± 1°C. Guppies were fed flake paste (TetraMin® Tropical Flakes, Spectrum Brands, Inc., Cincinnati, Ohio, USA) in the morning and brine shrimp nauplii (*Artemia* spp.) in the afternoon. Quantities were scaled by age and approached *ad libitum.* Wild-caught guppies were allowed to mature before being randomly crossed to produce a G_1 generation. The G_1 broods of each female were sexed at the age of 29 days by noting the presence of melanophores on the abdomen (following Reznick 1982), and one son and one daughter were randomly selected from each female and reared to adulthood. Mature G_1 females were randomly crossed with unrelated G_1 males to create a G_2 generation. Within 24 hours of birth, we divided each G_2 brood into four

1.5-liter flow-through tanks and randomly assigned them to one of four treatments: high-food no-predator, low-food no-predator, high-food predator, and low-food predator. Fish in the "no-predator" treatment experienced recirculating water lacking chemical predator cues. In the "predator" treatment, the water circulated through a tank containing a pike cichlid (*Crenicichla frenata*) (Torres-Dowdall et al. 2012; Handelsman et al. 2013, Ruell et al. 2013). This cichlid was fed guppies so that fish in this treatment experienced chemical cues from the cichlid predator as well as alarm cues from conspecifics. Fish in the "low-food" treatment received the same foods at the same time as the high-food treatment, but the quantity was approximately half that of the high food treatment. We sexed guppies when they were 29 days old (see above). One male from each family and treatment was placed in an individual 1.5-liter flow-through tank in his respective treatment: high-food no-predator, low-food no-predator, high-food predator and low-food predator.

Gonopodium measurement

Gonopodium length was measured at the point each male reached sexual maturity. We considered a male mature when the hood of the gonopodium was seen to be even with or just past the distal tip of the gonopodium (Figure 2.1b; Reznick 1990). On the day each male became mature, he was anesthetized, using MS222 (ethyl 3-aminobenzoate methane sulphonic acid salt, Sigma-Aldrich, St Louis, MO, USA), and photographed. We placed each male on a white background with his left side facing up, and carefully spread the gonopodium away from the body using a fine-tipped artist paintbrush. We took digital photographs using a Cannon EOS Rebel T3 digital SLR camera fitted with a 60mm macro lens (Canon U.S.A. Inc.) and illuminated with fixed full-spectrum florescent lights. Each image included a metric ruler for

scale. We then used ImageJ64 (Schneider et al. 2012) to digitally measure standard length and gonopodium length to the distal tip. Gonopodium measurements were made by drawing a line from the distal tip to a linear approximation of the intersection of the base of the gonopodium with the body (Figure 2.1a). EDB performed all measurements. Each photo was measured twice ($r = 0.83$ for gonopodium length; $r = 0.99$ for standard body length) and the mean values for standard length and gonopodium length were used.

Analysis

To test for the evolution and plasticity of gonopodium length, we used a linear mixed model with body size, population, food treatment, and predation treatment as possible fixed effects as well as all 2-way interactions (R package lmer; Bates et al. 2005; R Core Team 2013). We then performed backward elimination using the "anova" command with Kenward-Roger approximation (R package pbkrtest; Halekoh and Hojsgaard 2014) to sequentially remove the effect with the largest p-value greater than 0.05 (R package lmerTest; Kuznetsova et al. 2015). To account for the relatedness of brothers across treatments, we included family as a random effect in all models. With the exception of male age, we did not remove any of the fixed effects since they were part of the experimental design. Our final linear mixed model included body size, population, food treatment, and predation treatment as fixed effects as well as an interaction between body size and the food treatment. Visual inspection of the predicted-versus-residual-plot verified the fit of the linear model; thus we did not use a nonlinear model or log transform gonopodium length or body size (though model results from log-transformed data were the same as those presented below). To compare the introduced populations to the source population, we performed a post hoc contrast (R package multcomp; Hothorn et al. 2008).

Results

Gonopodium length ranged from 3.08 to 4.16mm, and the raw means were 3.69±0.02mm (x±SE) for the source population and 3.55±0.01mm for the introduced populations. The final model included family as a random effect and the following fixed effects: body size, population, food treatment, predation treatment, and an interaction between body size and food treatment. To investigate evolution, we looked at the effect of population in the model. Males in three of the four introduced populations had shorter gonopodia than the source population (Table 2.1, Figure 2.2), which was supported by a post hoc contrast that showed the pooled introduced populations had smaller gonopodia than the source population after accounting for body size ($z=-3.52$, p=0.0004).

To investigate developmental plasticity, we looked at the effects of the food treatment and the predation treatment. Although males reared with predator cues seemed to have shorter gonopodia (without cue = 3.63±0.02; with cue = 3.56±0.02), this was associated with a corresponding reduction in body length (without cue = 15.02±0.07; with cue = 14.26±0.06). Therefore, there was no effect of the predator cue rearing treatment on gonopodium length after accounting for differences in body size (Table 2.1). Conversely, gonopodium length was maintained in males reared under low food levels (high food = 3.61±0.02; low food = 3.58±0.02) despite a reduction in body length (high food = 14.92±0.07; low food = 14.33±0.06). Therefore, gonopodia were significantly longer in the low food treatment after accounting for body size (Figure 2.3A). There was also an interaction between body size and food treatment (Table 2.1) indicating that gonopodium length increased more strongly with body size (with a steeper slope) for males in the low food treatment than males in the high food treatment (Figure 2.3B).

32

Discussion

In-depth single species studies are critical for understanding selection on genitalia (Arnqvist 1997). Our study is the first to investigate the evolution and plasticity of genitalia by combining an experimental introduction with a common garden, split-brood rearing design. We documented the evolution of the gonopodium in only 2-3 years (8-12 generations), with repeated reduction in gonopodium length in replicate populations introduced to environments with reduced predation pressure. The split-brood design allowed us to measure developmental plasticity of male genitalia, which revealed that predator cues in the rearing environment did not affect gonopodium length beyond a reduction parallel to that for body length; however, there was an effect of food levels on gonopodia after correcting for body size, as males reared under low food maintained a similar gonopodium length to those reared under high food, despite a reduction in body size.

The introduced populations evolved smaller genitalia for a given body size in environments with reduced predation pressure compared to the high predation source population. While this experiment was not designed to determine the type of selection acting in this system, the direction of the evolved changes in gonopodium length allows us to discuss potential drivers. Some have argued that natural selection from predators is an important driver of genital evolution leading to smaller copulatory organs to increase swim performance, with some support in mosquito fish, *Gambusia hubbsi* (Langerhans et al. 2005; Langerhans 2011; Heinen-Kay and Langerhans 2013). We did not find support for this hypothesis since the source population had longer gonopodia than the descendant populations that evolved with reduced predation risk, suggesting that predation risk does not select for shorter gonopodia in *P. reticulata*. Regarding sexual selection, precopulatory female choice and sexual conflict generate opposite predictions:

33

that females should prefer longer gonopodia and exhibit greater mate choice in low predation environments (Brooks and Caithness 1995; Kahn et al. 2009) or that reliance on sneak copulations in high predation environments selects for longer gonopodia (Evans et al. 2011). We found longer genitalia in the source population that coexists with major predators, consistent with the sexual conflict hypothesis (Gasparini et al. 2011; Arnqvist and Rowe 2002) since forced copulation rates are higher in high predation environments (Luyten and Liley 1985; Magurran and Seghers 1994; Evans et al. 2011).

When we compare the source population to the introduced populations, the average gonopodium length changed from 3.69±0.02mm (x±SE, source) to 3.55±0.01mm (introduced) in 8-12 generations, a reduction of almost 4% in 2-3 years. While rapid, the magnitude of change that we detected is comparable to other studies of contemporary morphological evolution. Selection from human harvest on multiple species of wild fish populations has caused rapid evolution of morphological traits with an average change of 18% over 30 years (Darimont et al. 2009). Comparative work has suggested that genital evolution is much faster in species like the guppy that mate multiply, perhaps because of the strength of sexual selection (Arnqvist 1998), which may be stronger than viability selection in some systems (Siepielski et al. 2011). We did not detect an interaction between population and body size, indicating that selection in a new environment did not change the relationship (slope) between gonopodium length and body length and supporting the idea that allometric relationships can be difficult to shift evolutionarily (Huxley 1932; Egset et al. 2012).

We found no effect of predator cues in the rearing environment on gonopodium length after accounting for body size, because predator cues caused a parallel reduction in both body length and gonopodium length. It is possible that predator cues caused an overall stress response

that reduced male growth and body size along with gonopodium length. Predation risk has been previously shown to reduce body size (Krause and Liesenjohann 2012) and growth (Handelsman et al. 2013) in guppies. It is unclear how this overall reduction in gonopodium and body length with predator cues might affect fitness. Previous work attributed shorter gonopodia to reduced success with forced copulation (Evans et al. 2011) and female choice (Brooks and Caithness 1995; Kahn et al. 2009), but these studies focused on differences in gonopodia relative to body length, not the parallel decline in gonopodium and body length we observed with predator cues. Smaller size at maturity may be adaptive for survival in environments with predators, suggesting this plastic reduction in size with predator cues may have fitness benefits (e.g., Reznick 1990; Abrams and Rowe 1996).

We expected gonopodium length to decrease with reduced food levels in the rearing environment if it is a condition-dependent trait (Arnqvist and Thornhill 1998). On the contrary, the mean gonopodium length remained relatively constant (high food = 3.61±0.02; low food = 3.58±0.02) despite a reduction in body length with low food levels (high food = 14.92±0.07; low food = 14.33±0.06), causing an increase in gonopodium length relative to body size under low food (Figure 2.3A). Thus, it appears that body size declines with reduced food levels but gonodium length does not, perhaps because males must maintain gonopodium length in order to maintain reproductive success. This may be particularly important if poor-condition males rely on forced copulations rather than energetically costly displays (Nicoletto 1993); in that case the maintenance of gonopodium length under low resource conditions may allow males of varying condition to have comparable reproductive success through alternative mating tactics (Gross 1984). However, this result is surprising given the parallel reduction in body length and gonopodium length we observed with predator cues in the rearing environment. It is possible that

body size and genitalia change in parallel under certain stressors, but not under others. Our additional finding of an interaction between body length and food levels indicates that the relationship between body size and gonopodium length (the slope) depends on resources, with food limitation causing a greater investment in gonopodium length with increasing body size (Figure 2.3B). Food restriction during development may alter the allometric relationship between genitalia and body size through insulin pathways (Snell-Rood and Moczek 2012; Koyama et al. 2015).

Future work should measure additional features of the gonopodium, particularly aspects of the distal tip, which may be driven by other selective forces like cryptic female choice (Rowe and Arnqvist 2012) and predation pressure (Heinen-Kay and Langerhans 2013). Additionally, gonopodia are only one half of the story, and female genitalia should be studied simultaneously to tease apart the drivers of genital evolution (Anderson and Langerhans 2015). Finally, we assume that sexual selection and male mating tactics differ between high and low predation environments (Luyten and Liley 1985; Magurran and Seghers 1994) and that this drives selection on gonopodia (Reynolds et al. 1993, Evans et al. 2011), but future work should investigate the relationship between gonopodium length and mating behavior in these populations and treatments.

Most previous studies have compared populations by measuring genitalia in wild caught individuals, making it difficult to infer whether genital variation stems from genetic differences caused by natural selection in different environments or the plastic effects of being reared in different environments. The use of an experimental introduction and common garden rearing design allowed us to document rapid and repeated evolution of gonopodia in populations introduced to a new environment. The decrease in gonopodium length associated with a

reduction in predation pressure suggests that longer gonopodia are advantageous in environments with predators where they might facilitate forced copulations, providing support for the sexual conflict hypothesis. While we detected genetic variation among populations, we also detected developmental plasticity in response to the rearing environment. The plasticity underlying genitalia is often ignored in evolutionary studies but may be important in explaining some of the observed variation in genitalia.

Contributors

Figure 2.1. From photographs we measured standard length (white line, image a) and gonopodium length (solid black line, image b). 1. Distal tip 2. Hood.

Table 2.1: Results from the linear mixed model where each of the introduced populations is compared to the source population (intercept). P-values less than 0.05 are indicated by an asterisk.

	Estimate	Standard Error	t-value	p-value
Intercept	2.39	0.28	8.66	<0.0001*
Standard body length	0.08	0.02	4.76	<0.0001*
Intro1 (LL)	-0.08	0.03	-2.38	0.02*
Intro2 (UL)	-0.08	0.03	-2.52	0.01*
Intro3 (IC)	-0.04	0.03	-1.17	0.25
Intro4 (IT)	-0.15	0.04	-4.42	<0.0001*
Low food treatment	-0.90	0.34	-2.64	0.01*
Predation treatment	0.02	0.02	0.88	0.38
Low food * body length	0.07	0.02	2.79	0.006*
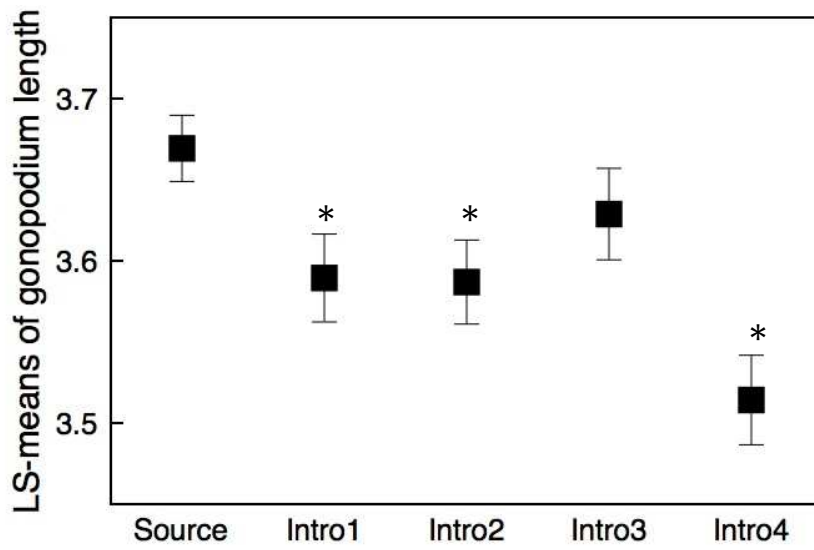
Figure 2.2: Least squares means ± SE are shown for gonopodium length (mm) in the source population and the four introduced populations from the final model (Table 1). Populations that differ significantly from the source population are indicated with an asterisk.
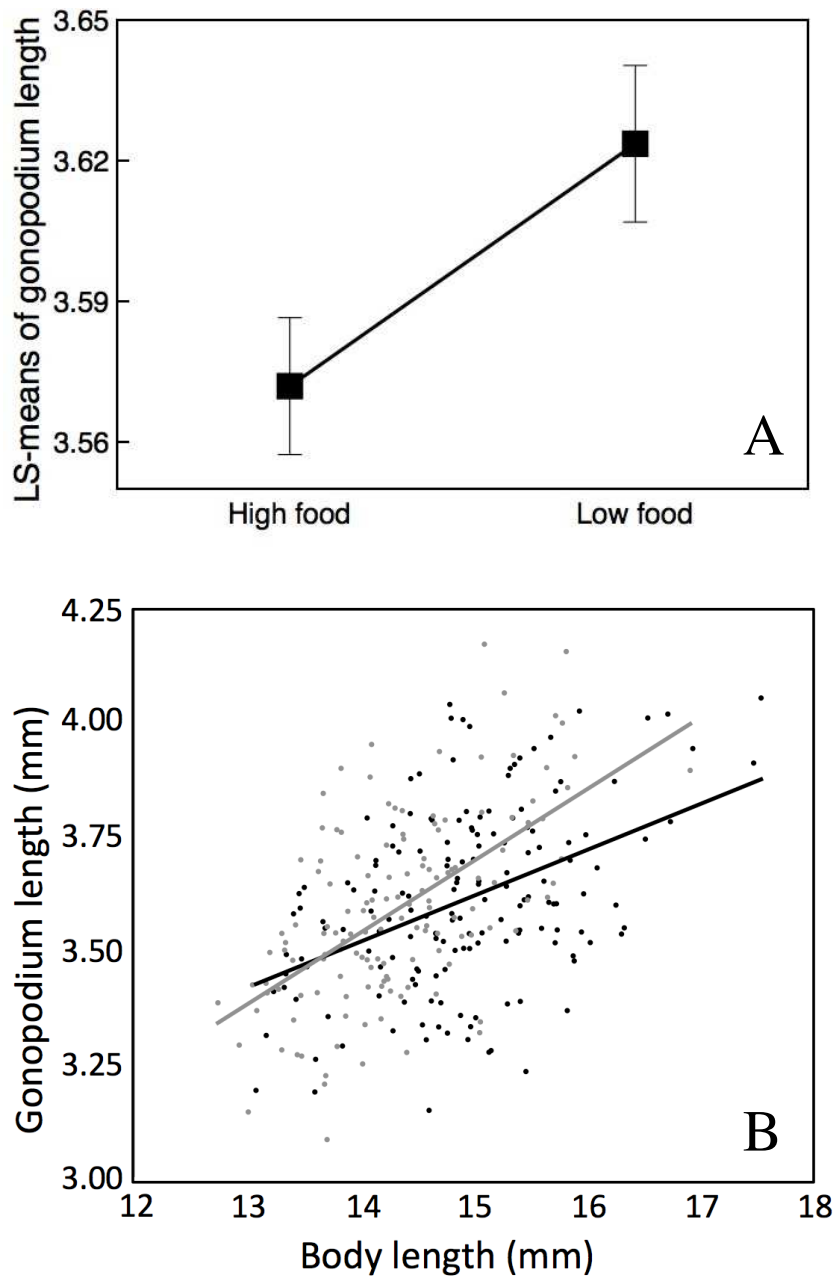
Figure 2.3: A) The least square means of gonopodium length from the model are shown for the high-food and low-food rearing treatments. B) Raw data are plotted showing the relationship between gonopodium length (mm) and standard body length (mm) for the low food (grey) and high food (black) treatments.

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III. DEVELOPMENTAL PLASTICITY, CONTEXTUAL PLASTICITY, AND THE EVOLUTION OF MATING BEHAVIOR

Summary

In the face of rapidly changing environments, behavior is expected to change before other traits, and initial changes are thought to be plastic. Plasticity may limit adaptive evolution by shielding phenotypes from selection, but it may also facilitate evolution by allowing animals to persist in a broad range of environments. Behavior can be developmentally plastic (shaped by the rearing environment, but fixed after development), or contextually plastic (labile and reversible in response to the immediate environment), allowing different modes of response to the environment. We studied Trinidadian guppies transplanted from a stream with many predators to four replicate streams lacking major predators. We measured evolution of mating behavior by collecting guppies from the source and introduction sites and rearing them in a common garden laboratory environment. We measured developmental plasticity by rearing siblings with varying predator cues and food levels. We assayed contextual plasticity by comparing individual behavior with and without conspecific alarm cues. Although we detected some evolutionary change and developmental plasticity of mating behavior, the patterns were not consistent across populations. However, mating behavior was contextually plastic in response to alarm cues, indicating an ability to rapidly shift behavior with changing conditions. We present one of the few empirical studies designed to explore evolution, developmental plasticity, and contextual plasticity in the same experiment.

Introduction

In the face of changing climates, habitat loss, and species invasions, it is increasingly important that we understand the way organisms adapt to new and changing environments (Stockwell et al. 2003; Losos et al. 2013). Initial changes may not be evolutionary genetic changes, but rather plastic responses, when a single genotype produces multiple phenotypes depending on environmental factors or individual experience (Reed et al. 2011; Sih 2013; Foster et al. 2015a). The role of plasticity in the evolutionary process remains controversial (Pigliucci 2005; Pfennig et al. 2010). On the one hand, adaptive plasticity (plasticity that is beneficial, allowing the expression of appropriate phenotypes in different environments; Gotthard and Nylin 1995) may limit evolutionary change by shielding genotypes from new selection pressures, essentially buffering environmental heterogeneity and directional selection by allowing populations to persist in novel environments without genetic change (Robinson and Dukas 1999; Sol et al. 2005; Fordyce 2006). Alternatively, it may facilitate evolution by allowing animals to persist in a broad range of environments and generating novel phenotypes that can become genetically fixed (West-Eberhard 1989; Price et al. 2003; Schlichting 2004; Ghalambor et al. 2007; Renn and Schumer 2013; Handelsman et al. 2014).

However, most models and discussions of plasticity's role in evolution fail to distinguish between types of plasticity (Piersma and Drent 2003; Dingemanse and Wolf 2013). "Plasticity" can describe phenotypes that vary depending on the rearing environment but are subsequently fixed, which has been called developmental plasticity (West-Eberhard 2003) or irreversible plasticity (Gabriel et al. 2005). On the other hand, animals may exhibit labile, reversible plasticity that depends on the stimuli surrounding an individual at a given time, which has been called phenotypic flexibility (Piersma and Drent 2003; Forsman 2015), reversible plasticity

(Gabriel et al. 2005), responsiveness (Sih and Bell 2008, Wolf et al. 2008), and activational plasticity (Snell-Rood 2013), hereafter contextual plasticity (Stamps and Groothuis 2010). Behavioral traits are often both developmentally and contextually plastic (Snell-Rood 2013; Stamps 2015), which is thought to allow animals to exhibit rapid responses to new environments (Wcislo 1989; West-Eberhard 1989; Sih et al. 2011; Sih 2013; Foster et al. 2015a).

Predictions about the role of plasticity in evolution become more complicated when we include both kinds of plasticity, developmental and contextual. Generally, environments with fine-scale temporal or spatial heterogeneity should select for contextual plasticity, because it allows for quick adjustments to changing conditions (Piersma and Drent 2003; Gabriel et al. 2005), though that contextual plasticity may be costlier than developmental plasticity, e.g., because of higher neural investment so that more pathways exist to allow rapid and reversible behavior shifts (Snell-Rood 2013). Compared to developmental plasticity, contextual plasticity may be more likely to buffer a population against evolutionary change in a new environment because it allows rapid shifts in phenotype at any stage of life (Wennersten and Forsman 2012). However, developmental plasticity may be more powerful in buffering phenotypes from selection if it allows new environmental cues to simultaneously shape the development of whole suites of traits important for survival, like life history, morphology, and behavior (Snell-Rood 2013). Thus either type of plasticity could hinder evolutionary change, but we lack clear predictions about their relative importance in shaping rates of evolution and we lack empirical studies that explicitly measure both kinds of plasticity and evolutionary change (Forsman 2015; Dingemanse and Wolf 2013).

We quantified patterns of evolution, developmental plasticity, and contextual plasticity of mating behavior in Trinidadian guppies, *Poecilia reticulata.* Guppies, small freshwater fish

native to the Caribbean, provide an excellent model organism for studying plasticity and adaptation for several reasons. First, they have well studied behavioral traits including male courtship and female preference for bright displaying males (Houde 1997, Liley 1966), though males may also attempt forced copulations (Matthews and Magurran 2000; Pilastro et al. 2007). Second, natural populations in Trinidad exist either with a suite of predators or without major predators creating natural geographic variation in selective pressure that shapes behavior. Presumably to reduce the risk of predation and overcome reduced female interest in mating, males from high predation environments perform fewer conspicuous courtship displays and more forced copulation attempts than males from low predation environments (Farr 1975; Endler 1987; Magurran and Seghers 1990; Magurran and Nowak 1991; Godin 1995; Rodd and Sokolowski 1995; Houde 1997). Third, guppy populations have shown rapid adaptation to changes in selective pressures (Seghers 1974; Reznick and Endler 1982; Magurran 2005), and these traits have been shown to have both a genetic (Farr 1975; Houde 1988,1992; Rodd and Sokolowski 1995; Brooks and Endler 2001a, b) and plastic basis (Breden and Stoner 1987; Gong 1997; Kelly and Godin 2001). For example, in the absence of predators, males display at higher rates and are less likely to attempt forced copulations than when a predator or predator model is present (Endler 1987; Magurran and Seghers 1990; Godin 1995), and males from high predation streams are more plastic in this response (Magurran and Seghers 1990). Finally, genetic evidence suggests that low-predation populations have been repeatedly founded by closely related downstream high-predation populations (Carvalho et al. 1991; Fajen and Breden 1992; Crispo et al. 2006), and experimental introductions of high predation guppies to low predation environments have been used to mimic these natural colonizations events (e.g., Travis et al. 2014). Thus guppies offer a unique opportunity to investigate phenotypic changes on short

timescales, as introduced guppies can be compared to ancestral source populations, and patterns are predicted to mimic differences that are naturally seen between high- and low-predation populations (Ghalambor et al. 2007).

We studied Trinidadian guppies transplanted from a stream with many predators to four replicate streams lacking major predators (Travis et al. 2014). We measured evolution of mating behavior by collecting guppies from the source and introduction sites (2-4 years post-introduction, depending on the population) and rearing them in a common garden environment. If the behavior of the introduced populations evolved in the new environment within 8-16 generations, we expected them to diverge away from the source population to be more similar to the classic low predation phenotype (more courtship displays and fewer forced copulation attempts; Luyten and Liley 1985; Magurran and Seghers 1994). We measured developmental plasticity in these same populations by rearing full brothers in the laboratory with varying exposure to predator cues and food levels. If guppy behavior is developmentally plastic with perceived predation risk, we expected exposure to predator cues during development to increase forced copulation attempts and decrease display rates (Endler 1987; Magurran and Seghers 1990; Godin 1995). If behavior is developmentally plastic in response to food availability, we expected a low food treatment to reduce overall mating effort because of the energetic tradeoff between foraging and courting (Abrahams 1993) and to particularly reduce the number of energetically costly sigmoid displays (Kolluru and Grether 2005; Kolluru et al. 2007). We assayed contextual plasticity by comparing individual behavior with and without the presence of conspecific alarm cues. If male guppy behavior is flexible with perceived predation risk, we expected the presence of alarm cues to increase forced copulation attempts and decrease sigmoid display rate (Endler 1987; Magurran and Seghers 1990; Godin 1995). We also expected an interaction between

53

rearing treatment (developmental plasticity) and acute exposure to alarm cue (contextual plasticity) if individuals reared with predator cues become sensitized and thus more responsive to chemical cues associated with predation. If the introduced populations exhibit adaptive plasticity (either developmental or contextual), thereby achieving the optimal phenotype without genetic change, we expected their behavior to be unlikely to evolve in the new environment (Robinson and Dukas 1999; Sol et al. 2005; Fordyce 2006). Finally, we measured mating behavior both with and without a competing male. While competition may increase the magnitude of male mating effort (Farr and Herrnkind 1974; Farr 1976), we expected to find similar effects of predation risk on mating behavior in these two separate experiments, providing additional support for the above predictions about evolution and plasticity.

Methods

Experimental introduction

Guppies were moved from a large river in Trinidad that contained predators to four headwater streams that did not previously contain guppies or major predators as part of a separate experiment to explore eco-evolutionary dynamics (Travis et al. 2014; Handelsman et al. 2013; Figure 3.1). The source site was a high predation location on the Guanapo River in Saint George, Trinidad that contains many predators (e.g., *Crenicichla frenata*, *Aequidens pulcher,* and *Hoplias malabaricus*). The four introduction sites previously contained only one gape-limited fish species, *Rivulus hartii,* which is not a major guppy predator (Mattingly and Butler 1994). Two of these introductions into low predation streams occurred in March 2008 (Lower Lalaja and Upper Lalaja; henceforth Intro1 and Intro2), and the other two occurred in March 2009 (Caigual and Taylor; henceforth Intro3 and Intro4).

Collection and husbandry

In March 2011, three years post-introduction for the Intro1 and Intro2 populations and two year post-introduction for the Intro3 and Intro4 populations, 40 juvenile guppies were collected from each of the four introduction sites as well as the high predation source location (Figure 3.1). Fish were transported to the laboratory at Colorado State University where they were reared in a common garden laboratory environment for two generations to minimize maternal and other environmental effects (rearing methods followed Handelsman et al. 2013, 2014 and were modified from Reznick 1982). Guppies were held in a flow through aquarium system fitted with 1.5-liter individual tanks on a 12:12 light:dark schedule at 27 ± 1°C and fed standardized amounts of flake paste (TetraMin® Tropical Flakes, Spectrum Brands, Inc., Cincinnati, Ohio, USA) in the morning and brine shrimp nauplii (*Artemia*) in the afternoon. Quantities were scaled by age and approached *ad libitum*. After wild-caught guppies became mature, they were randomly crossed to produce a G_1 generation. After reaching maturity, G_1 broods were randomly crossed with unrelated individuals to produce a G_2 generation. Within 24 hours of birth, we divided each G_2 brood into four 1.5-liter flow-through tanks and randomly assigned them to one of four treatments: high-food no-predator, low-food no-predator, high-food predator and low-food predator. Fish in the high-food treatment received the standard flake paste and brine shrimp diet that approached *ad libitum*. The low-food treatment received the same types of food at the same time, but the quantity was approximately half that of the high-food treatment. Fish in the no-predator treatment experienced recirculating water lacking chemical cues of predation. In the predator treatment, the water circulated through a tank containing a pike cichlid (*Crenicichla frenata*) (following Torres-Dowdall et al. 2012; Ruell et al. 2013; Handelsman et al. 2013). This cichlid was fed guppies daily so that fish in this treatment

experienced chemical cues from the cichlid predator as well as alarm cues from conspecifics. We sexed guppies at 29 days by noting the presence of melanophores on the abdomen (following Reznick 1982). One male from each family and treatment was placed in an individual 1.5-liter flow-through tank, with continued rearing in his respective treatment: high-food no-predator, low-food no-predator, high-food predator and low-food predator. In order to produce females for mating experiments, one female from each family was placed in a 10-liter recirculating group tank under no-predator conditions, and one female from each family was placed in a separate 10-liter recirculating group tank under predator conditions. Females from half of the families were held in two group tanks (with and without predator cues) and females from the other half of families were held in two separate group tanks (with and without predator cues). All females were maintained on a high food diet after they were placed in group tanks.

In March 2011, one year after the first collection, we resampled all of the streams in an identical way. This was three years post-introduction for the Intro1 and Intro2 populations and two years post-introduction for the Intro3 and Intro4 populations. We then repeated the exact same methods described above to rear animals for the experiments described below. Due to limited space in the laboratory and time constraints, we did not include each rearing treatment for each population and year in each of the experiments (see sample sizes in Table 3.1).

Mating trials

After G_2 males reached sexual maturity but one day before participating in mating trials, we placed one non-virgin female in each individual male tank for 12 hour to provide mating experience. The non-virgin females were not part of the experimental rearing design, but were additional females that had already participated in other mating experiments. To assess male

mating behavior with and without competition, we employed two frequently used approaches (Houde 1997): a 1x1 mating trial (involving one male and one receptive female; e.g., Reynolds and Gross 1992; Brooks and Caithness 1995) and a 2x2 mating trial (involving two males and two receptive females; e.g., Kolluru and Grether 2005; Grether et al. 2005). The virgin females used in the experiments were the G_2 lab-born fish reared in group tanks by treatment. Because we put half of the female families in one set of group tanks and the other half of families in another set of tanks and noted which families were present in each tank, we could ensure that males were not paired with siblings, though we did not know the family identity for each female.

Both the 1x1 and the 2x2 mating trials were conducted in 9.5-liter observation tanks filled with a thin layer of neutral gravel and approximately 7.5 liters of the same water the fish were reared in, either with or without predator cues. The tanks were covered with a blind that had viewing slits for observers and a full spectrum 40-watt bulb mounted 38 cm (1x1) or 34 cm (2x2) from the surface of the water.

For the 1x1 mating trials, we placed one experimental male and one unrelated virgin female from the same population and predator treatment in an observation tank, starting between 1900 and 2200 hours, after the lights had turned off for the day. We did not immediately observe the pair, but instead allowed them to interact overnight before observing them in the morning so that the female would be receptive but no longer a virgin; females remain receptive for up to five days following their first copulation (Liley 1966, Liley and Wishlow 1974, Houde 1997). This method results in more natural mating behavior since virgin females tend to mate quickly and indiscriminately (Houde 1988; Houde 1997). We conducted all trials between 700 and 1200 hours because guppy mating activity is highest in the morning (Endler 1987). We began each trial by turning on the full-spectrum bulb above the viewing blind in the otherwise dark room.

We began recording male behavior after the first mating behavior was observed and continued for 15 minutes. The trial was terminated and discarded if the male exhibited no mating behavior in 10 minutes. During the 15-minute trial, an observer recorded the following mating behaviors: the number of sigmoid displays (when the male positions himself in the visual field of the female, curves his body into an S-shape, splays his fins, and quivers in a stiff posture; Houde 1997), the number of forced copulation attempts (when the male approaches a female from behind and attempts to insert his gonopodium, intromittent organ, into her genital opening using a gonopodial thrust with or without making contact with the female; Houde 1997), and the amount of time spent following the female (when the male remains within four body lengths of a female and orients himself so that she is within his visual field).

The 15-minute 1x1 mating trials were broken into 3 observation periods of five minutes each. After the first five-minute observation period, we added 5mL of deionized water to the tank as a control for the addition of fluid, and continued observing for another five minutes. Thus the first 10 minutes of observation took place without the presence of conspecific alarm cues, and we coded both of these 5-minute periods as "before cue". To test for phenotypic flexibility in response to alarm cues, we then added 5ml of conspecific alarm cues through a separate tube and continued recording behavior for five more minutes ("after cue").

For those fish that participated in both experiments, the 2x2 mating trial took place between 700 and 1200 hours the day after the 1x1 mating trial. These males had a total of approximately 24 hours of experience with females (the initial 12 hours plus the 12 hours immediately prior to the 1x1 trial) followed by approximately 24 hours in isolation prior to the 2x2 mating trial in order to rebuild sperm reserves. Males that only participated in the 2x2 mating trial received identical experience, 24 hours of experience with a female and

approximately 24 hours in isolation prior to the 2x2 mating trial. Females from the 1x1 trials were also used in the 2x2 trials since females remain receptive for up to 5 days after their first copulation (Liley 1966). Males were never paired with the same female if they participated in both experiments.

For the 2x2 mating trial, we randomly selected two unrelated males and two unrelated receptive females from the same population and treatment. To distinguish individuals during the trial, we sketched the unique color patterns of both males. All four fish were placed in the 9.5-liter observation tank and allowed to acclimate for 10 minutes, but the females were held in a separate clear plastic container (13x7cm, water to depth of approximately 8cm) within the tank so that males could see the females without making contact. We then gently released the females and immediately began recording male behavior. Two observers recorded behavior, each following a focal male. We recorded the same behaviors as in the 1x1 experiments: the number of sigmoid displays, the number of forced copulation attempts, and the amount of time spent following a female. Like the 1x1 mating trials, the 2x2 mating trials lasted 15 minutes, with water injected after 5 minutes and conspecific alarm cues injected after 10 minutes.

To create the conspecific alarm cues for use in mating trials, we extracted alarm pheromones from the epidermis of sacrificed guppies (following Nordell 1998). These guppies were stunned in ice water and then euthanized via decapitation. We used razor blades to lacerate the epidermis in a crosshatched pattern, rinsed the skin with deionized water, and strained the solution through cheesecloth before collecting it in 5mL doses in syringes. These syringes were immediately frozen at -20°C for up to four weeks prior to experiments.

Statistical analyses

   We analyzed the 1x1 and 2x2 mating trials separately. We divided the response variables into two groups. First, following behavior (total number of seconds that a male followed a female, within four body lengths) was used as a measure of mating effort. Second, since males may perform both sigmoid displays and forced copulation attempts while attempting to mate with females, we used a bivariate approach by including both the number of sigmoid displays and the number of forced copulation attempts as response variables together in the same model as a measure of mating tactic. This resulted in a total of four models: two models of following behavior (1x1 mating trials and in 2x2 mating trials) and two models to predict mating tactic (both the number of sigmoid displays and forced copulation attempts) in 1x1 mating trials and in 2x2 mating trials.

   For all models, we used Bayesian linear mixed models (R package *MCMCglmm*; Hadfield 2010) in R (R Core Team 2013). Following behavior had a Gaussian distribution, and both of the behaviors in the mating tactic models had Poisson distributions. For all models, fixed effects included the population (to test for evolutionary responses), rearing treatment (to test for developmental plasticity), observation period during the assay either before alarm cues (including both of the first two 5-minute periods) or after alarm cues (the final 5-minute period; to test for contextual plasticity), and year (since the experiment was repeated for a second year). We tested for the presence of ongoing evolutionary change in the introduced populations (relative to the source) by fitting an interaction between year and population. However, this interaction failed to be significant (all $p > 0.5$) and so we removed it from the models. We also hypothesized that exposure to environmental cues during development could lead individuals to be more responsive (and flexible) once adults and therefore included an interaction between

rearing treatment and observation period. We included several random effects: family (repeated across rearing treatments), individual (repeated measures across observation periods within a mating trial), and trial number in the two models from the 2x2 experiments (to account for non-independence between paired males in the 2x2 mating trials).

All models were run for 401,000 iterations with a burn-in of 400 and a thinning interval of 1000 with a non-informative prior. Fixed and random estimates were estimated using Markov chain Monte Carlo (MCMC) sampling. Estimates were considered statistically significant if their 95% credible interval did not overlap zero. The bivariate models including both sigmoid displays (S) and forced copulation attempts (CA) yielded variance and covariance estimates for the two response variables from which we calculated correlations using the following formula:

$$\text{Correlation(S, CA)} = \frac{\text{Covariance(S, CA)}}{\text{Square root(Variance(S) * Variance(CA))}}$$

Results

Following behavior, reported as the total number of seconds out of 300 that a male spent following a female, was estimated as 71.05 for 1x1 mating trials and 65.79 for 2x2 mating trials (Table 3.2). Overall, the introduced populations did not differ from the source population in following behavior with two exceptions: Intro4 males spent more time following females than Source males in both 1x1 and 2x2 experiments, and Intro3 males spent more time following females than Source males in the 2x2 mating trial (Figure 3.2A and 3.2B). When these experiments were repeated in the second year, following behavior in the 1x1 mating trials was greater than in the first year (Table 3.2). There were no effects of the rearing treatments on following behavior except for a reduction in response to the low food treatment in the 1x1 mating trial (Figure 3.3A; Table 3.2). Males reduced their following behavior when alarm cue

was present compared to when alarm cue was absent (Figure 3.4A; Table 3.2). Finally, there was a significant interaction between rearing treatment and observation period in both experiments (Table 3.2); compared to brothers reared without predator cues, males reared with predator cues showed a greater decrease in following behavior in response to alarm cues in both 1x1 and 2x2 mating trials. This appears to be driven by initial differences since males reared with predator cues initially engaged in more following than males reared without predator cues, but then decreased to the same level after exposure to alarm cues (Figure 3.5).

Mating tactics, including sigmoid displays and forced copulation attempts, showed patterns similar to those seen in following behavior. The introduced populations did not diverge from the source population with a few exceptions: Intro1 males performed fewer sigmoid displays and more forced copulation attempts than Source males in both 1x1 and 2x2 experiments (Figure 3.2C, 3.2D, 3.2E, and 3.2F); Intro2 males performed more forced copulation attempts than Source males in both 1x1 and 2x2 experiments (Figure 3.2E and 3.2F); and Intro4 males performed more sigmoid displays than Source males in the 1x1 mating experiments (Figure 3.2C; Table 3.3). Compared to the first year of the experiment, in the second year males increased number of forced copulation attempts in 1x1 mating trials and the number of sigmoid displays in 2x2 mating trials (Table 3.3). There were no effects of rearing treatments on sigmoid displays or forced copulation attempts (Table 3.3). As seen with following behavior, the presence of alarm cues reduced the number of sigmoid displays and forced copulation attempts in all cases, except that sigmoid displays increased with alarm cues in the 2x2 mating trial (Figure 3.4B and 3.4C; Table 3.3). There was no interaction between developmental and contextual plasticity for mating tactics (Table 3.3).

We detected positive correlations between sigmoid displays and forced copulation attempts, such that individuals that performed more sigmoid displays also performed more forced copulation attempts (Table 3.4), but we did not detect a significant correlation at the family level. The correlation between an individual's number of sigmoid displays and forced copulation attempts was greater in the 2x2 mating trials (estimate = 0.84 and Credible Interval, CrI = 0.82, 0.85) than in the 1x1 mating trials (estimate = 0.26 and CrI = 0.10, 0.38; Table 3.4).

Discussion

By using an experimental introduction with a common garden rearing design, applying multiple rearing treatments during development, and exposing individuals to varying environments during behavioral observations, we were able to simultaneously measure evolutionary change, developmental plasticity, and contextual plasticity in guppy mating behavior. We found contextual plasticity in all behaviors (the time spent following a female, the number of sigmoid displays, and the number of forced copulation attempts) in both experimental contexts (mating trials with and without competition). We detected developmental plasticity in a reduction in following behavior in the 1x1 mating experiment in response to low food levels. We also detected an interaction between the two types of plasticity. Males reared with predator cues (developmental plasticity) showed a greater reduction in following behavior in response to alarm cues during the experiment (contextual plasticity) than males reared without predator cues. Finally, while we identified evolution of some mating behaviors in some populations introduced to novel streams in Trinidad, we did not find consistent evolutionary patterns that would suggest a singular adaptive response to a reduction in predation pressure or a simple relationship between plasticity and subsequent evolutionary change.

The presence of alarm cues reduced following behavior, sigmoid displays, and forced copulation attempts in both experimental contexts (1x1 and 2x2 mating trials). This may be a consequence of an overall reduction in activity, which is likely adaptive since reduced activity increases survival in the presence of predators (Lima and Dill 1990). Regarding mating tactics, we expected males to reduce the number of sigmoid displays performed in the presence of alarm cues since guppies have been shown to display less in the presence of predator models (Godin 1995) and live predators (Magurran and Nowak 1991), and a reduction in courtship in response to predatory threats is a general strategy seen across taxa (e.g., in water striders Sih et al. 1990 and in túngara frogs Ryan 1985). Males did reduce rates of sigmoid display in the 1x1 mating trials as expected, but sigmoid display rates increased in the 2x2 experiments. We might expect male-male competition in the 2x2 trials to affect display rates since previous studies have found that guppies display more in the presence of a rival male (Farr and Herrnkind 1974; Farr 1976) and display rates tend to be correlated between competing males (Farr 1980). However, it was surprising that the effect of competition resulted in an increase in display rates in the presence of alarm cues, which could increase conspicuousness to a predator. The relationship between male courtship, male-male competition and contextual plasticity in response to predation risk would be an interesting area for future work to determine if males engage in greater risk-taking behavior in the presence of rivals.

We expected males to increase their rates of forced copulation in the presence of alarm cues as has been shown previously in guppies in response to predator models (Godin 1995) and live predators (Magurran and Seghers 1990; Magurran and Nowak 1991). In both 1x1 and 2x2 mating trials, males reduced the number of forced copulation attempts in the presence of alarm cues, opposite our prediction. However, this may be related to the reduction in following

64

behavior and display rate with alarm cues, reflecting an overall adaptive reduction in activity level in the presence of a predator as a general strategy to increase survival (Lima and Dill 1990). Additionally, chemical alarm cues may evoke different responses than visual cues of a predator. It has been suggested that males may increase forced copulation attempts in the presence of predators because females are distracted by the predator and engaged in predator inspection (Magurran and Nowak 1991; Evans et al. 2002). If male mating behavior is driven by female antipredator behavior (Evans et al. 2002), it is possible that males did not increase rates of forced copulations because females responded differently to our chemical cue as compared to the visual predator cues used in other studies.

Males reduced following behavior in response to the low food rearing treatment in the 1x1 experiment. This developmental plasticity is in the direction we would predict since there should be an energetic tradeoff between foraging and mating, and mating behavior can be costly (Abrahams 1993; Blanckenhorn et al. 1995). Although males were not investing in foraging during our mating trials, as there was no food in the observation tank, males reared under low food levels may have experienced energetic constraints that limited their investment in mating (Fraser et al. 2004). However, we did not see a reduction in following behavior in response to the low food treatment in the 2x2 experiment, where males appeared to invest in low levels of following regardless of food levels. The food treatment did not affect male mating tactics, similar to the findings of other studies (Kolluru and Grether 2005). While males reared with predator cues were more contextually plastic, we did not detect overall differences in mating behavior in response to predator cues in the rearing environment. This differs from most other work in this system where predator cues in the rearing environment shifted the expression of traits including cerebral laterality (Broder and Angeloni 2014, Chapter 1), metabolism (Handelsman et al. 2013),

65

color development (Ruell et al. 2013), cortisol levels (Fischer et al. 2014), and gene expression (Ghalambor et al. 2015). We may not have detected developmental plasticity in this study if reproductive behavior is under strong selection to be reversible, or contextually plastic (Wcislo 1989), which would be expected if the mating environment is variable over shorter but predictable timescales (Gabriel et al. 2005; Snell-Rood 2013). Similar patterns were found in threespined stickleback where morphology and life history were developmentally plastic and irreversible while behavior was generally more flexible and contextually plastic (Foster et al. 2015b).

We detected an interaction between developmental and contextual plasticity for following behavior (Figure 3.4). Males from both rearing treatments reduced following behavior to the same low level even though males reared with predator cues initially performed more following behavior. This suggests that there may be an optimal level of following behavior in the presence of a predation threat, which resulted in a steeper reaction norm for males reared with predator cues. This interaction highlights the importance of designing experiments that consider both developmental and contextual plasticity and their relationship.

We saw some evolutionary changes in the four guppy populations introduced to a low predation environment. Compared to males from the source population, Intro1 males attempted more forced copulations and performed fewer courtship displays in both 1x1 and 2x2 mating trials; Intro2 males attempted more forced copulations in both mating trials; Intro3 males exhibited more following behavior in the 2x2 mating trial; and Intro4 males exhibited greater following behavior in both types of mating trials and performed more courtship displays in 1x1 trials. Though all four experimental populations were introduced to a low predation environment, these evolutionary changes were inconsistent and did not match our expectation for an increase

in courtship displays and a reduction in forced copulation attempts. Thus, the evolution that we documented may not have been a response to selection caused by reduced predation risk. Instead, these changes could be due to random processes or because of selective forces that were unique to each environment. Intro1 and Intro2 had an additional year in which to evolve, though differences were not consistent between these populations and those introduced one year later, Intro3 and Intro4. It is possible that behavioral patterns might converge across these populations given more time to evolve in these low predation environments. The patterns we observed may be representative of general patterns of behavioral evolution; it has been suggested that because it is often very plastic, behavior is more evolutionarily labile than morphological or life history traits, evolving more rapidly but less directionally over time (Gittleman et al. 1996; Blomberg et al. 2003).

We observed positive correlations between the number of sigmoid displays and the number of forced copulation attempts that males performed (Table 3.4), which were significant for individuals but not families. This positive relationship at the level of the individual suggests that there is not a trade-off between courtship and forced copulations as we originally predicted, and that investment in both mating behaviors can increase together, as shown by Fischer et al. (2016). The correlation differed depending on the social environment, either with or without competition. Numbers of sigmoid displays and forced copulation attempts were more highly correlated when a competitor was present compared to when a competitor was absent (Table 3.4). Correlations among morphological traits (Handelsman et al. 2014) and behavioral traits (Bell and Sih 2007; Fischer et al. 2016) have been shown to be plastic in response to the rearing environment, but here we demonstrate plasticity in trait correlations in response to the immediate social environment. This could have implications for evolution since plasticity of trait

67

correlations can evolve (Fischer et al. 2016) and may shape future evolutionary patterns (Handelsman et al. 2014).

Ours is one of the first studies to simultaneously investigate developmental plasticity, contextual plasticity, and evolution of mating behavior, which uncovered consistent contextual plasticity, interesting effects of the social environment, an interaction between developmental and contextual plasticity, and inconsistent but rapid evolution of mating behavior. Although the evolutionary patterns were not consistently related to the plasticity of the behavioral trait, the high levels of contextual plasticity we observed might have allowed guppies to adjust their behavior when they encountered an environment with reduced predation risk and may have buffered the population from directional, adaptive evolutionary change. This relaxed selection caused by contextual plasticity may have even facilitated non-adaptive random evolution leading to the diverse evolutionary trajectories we observed across populations. Future work to integrate plasticity and evolution would ideally continue to incorporate different types of plasticity (Piersma and Drent 2003; Dingemanse and Wolf 2013), with the understanding that developmental and contextual plasticity may behave differently. Selection may act separately on these two types of plasticity, may favor one over the other (Snell-Rood 2013), and these patterns of plasticity may shape subsequent evolution in different ways (Wennersten and Forsman 2012; Snell-Rood 2013). Additionally, experiments designed to measure developmental plasticity may actually be measuring contextual plasticity if they are conducted in conditions that match the rearing environment. Additional research could also simultaneously investigate morphological and life history traits as well as behavioral traits. Because these classes of traits are not independent (e.g., Losos 1990), there may be complicated interactions between plasticity and evolution (Forsman 2015), particularly since patterns of plasticity (developmental vs. contextual)

68

may differ across suites of traits (Foster et al. 2015b). However, this knowledge is important for understanding population persistence in new and changing environments (Dingemanse and Wolf 2013).

Contributors

Table 3.1: Sample sizes for each population and treatment for 1x1 and 2x2 mating trials for years 1 and 2.

Experiment	Year	Population	No predator High food	No predator Low food	Predator High food
1x1	Year 1	Source	23		20
		Intro 1 (LL)	13		
		Intro 2 (UL)	13		
		Intro 3 (IC)	10		11
		Intro 4 (IT)	12		11
	Year 2	Source	22	18	21
		Intro 1 (LL)	14	18	
		Intro 2 (UL)	21	20	
		Intro 3 (IC)	23	14	18
		Intro 4 (IT)	18	19	20
2x2	Year 1	Source	24	20	20
		Intro 1 (LL)	13	11	
		Intro 2 (UL)	13	1	
		Intro 3 (IC)	10	8	11
		Intro 4 (IT)	13	13	11
	Year 2	Source	16	8	22
		Intro 1 (LL)	18	16	
		Intro 2 (UL)	22	20	
		Intro 3 (IC)	21	13	18
		Intro 4 (IT)	20	18	20

Table 3.2: Model output for mating effort (following behavior) from two separate models of 1x1 mating trials and 2x2 mating trials. All estimates are relative to the intercept (e.g., in the 1x1 mating trial, Intro1 males spent 71.05 + 5.00, or 76.05 seconds following the female).

		1x1 mating trial	2x2 mating trial
Trait	Effect	Posterior mean of effect (95% CrI)	Posterior mean of effect (95% CrI)
Following	Intercept	71.05 (40.77, 101.21)	65.79 (38.95, 94.30)
	Intro1 (LL)	5.00 (-36.47, 41.87)	-7.31 (-44.51, 26.86)
	Intro2 (UL)	-14.29 (-53.99, 26.25)	-18.39 (-57.75, 17.56)
	Intro3 (IC)	8.70 (-21.08, 43.62)	47.98 (17.17, 79.30)*
	Intro4 (IT)	44.62 (10.50, 76.66)*	44.34 (11.84, 70.07)*
	Treatment 2 (reared with predator cues)	-7.00 (-38.96, 28.34)	-15.65 (-48.18, 11.63)
	Treatment 3 (reared with low food)	-45.60 (-78.65, -7.26)*	8.65 (-20.46, 33.03)
	Observation Period (before alarm cues)	37.07 (21.92, 48.80)*	26.49 (14.79, 38.53)*
	Year (2)	46.21 (18.93, 72.74)*	16.85 (-5.62, 38.84)
	Treatment 2 x Observation Period (reared with pred cues x before alarm cues)	26.3 (5.21, 46.07)*	28.72 (7.45, 47.60)*
	Treatment 3 x Observation Period (reared with low food x before alarm cues)	15.78 (-7.03, 42.62)	5.79 (-13.22, 24.31)

*The 95% Credible Interval (CrI) indicates the effect is significantly different from zero.

Table 3.3: Model output for mating tactics from two separate bivariate models of 1x1 mating trials and 2x2 mating trials that each included both sigmoid displays and forced copulation attempts as response variables. Both behaviors have Poisson distributions resulting in posterior estimates that are on a log scale. All estimates are relative to the intercept, and 95% credible intervals (CrI) are in parentheses.

		1x1 mating trial	2x2 mating trial
Trait	Effect	Posterior mean of effect (95% CrI)	Posterior mean of effect (95% CrI)
Sigmoid displays	Intercept (Source population)	-0.99 (-1.67, -0.40)	-0.36 (-0.77, 0.11)
	Intro1 (LL)	-1.16 (-2.01, -0.31)*	-0.87 (-1.48, -0.23)*
	Intro2 (UL)	-0.80 (-1.63, 0.05)	-0.59 (-1.17, 0.04)
	Intro3 (IC)	-0.23 (-0.87, 0.52)	0.29 (-0.21, 0.82)
	Intro4 (IT)	0.72 (0.06, 1.37)*	0.18 (-0.33, 0.66)
	Treatment 2 (reared with predator cues)	-0.39 (-0.91, 0.18)	-0.29 (-0.83, 0.11)
	Treatment 3 (reared with low food)	-0.43 (-1.05, 0.20)	-0.09 (-0.47, 0.31)
	Observation Period (before alarm cues)	0.35 (0.10, 0.57)*	-0.29 (-0.50, -0.11)*
	Year 2	-0.14 (-0.65, 0.45)	0.53 (0.11, 0.88)*
	Treatment 2 x Observation Period (reared with pred cues x before alarm cues)	0.35 (-0.04, 0.74)	0.29 (-0.02, 0.63)
	Treatment 3 x Observation Period (reared with low food x before alarm cues)	0.20 (-0.23, 0.62)	-0.02 (-0.29, 0.28)
Copulation attempts	Intercept (Source population)	-2.00 (-1.67, -0.40)	-0.18 (-0.58, 0.21)
	Intro1 (LL)	1.48 (0.50, 2.38)*	1.02 (0.31, 1.60)*
	Intro2 (UL)	1.08 (0.19, 1.97)*	0.73 (0.11, 1.37)*
	Intro3 (IC)	0.05 (-0.67, 0.84)	-0.03 (-0.58, 0.54)
	Intro4 (IT)	0.07 (-0.60, 0.80)	0.27 (-0.32, 0.75)
	Treatment 2 (reared with predator cues)	-0.21 (-0.95, 0.48)	0.16 (-0.34, 0.75)
	Treatment 3 (reared with low food)	-0.51 (-1.37, 0.30)	0.12 (-0.35, 0.55)
	Observation period (before alarm cues)	0.53 (0.14, 0.87)*	1.26 (1.01, 1.51)*
	Year 2	1.08 (0.51, 1.74)*	-0.30 (-0.72, 0.07)
	Treatment 2 x Observation Period (reared with pred cues x before alarm cues)	0.46 (-0.19, 1.07)	0.05 (-0.36, 0.48)
	Treatment 3 x Observation Period (reared with low food x before alarm cues)	0.51 (-0.11, 1.19))	0.20 (-0.18, 0.58)

*The 95% Credible Interval (CrI) indicates the effect is significantly different from zero.

Table 3.4: Mating tactic variance, covariance, and correlation (with 95% credible intervals, CrI, in parentheses). Individual, family, and residual variance (V) and covariance (COV) components as well as correlations are shown for bivariate models (1x1 and 2x2) of mating tactic behaviors (S = sigmoid displays and CA = copulation attempts).

Effect	Parameter	1x1 mating trial Posterior estimate (95% CrI)	2x2 mating trial Posterior estimate (95% CrI)
Individual	V_S	2.42 (1.53, 3.30)	1.15 (0.74, 1.56)
	V_{CA}	1.45 (1.01, 1.88)	1.40 (1.03, 1.74)
	$COV_{S,CA}$	0.49 (0.12, 0.95)	1.06 (0.74, 1.35)
	$Correlation_{S,CA}$	0.26 (0.10, 0.38)	0.84 (0.82, 0.85)
Family	V_S	0.71 (0.08, 1.38)	0.35 (0.11, 0.66)
	V_{CA}	0.22 (0.05, 0.41)	0.16 (0.06, 0.30)
	$COV_{S,CA}$	0.18 (-0.08, 0.49)	0.06 (-0.07, 0.25)
	$Correlation_{S,CA}$	0.46 (-1.26, 0.37)	0.25 (-0.86, 0.56)
Trial	V_S		0.40 (0.13, 0.68)
	V_{CA}		0.30 (0.11, 0.51)
	$COV_{S,CA}$		0.00 (-0.20, 0.08)
	$Correlation_{S,CA}$		0.00 (-1.67, 0.14)
Residual	V_S	0.31 (0.19, 0.45)	0.24 (0.15, 0.34)
	V_{CA}	0.56 (0.39, 0.73)	0.34 (0.27, 0.40)
	$COV_{S,CA}$	0.00 (-0.13, 0.11)	0.00 (-0.07, 0.05)
	$Correlation_{S,CA}$	0.00 (-0.48, 0.19)	0.00 (-0.35, 0.14)
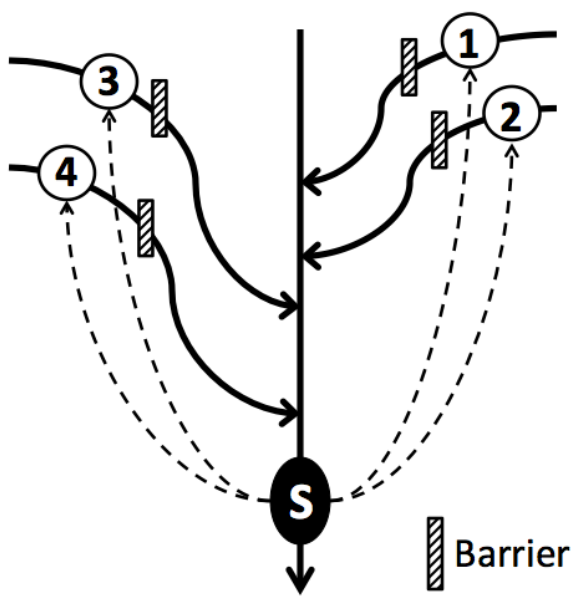
Figure 3.1: Schematic showing the streams in Trinidad where guppies were introduced from a high predation source population (S) to four replicate low predation streams (1, 2, 3, and 4) that previously lacked guppies because of barriers to upstream movement.
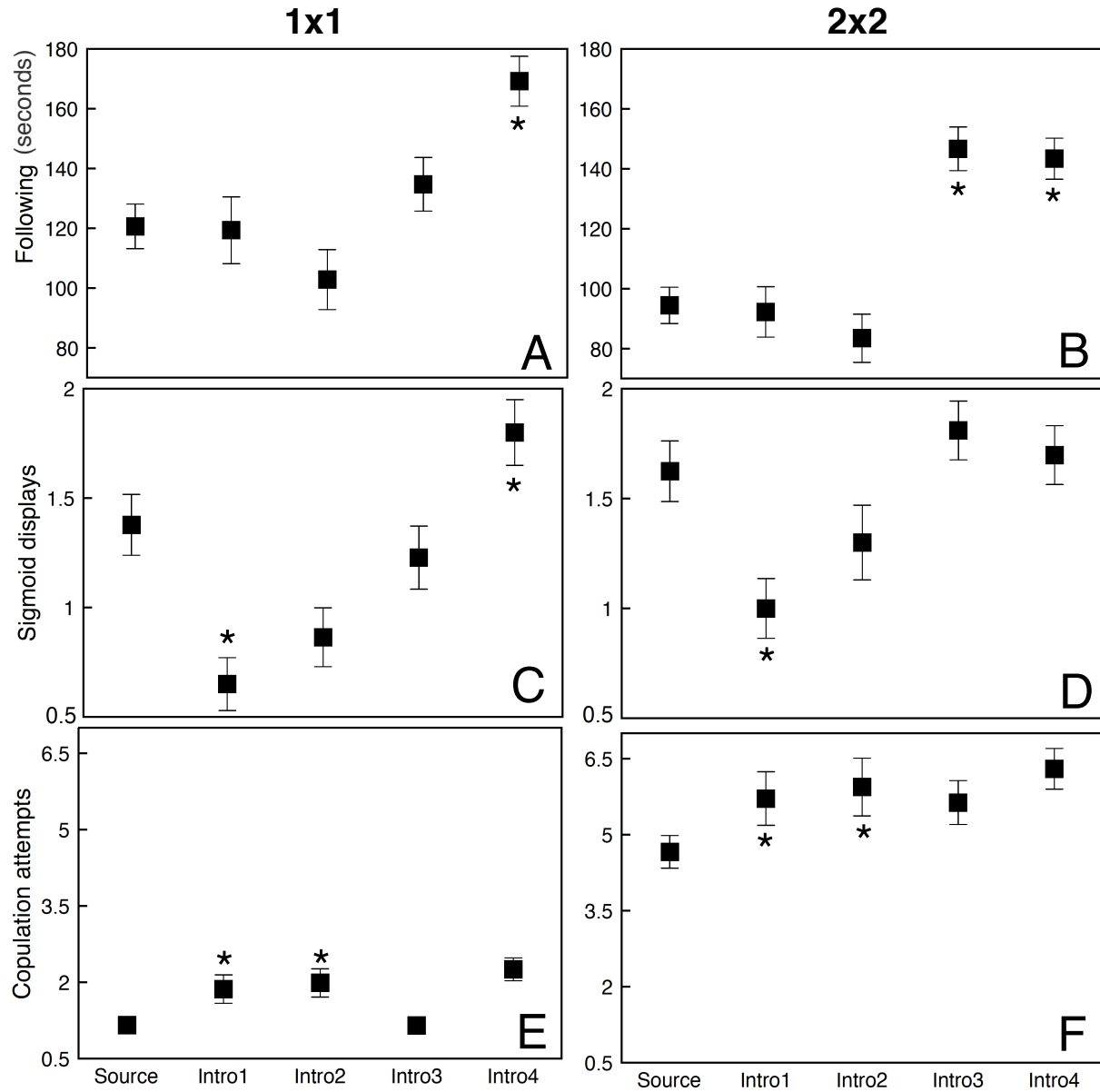
Figure 3.2: Evolution results showing means ± SE of the raw data for all populations in the 1x1 mating trials (left column A, C, E) and the 2x2 mating trials (right column B, D, and F) for all three behaviors: time spent following a female (A and B), number of sigmoid displays (C and D), and number of forced copulation attempts (E and F). Significant divergence from the source population is indicated with an asterisk.
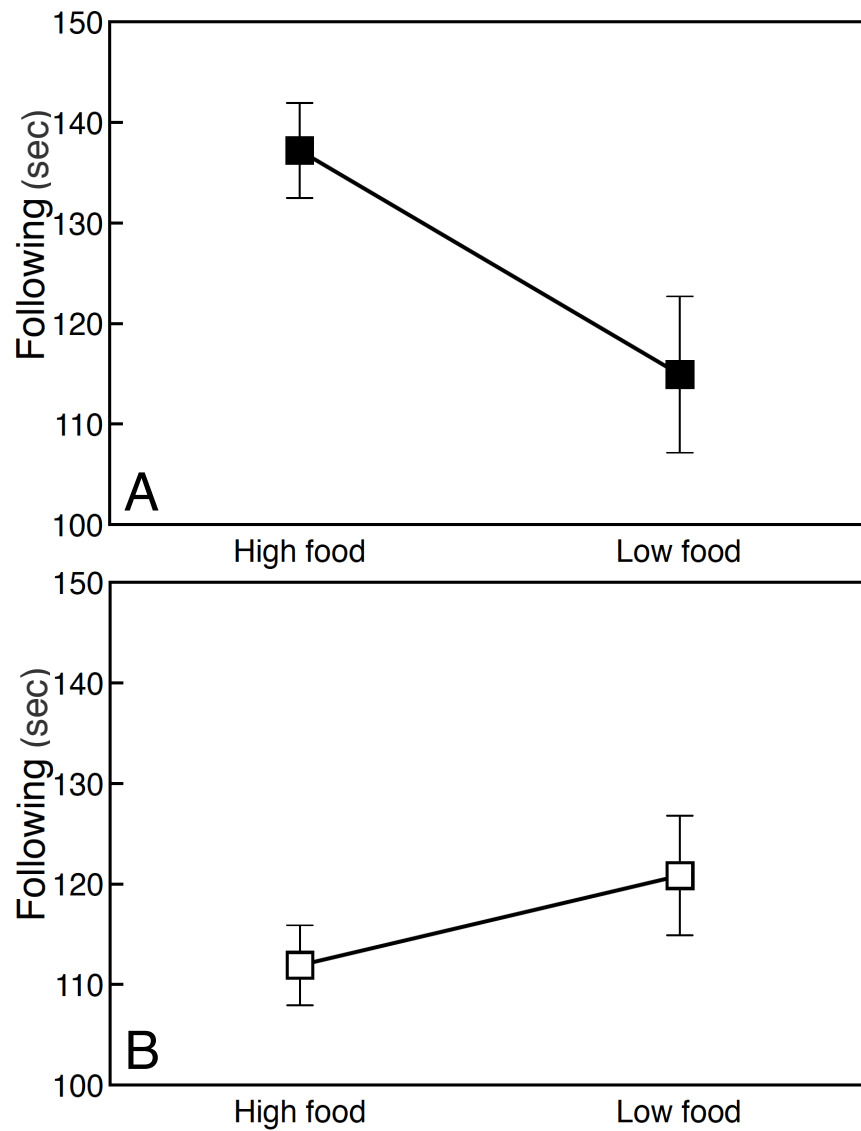
Figure 3.3: Developmental plasticity results showing means ± SE of the raw data for mating effort (time spent following a female) for males reared in high and low food treatments pooled across all populations. Results are shown for both the 1x1 mating trials (A) and the 2x2 mating trials (B).
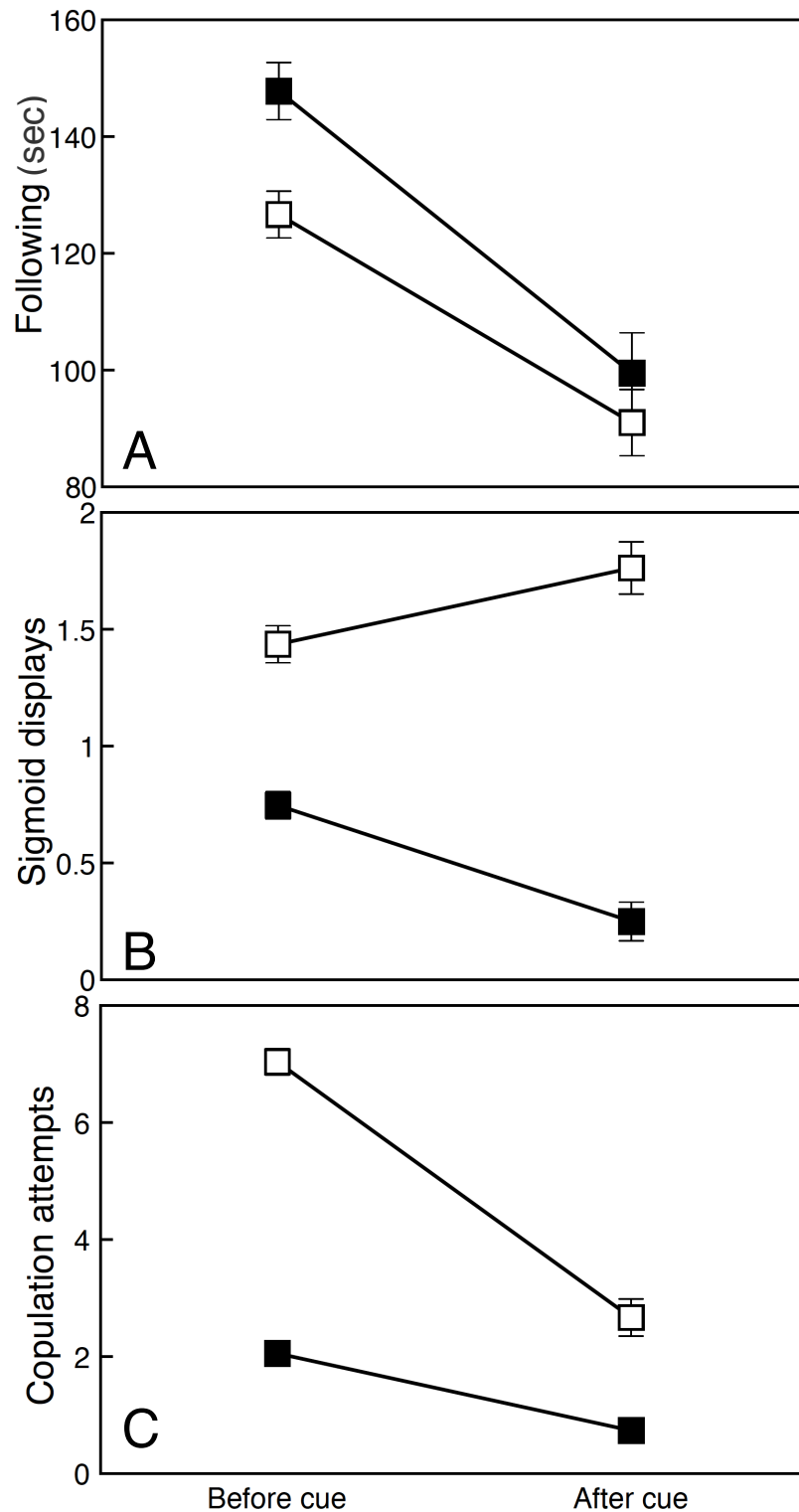
Figure 3.4: Contextual plasticity results showing means ± SE of the raw data for time spent following a female (A), number sigmoid displays (B), and number of forced copulation attempts (C) **before** and **after** exposure to alarm cues. Results are shown for both the 1x1 mating trial (filled symbols) and the 2x2 mating trial (open symbols).

Figure 3.5: Interaction between developmental and contextual plasticity. Means ± SE of the raw data for following behavior are shown for the interaction between period (before and after exposure to alarm cues) and rearing environment, either with predator cues (grey triangles) or without predator cues (grey circles). Results are shown for both the 1x1 mating trial (A) and the 2x2 mating trial (B).

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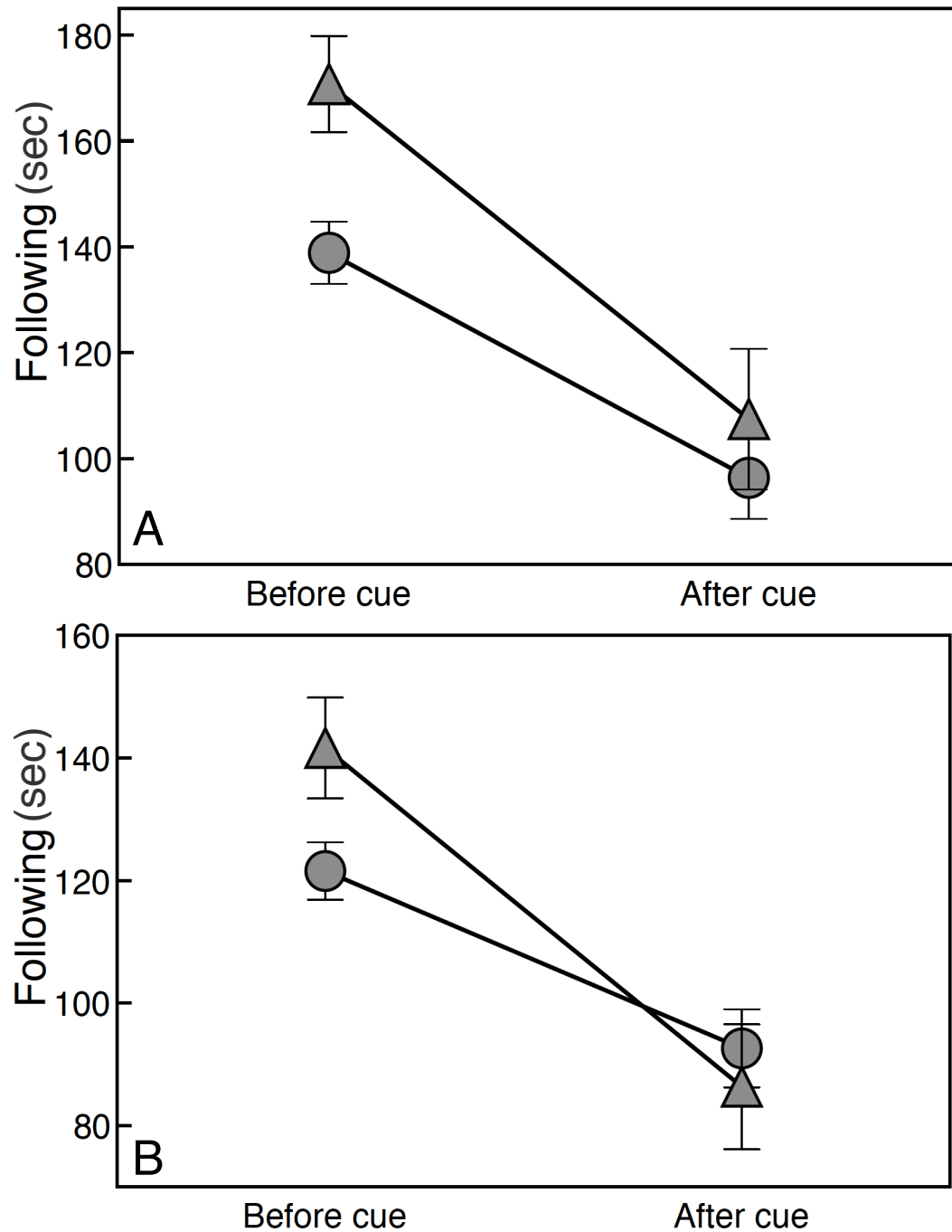Abrahams, M. V. (1993). The trade-off between foraging and courting in male guppies. *Animal Behaviour*, *45*(4), 673-681.

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IV. AUTHENTIC SCIENCE WITH LIVE ORGANISMS CAN IMPROVE EVOLUTION EDUCATION

Summary

Despite advances in the study of evolution and its acceptance among scientists as a unifying explanation for biological processes, evolution is still rejected by a large portion of United States population. This is in part due to the challenges educators face when trying to overcome students' preexisting beliefs about evolution, which can bias assimilation of information and inhibit learning. We propose that the most effective way to overcome such belief persistence is through an engaging, hands-on inquiry approach that mimics the scientific process used to study evolution. While this teaching approach, known as authentic science, has gained recognition for its effectiveness in the classroom, it has not been widely applied to teach evolution. We describe how an authentic science approach can be used to teach evolution by natural selection, and provide a formula for the development of such programs. Following this formula, we developed a program using Trinidadian guppies and implemented it in 7th grade classrooms in Colorado. Pre- and post-program assessments revealed significant increases in both the understanding and acceptance of evolution among participants. Authentic science experiments using locally adapted populations of live organisms may be able to overcome belief persistence and improve evolution education.

Introduction

A large proportion of the United States public rejects evolution as a unifying explanation for biological processes (Miller et al. 2006), yet evolutionary approaches are becoming

increasingly important in solving the world's most pressing problems such as improving global food security, preventing and treating diseases, coping with climate change, and preserving ecosystems (Losos et al. 2013). Coupled with mediocre scientific literacy scores (Kelly et al. 2013), this highlights a failing of the U.S. education system to effectively communicate evolutionary principles, including the process of evolution by natural selection.

Students in the United States are not typically exposed to evolution through formal teaching until they reach middle school (Wagler 2012). While it is recognized that knowledge must be constructed based on existing knowledge (Ausubel et al. 1978; Bransford et al. 2000), evolution educators must also facilitate construction of knowledge in the face of preset beliefs that students develop from experiences in primary school, at home, and from religious influences (Alters and Nelson 2002; Bloom and Weisberg 2007; Williams 2009). Preexisting beliefs have been shown to bias assimilation of information and inhibit learning (Lord et al. 1979; Anderson et al. 1980; Bloom and Weisberg 2007; Kahan 2010).

Belief persistence may be overcome by maximizing student engagement (Dole and Sinatra 1998; Nelson 2008), exposing students to causal evidence (Slusher and Anderson 1996; Nelson 2008; Kampourakis and Zogza 2009), and by holding students accountable for conclusions drawn based on evidence (Tetlock 1983). Thus, an effective teaching approach would be modeled after the scientific process where students ask fundamental questions, collect data, and draw conclusions based on their results. Such a teaching approach is referred to as *authentic science,* and it is a style of inquiry teaching in which students engage in the scientific process as if they were practicing scientists (Chinn and Malhotra 2002; Buxton 2006). It has been suggested that an inquiry approach should be used to teach evolution (Scharmann 1994;

90

Farber 2003; Scharman 2005; Robbins and Roy 2007) since it may increase both knowledge and acceptance of evolution (Robbins and Roy 2007).

While there have been calls to design programs that specifically attempt to overcome preset beliefs associated with evolution (Kampourakis and Zogza 2009), few authentic science programs have been developed for teaching evolution by natural selection (but see Bordenstein et al. 2010). Several case study inquiry programs have been developed (Farber 2003; Passmore et al. 2005; Desantis 2009), but they do not involve hands-on authentic science experiences. Unlike other foundational scientific principles that can be demonstrated in the classroom, an authentic science approach to teaching evolution is complicated by the problem of how to demonstrate a process that occurs over long timescales. How can students ask evolutionary questions, collect data on evolutionary processes, and draw their own conclusions about evolution during a typical one-week lesson? This may be accomplished using fast-growing organisms like bacteria or viruses (Bordenstein et al. 2010) or through simulations like computer games (e.g., www.pbs.org/wgbh/evolution/sex/guppy and http://ats.doit.wisc.edu/biology/ev/ns/t3.htm). While it has been argued that computer simulations may mimic the scientific process and facilitate conceptual change (Smetana and Bell 2012), experiences with living organisms are more engaging (Allen 2004). Here we propose an authentic science method to teach evolution by natural selection using live animals and then provide an example of its effective use in seventh-grade classrooms in Colorado.

Methods

General program design and overview

Rather than trying to observe ongoing evolutionary change in the classroom (as has been attempted using bacteria; Bordenstein et al. 2010), students can instead investigate adaptively divergent populations of organisms that represent the outcome of past evolutionary change, much like the examples used by Charles Darwin in presenting his case for evolution by natural selection (Darwin 1872). Populations of living organisms that differ from each other in obvious ways because they have become locally adapted to divergent environments provide a powerful empirical framework to explore the conditions necessary for natural selection to occur. In particular, by quantifying variation within and between populations, with foundational knowledge of inheritance, locally adapted divergent populations can be used to illustrate four important concepts that students must grasp in order to understand evolution by natural selection: 1) variation exists within and among populations; 2) much of that variation is inherited through genes; 3) selection determines which individuals pass on their genes to the next generation; and 4) over time this leads to genetic changes in a population, or evolution. These concepts are often reduced to four words (Variation, Inheritance, Selection, and Time) and are commonly represented by the abbreviation VIST (www.evolution.berkeley.edu). An authentic science approach allows students to make observations and conduct experiments to discover ideas 1- 3 and can conclude with thought exercises about outcomes over time, idea 4, and how this leads to the observed differences among populations before them.

The first objective of the program is to engage the students in the scientific process, which begins with observation. Before introducing students to the terms evolution or natural selection, they can be engaged by making observations of a plant or animal system and noting

differences within and among populations. Live organisms are particularly engaging (Allen 2004) and it is important to maximize engagement in order to overcome belief persistence (Dole and Sinatra 1998). Almost any plant or animal system can be used as long as there are obvious, quantifiable phenotypic differences that can be intuitively connected to a source of natural selection (Figure 4.1a). Whenever possible, it is helpful to use a familiar or local species, or to even use domesticated organisms, since students are more likely to engage if they feel a connection to the content because of past experiences (Dole and Sinatra 1998).

Once students are familiarized with the contrasting environmental context of where the populations are from (e.g., sunny vs. shady, with and without predators, Figure 4.1) they can begin to ask questions and propose explanations for why differences might exist among the populations. They can then design observational or experimental studies to test their hypotheses. These studies should quantify the variation in traits of interest within and among populations (Figure 4.1a), explore the evidence for inheritance (Figure 4.1b), and demonstrate how selection acts on variation via differential survival or reproduction in response to environmental variation (Figure 4.1c).

At this stage, students have engaged in authentic science by generating hypotheses and predictions for why populations might differ in traits of interest, and have observed and collected data on how traits vary within and between populations. Students can then use their own data and observations to test their own hypotheses within the VIST framework. 1) Students can explore the concept of variation (V) by examining plots of their data (e.g., frequency distributions of measured traits like plant height or fish coloration) and visualizing that *there is variation among individuals within a population and between populations* (Figure 4.1a). Sources of genetic and environmental variation can also be introduced and discussed (e.g., Broder et al. 2016). More

93

generally, discussions of the observed results should emphasize that variation within and between populations is commonly found in almost all organisms, and this can be illustrated with familiar visual examples of other plants and animals. 2) Students can then apply their knowledge of inheritance (I), by focusing on *heritable variation* (Figure 4.1b) using traits from the system being studied as well as familiar examples (e.g., domestic animals). A review of Mendelian genetics is important since an emphasis on genetics in evolution programs can promote conceptual change (Kampourakis and Zogza 2009). 3) Students can then be introduced to the idea of selection (S), that *not all individuals are equally likely to survive and reproduce depending on the environment.* If students have carried out experiments in the class, they can discuss how certain traits increase or decrease the likelihood of surviving and reproducing in various environments. For example, students might explore how variation in light levels shape leaf size or survival in low light conditions in plants, or how predation regime influences color patterns in fish (e.g., background matching may provide a selective advantage to prey fish if predators are present; Figure 4.1c). However, if such experiments are not possible, then students can discuss familiar examples of natural selection in the wild (disease, drought, etc.). 4) Finally, students can consider the consequences of these findings over time (T), that *this variation in survival and reproduction leads to changes in allele frequencies across generations* (Figure 4.1d). There are many ways to make this last point, including online tools, computer simulations, and thought exercises. The main conclusion the students should draw is that genetic change over time is an inevitable outcome when selection acts on heritable variation.

Following a discussion of variation, inheritance, selection, and time, the final step is to describe this process as evolution by natural selection. By defining the process of evolution as a change in allele frequencies within a population over time, students must reconcile their

94

preconceived ideas of evolution with the conclusions they reached examining their own results. We feel this point is critical. By allowing students to ask their own questions, collect their own data, and come to their own conclusions about evolutionary concepts prior to introducing the term evolution, we hope that students can overcome preset beliefs, leading to increased acceptance and understanding of evolution.

Specific program details

We developed and implemented a program based on the above framework to 7th-grade students at Windsor Middle School in Windsor, CO and Severance Middle School in Severance, CO. Evolution by natural selection was illustrated using live Trinidadian guppies, (*Poecilia reticulata)*. Guppies are a model system in evolutionary biology for studying natural selection in the wild (Reznick et al. 1990; 1997), and are also familiar to many students via the pet trade. We used guppies sourced from three populations: 1) wild guppies collected from a stream where predation from larger fish is very high, and individuals exhibit a suite of genetically based morphological traits (e.g., reduced male coloration) and behavioral traits (e.g., faster escape responses) known to reduce predation risk; 2) wild guppies collected from a stream where most predators are absent, and individuals exhibit traits that reflect reduced predation risk (e.g., increased male coloration, more courtship displays, slower escape responses); and 3) domesticated guppies from a local pet store that exhibit exaggerated traits (e.g., ornate colors, elongated fins and tails, naive behaviors towards predators) that have been artificially selected. Students generated hypotheses to explain differences they observed among guppy populations (e.g., male body coloration) and designed two experiments: 1) observations of mating behavior to test that male color provided a mating advantage, and 2) a predator encounter experiment to test if dull colored males were more cryptic. We compiled the data from the entire class, and

95

each student made figures summarizing both experiments and evaluated the results in light of their hypotheses.

We followed the student experiments with discussions of their data and experiences to introduce our four concepts. 1) *Variation*: the coloration and number of courtship displays performed by male guppies was variable both within and among populations. 2) *Inheritance:* a Mendelian Punnett-square approach illustrated how genes for bright coloration and high rates of courtship are passed on to offspring; 3) *Selection:* results from the predator encounter experiment, where domestic guppies were six times more likely to be depredated than males from the two wild populations, illustrated a mechanism of natural selection; and results from the mating experiments, where females were more interested in bright males, demonstrated a mechanism of sexual selection. We also discussed how selective breeding for the pet trade produced the exaggerated coloration of domestic guppies, to explain artificial selection. Finally, we discussed how particular traits should affect fitness in the three environments. 4) *Time:* a thought exercise, where students imagined that males could have alleles that code for bright ("A"), or dull ("a") coloration, allowed them to predict how the ratio of "A" to "a" alleles might change over time in the population under different scenarios.

Assessment

We implemented this program in April 2012 and April 2013 at Severance and Winsor middle schools. All participants were 7th-grade students associated with two teachers (SW and KDK), and the guppy program described above replaced their regular evolution by natural selection unit. We administered pre- and post-program assessments in 2013 to five of KDK's classes at Severance and four of SW's classes at Windsor (n=204 total students). To estimate

knowledge of evolution by natural and artificial selection, we used the 7-question multiple-choice test administered each year to Windsor Middle School 7th-grade students (Figure 4.2). These questions were written by one of the teachers (SW) based on the suggested learning outcomes for microevolution education in the Colorado Academic Standards (2009). The presenters of the program (EDB, CKG, and LMA) did not modify these questions, ensuring that the questions were not influenced by the program. We were unable to supplement this with a published assessment, because of a lack of tools in the literature appropriate for this age group at the time of this study. Other assessments exist for high school and college students, including The Knowledge of Evolution Exam (Moore et al. 2009) and the Measure of Understanding of Macroevolution (Nadelson and Southerland 2010), were deemed inappropriate given their Flesch-Kincaid grade-level scores of 8.9 and 9.3 respectively (Flesch 1948).

To measure acceptance of evolution, we selected four questions from the MATE Instrument that uses a Likert-scale to indicate agreement with various statements (Rutledge and Sadler 2007; Figure 4.2). We recognize the limitations of using only a subset of the MATE Instrument, but we had to exclude questions that mentioned religion or beliefs, because the teachers (SW and KDK) felt that it was a violation of their teaching agreement to include such questions. This assessment was granted exemption by the CSU Human Subjects Approval Board (IRB ID 038-14H).

To analyze the results, we performed two repeated measures ANOVAs; the first used the average scores from the seven multiple-choice questions (knowledge) and the second used the average scores from the four likert-scale questions (acceptance). In both analyses, the test (pre or post) was a fixed effect. We also included random effects of individual student (n=204) nested

within classroom (n=9) nested within teacher (n=2). We excluded students that did not have both a pre- and post-program assessment.

Results

Students who participated in our program increased both their knowledge (Figure 4.3a) and acceptance (Figure 4.3b) of evolution based on their pre- and post-program scores. Evolution knowledge test scores increased from 65.8 ± 1.4 ($x\pm SE$) on the pre-test to 79.7 ± 1.4 on the post-test out of 100 possible points ($F_{1,203}=87.00$, $p<0.0001$). The least squared means accounting for random effects increased from 66.0 ± 1.4 to 79.7 ± 1.4 (Figure 4.3a). The acceptance of evolution scores, which could range from 1 to 5, increased from an average of 3.07 ± 0.04 ($x\pm SE$) on the pre-test to 3.21 ± 0.05 on the post-test ($F_{1,197}=7.49$, $p=0.007$). The least squared means accounting for random effects increased from 3.07 ± 0.04 to 3.20 ± 0.04 (Figure 4.3b). Though there was variation among classrooms and schools, the observed patterns did not appear to be influence by just one classroom or school as most of the nine participating classrooms showed similar increases in scores on the knowledge assessment (Figure 4.3c) and the acceptance assessment (Figure 4.3d).

Conclusions

The persistent rejection of evolution by a significant portion of the United States population in the face of educational reforms has been called the "single greatest failure of science education in the history of the US" (Rice et al. 2011). We present an authentic science approach that is flexible in its structure, but shares the common elements of VIST to teach evolution by natural selection using populations adapted to different environments.

Our use of this approach resulted in positive outcomes, as students appeared to overcome belief persistence by indicating a greater acceptance of evolution and also exhibited increased knowledge of evolution by natural selection. For comparison, a teacher at Windsor Middle School administered our pre- and post-assessments, but did not participate in the program, teaching the same material at the same time using traditional approaches like readings, hands-on activities, and interactive worksheets. Those three classrooms of students (n=59 students) showed no change in knowledge test scores (x±SE; 61.4±3.1 pre-test and 61.5±3.2 post-test) or acceptance scores (x±SE; 3.0±0.07 pre-test and 2.9±0.07 post-test). This example supports claims that traditional teaching methods are ineffective at teaching evolution (Alters and Nelson 2002).

It is important to note that we cannot isolate any one factor as being responsible for the differences in student performance or acceptance. It is likely a combination of the authentic science approach, the VIST framework, the use of live animals, and the presence of scientists in the classroom. Nevertheless, such results suggest that similar approaches can be a highly effective means for teaching evolution. In addition to potentially increasing knowledge and acceptance of evolution, an authentic science approach has the benefit of increasing student understanding of the nature of science more broadly, which is positively associated with the acceptance of evolution (Lombrozo et al. 2008). As funding agencies push for greater effort on broader impacts, we encourage scientists to consider the significant impact on knowledge and acceptance of evolution that might be made through collaborations with K-12 educators to teach evolution and develop authentic science programs more generally.
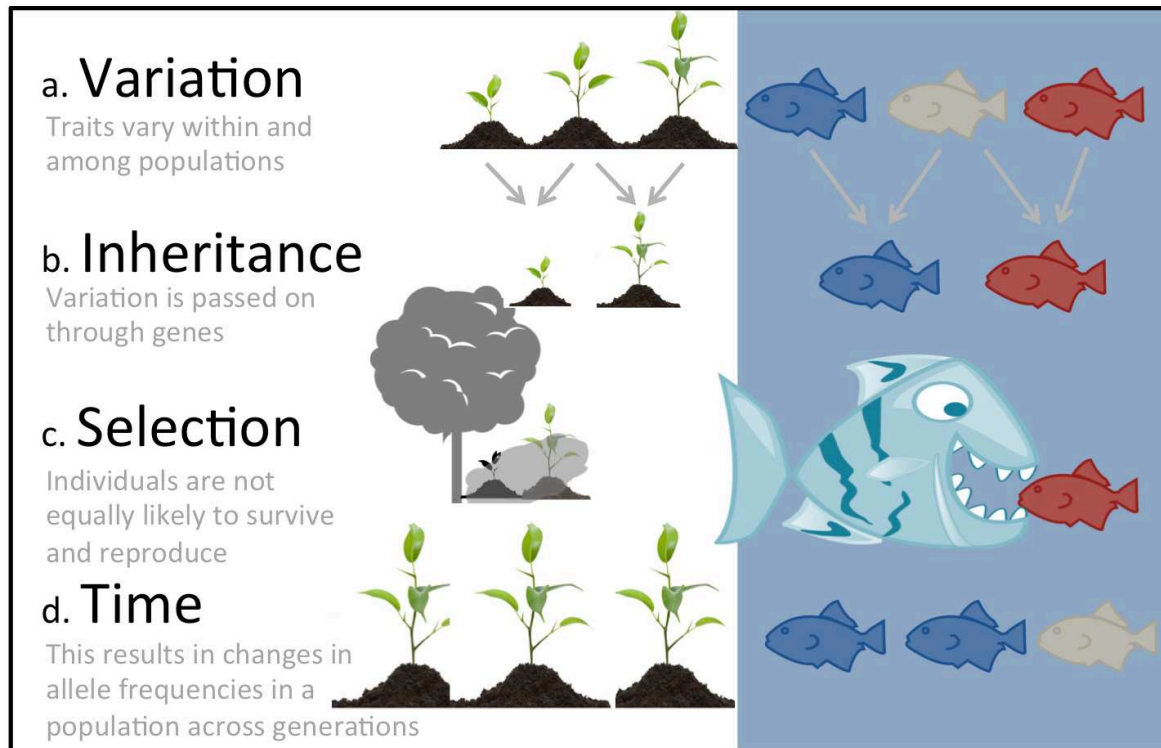
Contributors

Figure 4.1: Schematic showing how VIST can be applied to a hypothetical plant and animal system.

ASSESSMENT
Thank you for participating in this short quiz. Remember that you will not be graded on this quiz. But please do your best to answer the questions.

1. Which of the following is NOT an example of an adaptation?
   a. Desert foxes have very large ears to release extra body heat.
   b. Guppy males display their colors to attract females for mating.
   c. Polar bears have a layer of fat under their fur to keep them warm in icy waters.
   d. Chimpanzees prefer oranges over apples because they like the flavor.

2. Which is NOT needed for natural selection to take place?
   a. Some animals must die or fail to reproduce based on their environment.
   b. There must be human impact on the environment.
   c. There must be variation (differences).
   d. Traits must be inherited (passed to offspring).

3. There are many breeds of dogs today, from the Chihuahua to the Labrador. These were developed through which process?
   a. Artificial selection
   b. Extinction
   c. Natural selection
   d. Speciation

4. In ARTIFICIAL selection, how are animals selected to pass on their genes?
   a. By the breeder
   b. By wild predators
   c. By female choice
   d. Both b and c, depending on the environment

5. In NATURAL selection, how are animals selected to pass on their genes?
   a. By the breeder
   b. By wild predators
   c. By female choice
   d. Both b and c, depending on the environment

6. If only the tallest animals on an island can reach the treetops and get enough food to survive, what will be true of the next generation of animals?
   a. There will be more TALL individuals.
   b. They will be more SHORT individuals.
   c. They will be THE SAME as the previous generation.
   d. No prediction can be made, genes are just random.

7. If female birds on an island prefer birds with bright feathers, what will be true of the next generation of birds?
   a. There will be more BRIGHT feathered males.
   b. There will be more DULL feathered males.
   c. They will be THE SAME as the previous generation.
   d. No prediction can be made, genes are just random

8. On this question there is no right or wrong answer. We are asking your opinion about several statements. Please fill in the circle indicating if you agree or disagree with the each statement.

	Strongly disagree	Disagree	Undecided	Agreed	Strongly agree
The theory of evolution cannot be tested scientifically	o	o	o	o	o
Evolution is a scientifically valid theory	o	o	o	o	o
Current evolutionary theory is the result of sound scientific research	o	o	o	o	o
The available data are unclear as to whether evolution actually occurs	o	o	o	o	o

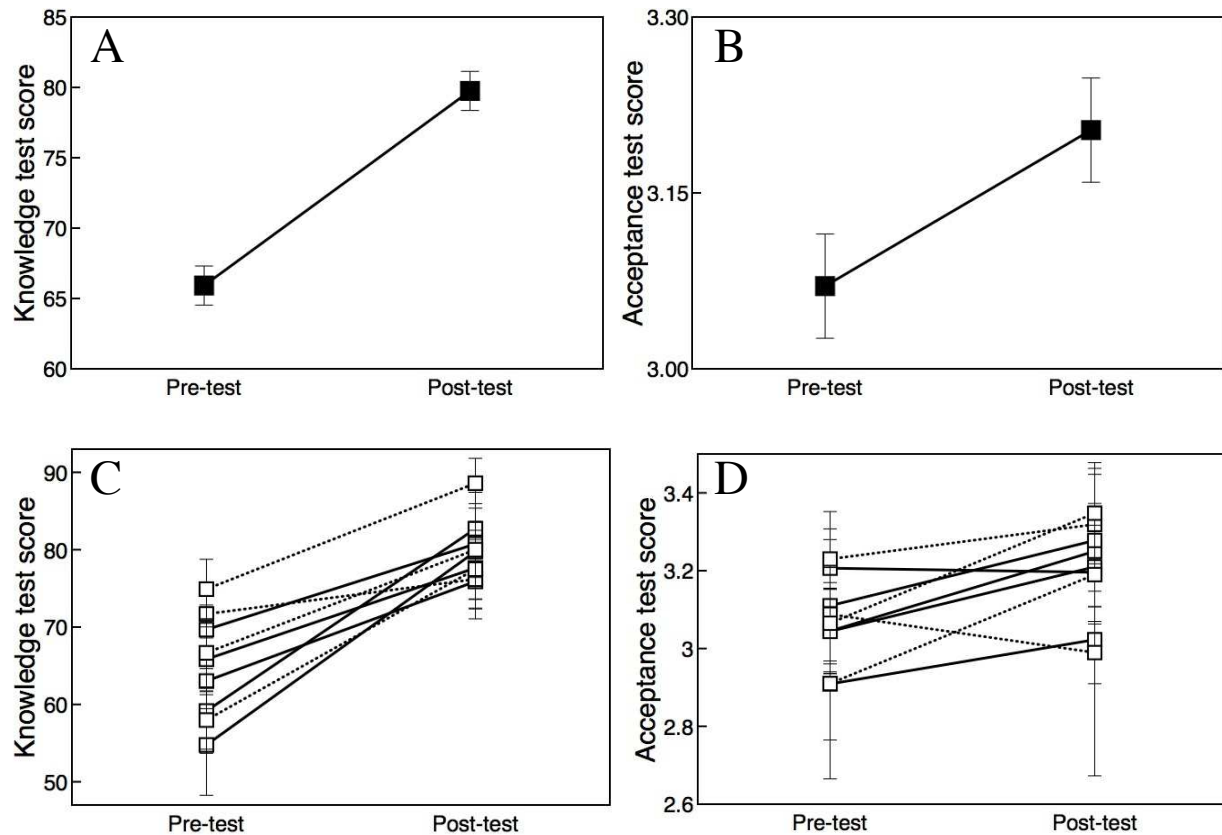Figure 4.2: Assessment that was administered before and after students participated in the program.

Figure 4.3: Assessment results: least squared means±SE are shown for two repeated measures ANOVAs comparing pre- and post-program test scores for program participants for (A) a measure of evolution knowledge, and (B) a measure of acceptance of evolution. The raw means are shown separately for the five classes from Windsor Middle School (solid lines) and the four classes from Severance Middle School (grey dashed lines) for the knowledge test scores (C) and the acceptance test scores (D).

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