

DADS MATTER: CAUSES AND CONSEQUENCES OF PLASTICITY IN PATERNAL
CARE

BY

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DISSERTATION

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ABSTRACT

Whether phenotypic plasticity can accelerate evolution is often debated.

Transgenerational plasticity (parental effects) are a particularly potent form of plasticity that occurs when the environment experienced by a parent influences offspring phenotypes, and theory suggests that parental effects can increase the speed of directional evolution. Parental effects might facilitate adaptation to new environments if parents are capable of ‘programming’ their offspring for the type of environment they are likely to experience. Therefore, it is possible that plasticity in one generation (changes in parental behavior, for example) might influence offspring in the next generation and may facilitate adaptive evolution and the colonization of novel environments, yet these ideas remain largely untested empirically.

In a wide range of organisms, including humans, mothers’ experiences can affect offspring morphology, physiology, and behavior. There is also an emerging literature showing that the way mothers behave toward their offspring can have a long-lasting influence on their offspring. Comparatively, a relatively unexplored possibility is that in species with paternal care, *fathers* adjust their parenting in response to stressors, and adjustments in care have long-term consequences for offspring (as has been shown for mothers).

Here, I report a series of studies examining how plasticity in father behavior might facilitate rapid adaptation in threespine stickleback fish (*Gasterosteus aculeatus*) via paternal effects. In threespine stickleback, the father is the sole provider of parental care, and parental care is necessary for offspring survival; without parental defense, nests would be depredated. As males are the sole provider of care, it is possible to separate post-fertilization paternal effects due to variations in paternal behavior from maternal effects. Parental behaviors include oxygenating eggs via fanning with pectoral fins and retrieving offspring. In stickleback, the marine ancestral

form is extant and is thought to have remained relatively unchanged. Multiple independently-derived freshwater populations have repeatedly diverged from this ancestral marine form, resulting in numerous replicates of derived populations, many of which are locally adapted. Therefore, the threespine stickleback system provides a good model for examining causes and consequences of plasticity in paternal behavior in response to predation risk.

First, I assessed the impact of the experience of parenting on a parent's behavior and physiology. While there is a rich literature documenting physiological and behavioral changes that organisms undergo as they become parents, there are little data in either humans or nonhuman animals that test the intuitive hypothesis that becoming a parent influences personality traits (behaviors that are variable among individuals and consistent within individuals over time). Life history theory predicts that males should be less risk-averse after successfully parenting, and the neuroendocrinology of parenting suggests that parenting could reorganize the hormonal landscape and behavior of fathers. Using a controlled longitudinal study, I randomly assigned males to either the experimental (reproduced and parented) or control (did not reproduce and parent) group, and repeatedly measured a personality trait ('boldness') and 11-ketotestosterone (11-kT, the major androgen in fishes) in individual males. In the control group, males became more bold over time. However, in the experimental group, boldness did not change. Further, 11-kT changed dramatically in the experimental group, and changes in 11-kT in parents were associated with boldness after parenting ceased. Further, males that parented showed greater among-individual variation in 11-kT, suggesting a potential mechanism driving natural variation in parenting behavior. This study is one of the first to test proximate and ultimate explanations for changes in personality as a function of a major adult event – reproduction and parenting.

Second, I examined plasticity in paternal behavior in both the lab and field. Using a within-subject design, I randomly assigned wild-caught males from Putah Creek, CA to either the “predator-exposed” or “unexposed” treatment group and allowed them to spawn. Three days post-fertilization, I introduced a model rubber sculpin (a fish predator present in Putah Creek) into the tank of “predator-exposed” males for two minutes. Males in the “unexposed” treatment did not experience the predator. Males were then allowed to parent normally and complete their clutch. I then moved males into new tanks and males that were initially in the “predator-exposed” treatment were assigned to the “unexposed” treatment, and vice versa. I found that males exhibited natural variation in parenting behavior, and consistently differed from one another both within and across clutches. Further, males exposed to predation risk reduced fanning behavior for two days, and then resumed normal fanning for the remainder of the nesting cycle. This demonstrated that males plastically adjusted their parenting behavior in the presence of a predator.

I also examined plasticity in parenting behavior in the field. I marked parenting males in the Navarro River, CA that had eggs in the nest with flags. I measured undisturbed parenting behavior, and then I presented males with a caged live gravid female (representing courtship opportunity), a live conspecific male (representing territorial intrusion), and a live sculpin (representing predation risk), with one hour between each stimuli. I found that males showed natural variation and consistently differed from one another in parenting behavior as in the lab. Males also exhibited behavioral plasticity by reducing fanning while stimuli were present. Further, I found that males that were more attentive to the stimuli fanned the nest more often, suggesting the presence of a behavioral syndrome.

I then assessed the consequences of fathers' plasticity by examining the impact of parental experience with predation risk on offspring phenotypes. I compared the morphology, behavior, and physiology of adult offspring that were reared by fathers that either had or had not been exposed to predation risk during the time when they were providing care. I found that offspring of predator exposed fathers were smaller, in worse body condition, showed duller nuptial coloration, and were less active than offspring of unexposed fathers. Fathers' experience with predation risk also induced a sex-based difference in cortisol concentration, such that daughters had higher cortisol than sons in response to predation risk (offspring of unexposed fathers showed no difference in cortisol concentration between sons and daughters). These phenotypes matched those of stickleback from high-predation populations and juvenile stickleback exposed to predator cues. These results suggest that fathers might be capable of 'programming' their offspring for living in a high predation environment via short-term adjustments in paternal behavior in response to immediate predation risk.

Finally, I tested the hypothesis that behavioral plasticity by fathers in response to predation risk might have facilitated the adaptive radiation of threespined sticklebacks by comparing plastic responses in marine and freshwater populations. In freshwater, major predators on stickleback fry and juveniles are Odonate (dragonfly) larvae, a predator that is not present in marine populations, and which exerts important selective pressure on stickleback morphology. I collected fish from nine populations: two marine (ancestral), three freshwater of known age (2-30 generations, "new" freshwater), and four established freshwater. I induced plasticity in fathers by exposing them to a predator found only in freshwater. While derived populations showed antipredator responses, ancestral populations did not. When compared across populations, plasticity increased with population age (and thus predator familiarity).

Finally, I found greater variation in plastic responses in ancestral populations compared to derived populations. Altogether, my results suggest that, rather than ancestral populations showing greater overall levels of plasticity than derived populations, ancestral populations instead show greater standing variation in behavioral reaction norms, potentially providing different trajectories on which selection can then act. Taken together, these studies provide a comprehensive view of how plasticity both within and across generations can influence evolutionary patterns.

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CHAPTER 1: DOES BECOMING A PARENT INFLUENCE PERSONALITY TRAITS? A CONTROLLED EXPERIMENTAL TEST IN THREESPINE STICKLEBACK

ABSTRACT

While one of the hallmarks of personality traits is their consistency over time, we might expect personality traits to change during life history shifts. Becoming a parent is a major life history event, when individuals undergo dramatic behavioral and physiological changes. Here we employ a longitudinal experiment to ask whether personality changes in response to the experience of parenting in male threespine sticklebacks (*Gasterosteus aculeatus*). Life history theory predicts that males should be less risk-averse after successfully parenting, and the neuroendocrinology of parenting suggests that parenting could reorganize the hormonal landscape and behavior of fathers. We randomly assigned males to either the experimental (reproduced and parented) or control (did not reproduce and parent) group, and repeatedly measured a personality trait ('boldness') and 11-ketotestosterone (11-kT, the major androgen in fishes) in individual males. In the control group, males became more bold over time. However, in the experimental group, boldness did not change. Further, 11-kT changed dramatically in the experimental group, and changes in 11-kT in parents were associated with boldness after parenting ceased. Our study is one of the first to test proximate and ultimate explanations for changes in personality as a function of a major adult event – reproduction and parenting.

INTRODUCTION

The experience of reproducing and becoming a parent is one of the most important life history events for most organisms. While there is a rich literature documenting physiological and behavioral changes that organisms undergo as they become parents, there are little data in

either humans or nonhuman animals that test the intuitive hypothesis that becoming a parent influences personality traits (behaviors that are variable among individuals and consistent within individuals over time (Stamps & Groothuis 2010). Understanding the robustness of personality traits across critical lifetime events can shed light on their plasticity, causation, and evolution.

It is reasonable to suppose that personality traits might change as a function of reproduction and parenting because we know that parenting can have long term effects on behavior. For example, the experience of being a parent influences parenting behavior during subsequent breeding attempts (Clutton-Brock 1988; Reichert et al. 2012). What has not been explored, however, is whether the experience of becoming a parent influences *personality traits*, i.e. behaviors that are variable among individuals and consistent within individuals over time. Understanding the robustness of personality traits across critical lifetime events can shed light on their plasticity, causation, and evolution.

Here, we investigate the effects of reproduction and parenting on personality (boldness) in threespine sticklebacks (*Gasterosteus aculeatus*). In this species, all of the parental care that is necessary for offspring survival is provided by the father, and parenting is an energetically costly (Smith & Wootton 1999) yet critical experience for males that strongly influences fitness (Wootton 1984). Most freshwater sticklebacks live for one year, and are seasonal breeders. Boldness is an important axis of variation in this species – some individual sticklebacks are consistently relatively timid while others are bolder (Huntingford 1976), and this variation influences fitness (Bell & Sih 2007). Here, we measure boldness as willingness to forage under predation risk.

There are at least two nonmutually exclusive hypotheses to explain how and why boldness might change as a function of reproduction and parenting. First, according to life

history theory, investment in a current brood often comes at a cost to future reproduction, therefore, as the probability of future reproduction decreases, we might expect boldness to increase (Montgomerie & Weatherhead 1988; Clark 1994). Indeed, on average, risk-taking behavior is higher at the end of the breeding season than at the beginning (fish: Magnhagen & Vestergaard 1991, Candolin & Voigt 2003; birds: Pugesek 1983; insects: Rosenheim et al. 2008; mammals: Dammhahn 2012; but see Ukegbu & Huntingford 1988). However, cross-sectional studies that do not repeatedly measure the same individuals cannot tell us whether individuals change their behavior as a function of experience, or if changes reflect selection or dispersal, for example. Moreover, work to date has been observational (rather than manipulative); therefore we do not know the causal factors driving changes in boldness (i.e. experience, age, seasonality, etc.).

Another hypothesis (the 'physiological remodeling hypothesis') supposes that the dramatic neural (Russell et al. 2001; Franssen et al. 2011) and endocrine (Wingfield et al. 1990; Saltzman & Ziegler 2014) changes that accompany reproduction and parenting have long-lasting effects on subsequent behavior (see also Macbeth & Luine 2010; Cost et al. 2014; Logan et al. 2014). For example, physiological changes associated with parenting might influence personality traits if individuals do not return to a hormonal "baseline". This hypothesis assumes that changes in physiology are more dramatic in individuals that parent versus those that do not, and predicts that hormonal changes occurring over the course of parenting are associated with personality traits after parenting has ceased. Importantly, the life history and physiological hypotheses are not mutually exclusive; while the former offers an ultimate explanation, the latter offers a proximate one.

We evaluate evidence for the life history and physiological remodeling hypotheses by repeatedly measuring boldness before and after reproduction and parenting in male sticklebacks. A control group was also repeatedly measured for boldness, but did not rear offspring. By comparing individuals that parented (experimental) with the control group, we could ask whether changes experienced by males in the experimental group were specifically due to reproduction and parenting, or if they reflect confounding effects such as time, age, or seasonality. We first confirmed that our measures of boldness were personality traits, and then asked how the experience of becoming a parent influences boldness by comparing the average risk taking behavior between males in the experimental and control groups. To test the physiological remodeling hypothesis, we repeatedly measured excreted 11-ketotestosterone (11-kT), the main androgen in fishes associated with courtship and parenting (Pradhan et al. 2014), and examined how changes in 11-kT levels were related to boldness.

METHODS

Wild-caught stickleback males were introduced into separate housing tanks. One week later, males were phenotyped for boldness (the “Before” trials) in an observation tank (53 x 33 x 24 cm) with a 5x2 grid drawn on the front, a gravel bottom and plastic plants for refuge. A model great egret (*Casmerodius albus*) skull was attached over the observation tank. The egret skull was situated so that when it was released via a lever from behind a blind, the tip of the bill splashed the water surface. This stimulus simulated the sudden overhead attack of an egret searching for prey (Giles & Huntingford 1984).

A single male was transferred into the observation tank. Thirty seconds later, we added 10 live bloodworms directly under the egret skull. If the male did not approach the bloodworms

within five minutes ($N=35$ of 169 trials), he was given a score of one greater than the maximum *latency to eat* (301 seconds).

When the male approached within one body length of the bloodworms, we released the egret skull to splash the water twice in quick succession, and then affixed the skull so that it remained above the water (Bell 2005; Alvarez & Bell 2007). Following the simulated attack, we recorded three behaviors: time to resume eating following the predator attack (*latency to eat*), number of pecks at the bloodworms (foraging under risk, *pecks at food*), and total number of squares moved (activity under risk, *squares moved*) for five minutes from behind a blind.

Each male was observed three times with 24 hours between trials and measured for standard length and weight after the third trial.

Experimental and control groups

Males were randomly assigned to either the experimental or control group. Males in the experimental group were paired with a male from the control group. Individual males were introduced into 9.5L tanks. Each tank contained a refuge, an open plastic box filled with fine sand, gravel, and filamentous algae for nest building.

Once both the control and experimental males within a pair had built nests, a gravid female was selected at random, weighed, placed in a long-necked flask and introduced to the paired control male for five minutes. This allowed the male to interact with and court the female, but not spawn. We then placed the female directly into the tank of the experimental male. We subtracted female weight after spawning from weight prior to spawning to estimate egg mass. We acknowledge that the experience of reproduction and parenting were confounded in this experiment. However, if we had attempted to separate the two, we would have had to

experimentally depredate the control males' nests, which could have influenced their subsequent behavior.

Five days after fry hatched, we transferred the experimental male and his paired control male to new separate housing tanks. One week later, males were measured for boldness once per day for three days (the "After" trials). In total, we measured boldness of $N=10$ experimental and $N=10$ control males which completed both the Before and After trials ($N=6$ trials per individual).

Measuring 11-kT

11-kT excreted in water was measured at four time points using EIA: Before (immediately following the second Before trial), with eggs in the nest (three days after spawning), with fry in the nest (three days after fry hatched), and After (immediately following the second After trial). Control males were measured for 11-kT at the same time as their paired experimental male.

Data analysis

To confirm that our measures of boldness were personality traits, we estimated the repeatability of boldness during the Before trials. We combine data from males in the control and experimental groups for this analysis, as they had all received the same experience at this time. To test if parenting influenced rank-order stability (among-individual variation) in boldness, we estimated repeatability across the Before and After trials for the two treatment groups separately. We used generalized linear mixed models with Markov chain Monte Carlo (MCMC) estimation for all repeatability analyses using MCMCglmm (Hadfield 2010) in R v. 3.0.1 (<http://www.r-project.org>).

To determine whether reproduction and parenting influenced mean-level stability of personality traits, we used linear mixed models (LMMs). To examine physiological changes associated with parenting and reproduction, we computed the difference in 11-kT release rate before and after parenting, as we were interested in long-term impacts of hormonal changes after parenting had ceased. We used Spearman rank correlations to examine associations between physiological changes and boldness.

RESULTS

Repeatability of boldness and 11-kT

During the Before trials, there were consistent individual differences in all behaviors measured (repeatability (R) [95% CI]: *latency to eat*: 0.39 [0.12, 0.63]; *pecks at food*: 0.18 [0.07, 0.44]; *squares moved*: 0.12 [0.05, 0.37]).

In general, males that were relatively bold during the Before trials were also relatively bold during the After trials (Table 1.1). A notable exception is *latency to eat*, which in the control group showed little among-individual variation and was not repeatable between the Before and After trials.

There were also consistent individual differences in 11-kT release rate (Table 1.1). Repeatability estimates of 11-kT did not statistically differ between the experimental and control treatment groups. However, relative to control males, males in the experimental group had both greater among-individual variation (i.e., were more different from one another) and greater within-individual variation in 11-kT.

Effects of parenting on mean level change

Boldness changed over time. Regardless of treatment, males were quicker to resume eating following a simulated predator attack during the After trials compared to the Before trials (Fig 1.1A). There was no evidence that the increase in boldness reflects habituation to the assay, as there was no effect of trial on behavior (Table 1.2).

The experience of reproduction and parenting also influenced boldness (Table 1.2). Specifically, there were significant treatment*stage interactions for *pecks at food* (Fig 1.1B) and *squares moved* (Fig 1.1C). Males in the control group increased activity (*squares moved*) in the After trials compared to the Before trials (paired t-test, $t_9=-2.61$, $P=0.03$), while males in the experimental group did not (paired t-test, $t_9=-1.72$, $P=0.12$, Fig 1.1C). Indeed, the general pattern is that control males became more bold over time, while the average behavior of experimental males did not differ between the Before and After trials.

11-kT release rates were higher in the experimental group compared to the control group (Table 1.2). Specifically, when experimental males had eggs or fry in the nest, they had higher 11-kT release rates compared to males in the control treatment, and this was maintained after parenting (Fig 1.2).

Hormonal changes and boldness

The boldness of experimental males after reproducing and parenting was related to the hormonal changes they experienced (Fig 1.3). Specifically, males that experienced an increase in 11-kT release rate foraged more under predation risk (*pecks at food*), while males that experienced a decrease in 11-kT release rate foraged relatively little ($r_s=0.80$, $N=10$, $P=0.009$). Foraging under risk was not related to hormonal changes in control males ($r_s=-0.44$, $N=10$,

$P=0.19$). Hormonal changes were not significantly related to either *latency to eat* or *squares moved* in either treatment.

DISCUSSION

We provide experimental evidence that personality traits can change, even in adults. According to all three measures of boldness, the control group became bolder over time. This result is consistent with correlative studies (Pugesek 1983; Magnhagen & Vestergaard 1991; Candolin & Voigt 2003; Rosenheim et al. 2008; Dammhahn 2012) showing that risk taking behavior increases over the course of the season, and is predicted by life history theory: animals take more risks as the end of the breeding season approaches because there are fewer opportunities for future reproduction (Montgomerie & Weatherhead 1988; Clark 1994).

We also provide experimental evidence that the experience of becoming a parent can influence personality traits. As in the control group, the experimental group resumed foraging faster (*latency to eat*) during the After trials compared to Before. However, according to two other measures of boldness (*pecks at food* and *squares moved*), the behavior of the experimental group did not change over time. We interpret foraging under predation risk and activity as behaviors that are more dangerous than quickly foraging after an attack because they are both conspicuous and require males to remain within reach of a sit-and-wait predator such as egrets. In contrast, quickly returning to eat after a predator attack does not necessarily incur high risk, as the animal could dart under cover after consuming the food item.

Why didn't the boldness of males in the experimental group change over time? One possibility is that there was positive feedback in males that had successfully raised a clutch: as their behavioral strategy had worked in the past, there was no reason to change that strategy.

Male sticklebacks often have multiple breeding attempts in a single season, and males that have one successful clutch are more likely to have another successful clutch (Wootton 1984). Another possibility is that neural and hormonal changes that occur during parenting might act as a proximate constraint on personality traits, channeling males along different trajectories.

Although experimental males did not change boldness over time *on average*, the experience of parenting made males more different from one another: males that had parented showed greater among-individual variation in *latency to eat* and 11-kT release rates than control males. There is evidence in humans that personality stability increases with age and experience, which is thought to reflect an increase in options that allow the expression of naturally occurring individual variation (Roberts & DelVecchio 2000). This can lead to individuals actively choosing environments that appeal to their personality (humans: Roberts & DelVecchio 2000; flies: Saltz 2011; stickleback: Pearish et al. 2013). It is possible that the experience of parenting in sticklebacks might do something similar, and reveal cryptic underlying individual variation.

We also found support for the physiological remodeling hypothesis. One of the assumptions of this hypothesis is that parenting is associated with dramatic physiological change. In the experimental group, 11-kT release rate increased 1.4-fold after there were eggs in the nest, and then dropped after hatching. In contrast, 11-kT release rate remained relatively stable over time in the control group. Other studies, including in sticklebacks (Pall et al. 2002), found that 11-kT levels peak while males care for offspring (Rodgers et al. 2006; Pradhan et al. 2014). Importantly, parents did not return to baseline, suggesting that they did not fully “recover” physiologically from the increase in 11-kT during parenting for at least one week and providing a potential mechanism to explain behavioral differences between males that parented and those that did not.

The extent to which a male experienced dramatic hormonal fluctuations was related to his behavior. Males that experienced a drop in 11-kT release rate following reproduction and parenting were more timid. In contrast, if a male experienced an increase in 11-kT release rate, he was more bold. For males that did not parent, changes in 11-kT release rates were not associated with boldness. It is unlikely that this is due to a “ceiling” effect in control males, as there was still variation in whether the control group experienced an increase or decrease in 11-kT release rate, albeit not as extreme as in the experimental group. In other fish species, males with high levels of androgens have greater paternity (Neff & Knapp 2009), and higher 11-kT levels are associated with larger brood sizes (Ros et al. 2009). Therefore, males with higher 11-kT levels after parenting may be more likely to resume breeding, and may therefore be more willing to take risks. Further studies examining the links between breeding experience, boldness, and hormone profiles are needed to elucidate mechanisms underlying changes in personality traits following the experience of parenting.

We found evidence for both proximate and ultimate causes of change in personality traits in stickleback as a function of a major life history event – the experience of becoming a parent. While studies in humans have suggested that experiences such as marriage, divorce, parenting, etc. influences personality traits (Jeronimus et al. 2014), such studies are by necessity correlational, and are often not longitudinal. By using a repeated measures design in which the same males were measured before and after a formative experience, and by comparing them to a control group that did not have that experience, we report here that becoming a parent can influence personality traits. It will be fascinating for future longitudinal and experimental studies to test whether other major adult experiences (pregnancy, dispersal, acquiring a territory, food

shortage, etc.) can influence personality traits in nonhuman animals and to develop proximate and ultimate explanations for how and why personality traits might change over time.

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TABLES

	<i>Experimental</i>	<i>Control</i>
<i>latency to eat</i>		
among	2.56 [0.81, 11.95]	0.008 [0, 3.99]
within	2.51 [1.50, 4.08]	3.22 [2.17, 5.96]
<i>R</i>	0.53 [0.14, 0.82]	0.003 [0, 0.52]
<i>pecks at food</i>		
among	3.13 [0.95, 19.88]	3.84 [0.95, 16.99]
within	16.48 [10.72, 29.23]	20.27 [12.91, 32.95]
<i>R</i>	0.18 [0.05, 0.56]	0.14 [0.04, 0.46]
<i>squares moved</i>		
among	67.77 [12.35, 319.71]	191.40 [38.33, 791.60]
within	200.77 [126.93, 366.01]	306.70 [193.90, 518.75]
<i>R</i>	0.25 [0.06, 0.63]	0.41 [0.14, 0.75]
<i>11-kT release rate (ng/g/min)</i>		
among	216.58 [61.00, 906.00]	8.86 [2.08, 41.91]
within	259.27 [155.78, 558.32]	67.72 [37.72, 112.10]
<i>R</i>	0.61 [0.19, 0.80]	0.11 [0.04, 0.40]

Table 1.1: Variance component (among- and within-individual) and repeatability estimates (*R*) of *latency to eat*, *pecks at food*, *squares moved* and 11-kT in experimental and control males. Numbers in brackets indicate 95% credibility intervals. Bold indicates significant repeatability.

<i>Factor</i>	<i>latency to eat</i>		<i>pecks at food</i>		<i>squares moved</i>		<i>11-kT release rate (ng/g/min)</i>	
	<i>F(df)</i>	<i>P-value</i>	<i>F(df)</i>	<i>P-value</i>	<i>F(df)</i>	<i>P-value</i>	<i>F(df)</i>	<i>P-value</i>
Treatment	0.18 (1,17.0)	0.67	0.12 (1,18.0)	0.74	0.06 (1,16.7)	0.81	5.11 (1,21.5)	0.03
Stage [†]	9.88 (1,91.1)	0.002	0.52 (1,69.4)	0.48	11.98 (1,66.9)	0.001	2.35 (3,49.4)	0.08
Treatment*Stage	0.15 (1,91.1)	0.70	4.70 (1,69.5)	0.03	4.97 (1,66.8)	0.03	0.80 (3,49.4)	0.50
Trial(Stage)	0.78 (4,91.1)	0.54	1.55 (4,68.9)	0.20	0.08 (4,66.3)	0.99	-	-
Length	0.001 (1,17.2)	0.99	3.18 (1,17.7)	0.09	0.05 (1,16.2)	0.83	0.73 (1,21.0)	0.40

[†]Stage has two levels for latency to eat, pecks at food, and number of squares moved (before and after) and four levels for 11-kT (before, with eggs, with fry, and after).

Table 1.2: Linear mixed model results. Bold indicates significant effects ($P < 0.05$).

FIGURES

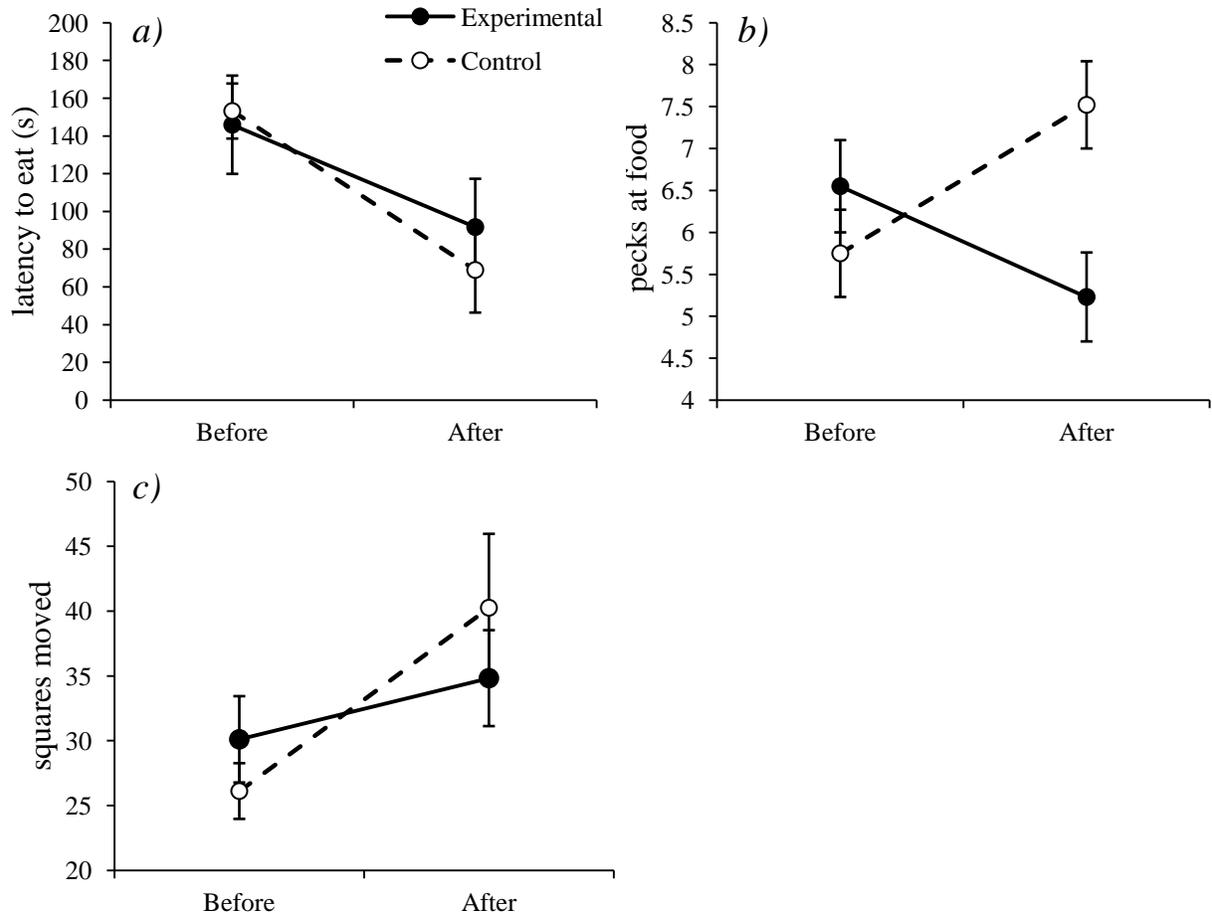


Figure 1.1: Boldness was influenced by stage and treatment. A) Males were quicker to resume eating following a simulated predator attack during the After trials. B) Males in the control group increased *pecks at food* After, while males in the experimental group did not change. C) Males in the control group increased activity under predation risk After, while males in the experimental group did not change. Error bars ± 1 SE.

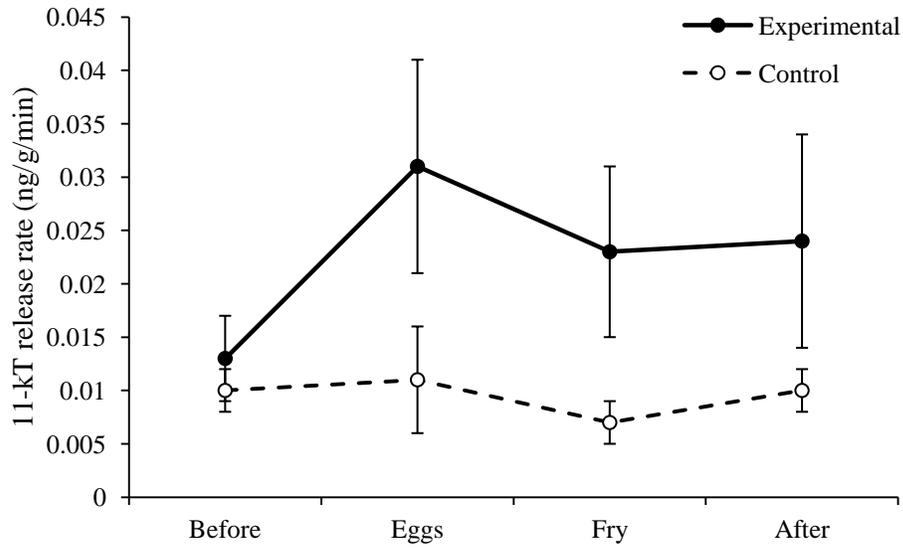


Figure 1.2: 11-kT release rate changed over time in the experimental group (solid line) versus the control group (dotted line). Error bars ± 1 SE.

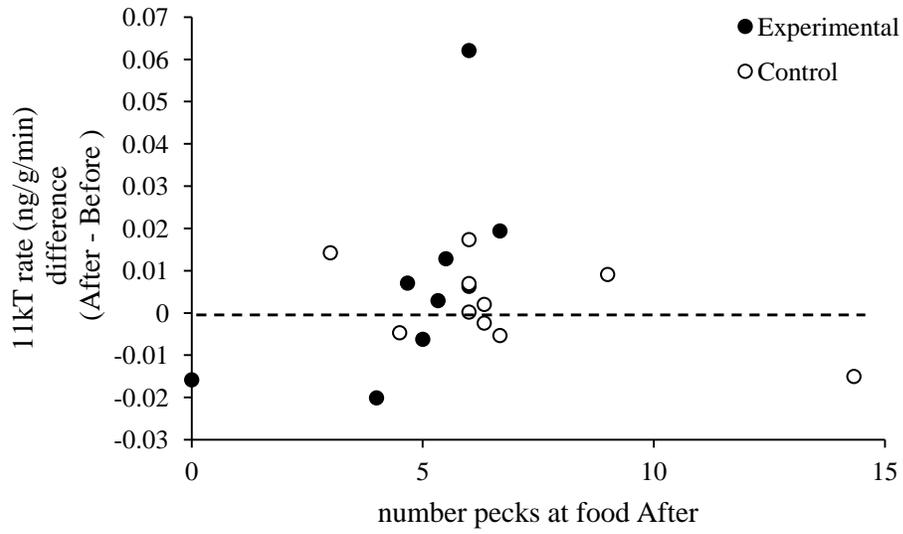


Figure 1.3: Shifts in 11-kT were correlated with boldness in the experimental group (closed circles). The dashed line indicates no change; points above the line indicate males with greater 11-kT After and points below the line indicate males with lower 11-kT After.

CHAPTER 2: CONSISTENT INDIVIDUAL DIFFERENCES IN FATHERING IN THREESPINED STICKLEBACK (*GASTEROSTEUS ACULEATUS*)¹

ABSTRACT

There is growing evidence that individual animals show consistent differences in behavior. For example, individual threespined stickleback fish differ in how they react to predators and how aggressive they are during social interactions with conspecifics. A relatively unexplored but potentially important axis of variation is parental behavior. In sticklebacks, fathers provide all of the parental care that is necessary for offspring survival; therefore paternal care is directly tied to fitness. In this study, we assessed whether individual male sticklebacks differ consistently from each other in parental behavior. We recorded visits to nest, total time fanning, and activity levels of 11 individual males every day throughout one clutch, and then allowed the males to breed again. Half of the males were exposed to predation risk while parenting during the first clutch, and the other half of the males experienced predation risk during the second clutch. We detected dramatic temporal changes in parental behaviors over the course of the clutch: for example, total time fanning increased six-fold prior to eggs hatching, then decreased to approximately zero. Despite these temporal changes, males retained their individually-distinctive parenting styles within a clutch that could not be explained by differences in body size or egg mass. Moreover, individual differences were maintained when males reproduced for a second time. Males that were exposed to simulated predation risk briefly decreased fanning and increased activity levels. Altogether, these results show that individual sticklebacks consistently differ from each other in how they behave as parents.

¹ This chapter appeared in its entirety in the journal *Current Zoology* and is referred to later in this dissertation as “Stein and Bell 2012”. Stein, LR, and Bell, AM. 2012. Consistent individual differences in fathering in threespined stickleback (*Gasterosteus aculeatus*). **58**, 45-52. This article is reprinted with permission of the publisher and is available from <http://www.actazool.org>.

INTRODUCTION

Consistent individual differences in behavior (“personality”) have been well-documented (Bell et al., 2009) and may have important fitness consequences (Dingemanse and Reale, 2005). Studies of animal personality have primarily focused on behavioral variation in traits such as aggressiveness, boldness and exploratory behavior (Reale et al., 2007). Fewer studies have examined whether how an individual behaves as a parent is part of an individual’s personality (but see Budaev et al., 1999). While there is evidence that variation in parental care is an important axis of inter-individual variation in a range of taxa, including mammals (Maestripieri, 1998; Meaney, 2001) and birds (Nakagawa et al., 2007), we know less about variation in parental care in species where males are the sole providers of parental care.

Threespined sticklebacks (*Gasterosteus aculeatus*) have become an important model system in the study of individual variation in behavior. Individual sticklebacks consistently differ in how they behave in ecologically-relevant contexts such as in the presence of a predator (Huntingford, 1976), during competition and other social interactions (Pike et al., 2008; Harcourt et al., 2009) and in a novel environment (Bell and Stamps, 2004). Another important context with a direct link to fitness in sticklebacks is parenting: in this species, parental care is necessary for offspring survival (Wootton, 1984) and is provided solely by the male. During the breeding season, male sticklebacks establish territories, build nests, and court females. After a female spawns, she leaves the territory and the male provides all of the parental care while defending the territory and nest from predators and intruders. During the incubation period (approximately 3 – 6 days), the male “fans” the eggs with his pectoral fins, providing oxygen and clearing carbon dioxide (Wootton, 1984) and removes rotten eggs and debris. After the eggs hatch, the male tends the fry for approximately 4 – 7 days until the fry are free-swimming, fanning the young

immediately after hatching and retrieving individuals that stray from the nest. In addition to physically protecting and meeting physiological requirements of his offspring, Tulley and Huntingford (1987) suggested that behavioral interactions between fathers and fry at the nest might also influence the development of his offspring's antipredator behavior.

While directly related to male's reproductive success, parenting in sticklebacks is costly: it is energetically expensive (Chellappa et al., 1989; Smith and Wootton, 2005) and exposes males to predation risk (Candolin, 1998). Moreover, while simultaneously rearing his offspring, male sticklebacks must also defend them and maintain a territory. Therefore we might not expect male sticklebacks to show within-individual consistency in parental behavior over time because they must be behaviorally flexible in order to manage several competing demands; this flexibility has been empirically demonstrated in other fishes (Hale et al., 2003; Lissaker and Kvarnemo, 2006). Another reason why we might not expect to see strong behavioral consistency is because male stickleback behavior changes dramatically over the course of the nesting cycle – rates of fanning, for example, change with the age of the eggs and fry (van Iersel, 1953).

On the other hand, studies in biparental systems have suggested that males behave consistently as parents. Indeed, some studies on birds have shown that males behave more consistently than their female mates, who adjust more readily to the needs of their offspring (Schwagmeyer and Mock, 2003; Nakagawa et al., 2007). A plausible cause of consistent individual differences in paternal behavior is a hormonal constraint; in other vertebrates, including some teleost fishes, tradeoffs between territory defense and parenting in males are mediated by androgens, where high levels of androgens increase defense behaviors and decrease parenting (Wingfield et al., 1990; Oliveira et al., 2002; McGlothlin and Ketterson, 2008; but see Rodgers et al., 2006; Dey et al., 2010). Given the well-documented variation in aggressive

behavior during territory defense in threespined sticklebacks (Huntingford, 1976), it is possible that males differ in how they resolve the tradeoff, with some males prioritizing territory defense while others prioritize paternal care.

Another important factor potentially influencing behavioral consistency in parenting is predation risk. Pressures imposed by predation might favor behavioral flexibility in parenting behaviors of prey if prey adaptively decrease nest-directed parental care such as incubation and nestling provisioning (Martin et al., 2000; Ghalambor and Martin, 2002) and increase nest defense (Lissaker and Kvarnemo, 2006; Cooke et al., 2008) under predation risk. However, if predation risk is constant and predictable, then we might expect animals to adopt a fixed behavioral strategy, if a fixed behavioral strategy minimizes risk of depredation (Tarwater and Brawn, 2008). Indeed, high levels of predation risk have been implicated in increasing behavioral consistency outside of the parental care context in threespined stickleback (Bell, 2005; Bell and Sih, 2007; Dingemanse et al., 2007).

Several authors have already suggested that individual stickleback males consistently differ in how they behave as fathers (van Iersel, 1953; Feuth-De Bruijn and Sevenster, 1982), in nest site preference (Moodie, 1971), and in size and composition of nests (Rushbrook et al., 2008). However, consistent variation in parental behaviors has not been explicitly examined. In this study, we assess whether wild-caught stickleback males differ in parental care, and whether they behave consistently across clutches in controlled conditions in the lab. We used a within-subject experimental design wherein males were bred twice (two “clutches”). During the first clutch, male parental behaviors were observed every day for approximately 10 days, the average length of a nesting cycle from spawning through fry dispersal. After completing the first clutch (clutch one), males were transferred to a new tank for the second clutch (clutch two) and

behavior was observed as in clutch one. Males were randomly assigned to the control or experimental treatment in clutch one, and experienced the other treatment in clutch two. The experimental treatment consisted of the introduction of a model nest predator (sculpin) to the tank. As theory predicts parental response to predation should increase as eggs approach hatching and fry develop (Cooke et al., 2008), we presented the model predator to experimental males on two occasions: once when eggs were near hatching, and again after the eggs hatched into fry. The experimental treatment was designed to simulate the presence of predation risk in the environment. We predicted that males exposed to the predator model would be more “cautious” in their parental behaviors after exposure to predation risk, reducing fanning behaviors and spending less time at the nest.

METHODS

Study population

Adult sticklebacks were collected from Putah Creek in April 2010. Putah Creek is a dammed, regulated freshwater stream in the Central Valley of California. While native fish predators are not common in this creek, prickly sculpin (*Cottus asper*) have been observed there and are a natural nest predator of the threespined stickleback (Pressley, 1981). Sticklebacks were shipped to the University of Illinois at Urbana-Champaign and kept in 83L (107 x 33 x 24 cm) mixed sex aquaria until the beginning of the experiment (May 2010) at 20 degrees Celsius on a summer (16L:8D) photoperiod. Water was cleaned via a recirculating flow-through system that consists of a series of particulate, biological, and UV filters (Aquaneering, San Diego, USA). 10% of the water volume in the tanks was replaced each day. Fish were fed a mixed diet consisting of frozen bloodworm, frozen brine shrimp, frozen Mysis shrimp *ad lib* each day.

Experimental set-up

Before being introduced into separate 9.5L (36 x 21 x 18 cm) tanks, 12 males were weighed and measured for standard length. Each tank had four equal-sized squares (9 x 9 cm) drawn on the front in sequence. Each tank contained a refuge (plastic "plant"), an open plastic box (13 x 13 x 3 cm) filled with fine sand, and filamentous algae for nest building. The floor of the tank was covered in coarse gravel. Dividers were placed between tanks to prevent visual interactions between neighboring males. Twenty females were housed across two 38L tanks (53 x 33 x 24 cm), each with at least one refuge. To induce gravidity, females were provided with extra bloodworm in addition to regular feeding.

Nest completion was determined when a visible opening in the nest was present. After nest completion, a gravid female was selected at random, patted dry, weighed, and placed with the male. If spawning did not occur within half an hour, the female was removed and a new gravid female was introduced after 24 hrs. Immediately after spawning, the female was removed, patted dry, and weighed again. The difference in female mass pre- and post-spawning provided a measure of total egg mass. Each male was presented with a unique female, and each female spawned only once.

Behavioral observations

Behavioral observations began the day after spawning. Between 1000 and 1300 every day, an observer (LS) recorded the following parental behaviors for five minutes: *Visits to nest*, the number of times the male was within half a body length from his nest while oriented toward the nest; *Total time fanning*, the total amount of time a male spent fanning within an observation

period; and *Total squares moved*, the total number of squares a male moved during the observation period. Observations were made once per day.

Experimental treatment

At the start of the first breeding attempt (clutch 1), six males were randomly assigned to the experimental treatment; the other six served as controls. Three days after spawning, experimental males were chased with a 4-inch rubber sculpin model (Jewel Bait Company) for two minutes to simulate a nest predation attempt. The model “nosed” the nest, circled the perimeter of the tank, and approached the nest again before being removed. This process was repeated again three days after the eggs hatched. For control clutches, we removed the top of the tank and gently splashed the water when the eggs were three days old, and when the fry were three days old to simulate the water disturbance caused when the sculpin model entered the tank. The simulated predation threat occurred after the daily behavior observation.

Observations ceased five days after the eggs hatched, and males were removed from their tank and placed in new individual tanks with nesting material and a refuge. Males were allowed to construct second nests and the entire process, including daily behavioral observations, was repeated (clutch 2); however, for the second clutch males previously exposed to the experimental treatment were now controls and vice versa. After males were removed from their fry a second time, they were placed in new individual tanks.

Data analysis

To quantify individual differences in behavior, we tested for the effect of treatment, individual male ID, and day in the nesting cycle (‘nesting stage’) on each behavior throughout

the nesting cycle using ANOVA. We used the mean squares from these ANOVA to estimate repeatability within a clutch; this allowed us to control for differences between nesting stages when evaluating individual differences (Lessels and Boag, 1987), with standard errors estimated as in Becker (1992). Treatment was never significant in the analyses of behavior over the entire nesting cycle (see Results), but the males were only exposed to predation risk during certain days rather than throughout the entire nesting cycle. Therefore we had a closer look at the effect of treatment on behavior by comparing the control and experimental group just on the days immediately following exposure to predation risk. To examine consistency in behavior across clutches, we computed average behavior during each clutch, and tested for correlations between the averaged values. We examined the effect of clutch (1st or 2nd breeding attempt), male standard length and egg mass on the averaged values. All statistics were performed using PASW 18 (SPSS Inc.).

RESULTS

Eleven males completed one clutch, eight males completed two clutches. Only one of the original twelve males aborted his clutch via cannibalization; these data were excluded from analysis. In the first clutch, five males were exposed to a predator model and six were controls. Of the eight males that completed two clutches, four were exposed to a predator model and four were controls during their second clutch. Males that did not complete two clutches either failed to build new nests or failed to spawn. There were no obvious differences between males that had one or two clutches, i.e. no significant differences in male length, egg mass, visits to nest, or total time fanning (independent samples t-test, $P > 0.5$ for all), except that males that had one clutch

visited the nest significantly more often (mean 5.36 ± 0.94 visits) than males that had two clutches (mean 2.98 ± 0.88 visits; independent samples t-test, $t_9 = 3.93$, $P = 0.025$).

For the analyses of individual differences in parental behaviors within a clutch, we focus on the data from the first clutch only ($n = 11$). During the five-minute observation periods, males engaged in multiple parental behaviors, including fanning and visiting the nest. On average, males spent one of the five minutes fanning the nest during the behavioral observation. Males were also active during the observation period, moving between areas of the tank on average approximately seven times in five minutes. Some of these behaviors, however, showed a distinctive pattern of temporal change across days throughout the nesting cycle (Figure 2.1, Table 2.1). For example, the *Visits to nest* as well as *Total time fanning* increased as the eggs approached hatching, and then rapidly decreased. Similar temporal changes were observed in clutch 2, e.g. increase in fanning before hatching, etc (data not shown).

While there were dramatic temporal changes in behavior within the breeding cycle, individuals showed distinctive parenting types. There was a significant effect of ‘individual’ on all of the behaviors measured, with repeatability within a clutch ranging from 0.22 ± 0.11 to 0.33 ± 0.12 (Table 2.1). There was no indication that a male’s parental behavior was influenced by the mass of the clutch within his nest (*Visits to nest*: $r = 0.309$, $P = 0.355$; *Total time fanning*: $r = 0.255$, $P = 0.450$; *Total squares moved*: $r = 0.418$, $P = 0.201$) or his body size (*Visits to nest*: $r = -0.068$, $P = 0.841$; *Total time fanning*: $r = -0.416$, $P = 0.204$; *Total squares moved*: $r = -0.123$, $P = 0.718$). When the males reproduced for a second time, they retained their individually distinctive parenting type (Figure 2.2). For example, male 10, which had a high number of *Visits to nest* during clutch 1, also visited the nest often during clutch 2. Figure 2.2 shows the correlation between the average *Visits to nest* in clutch 1 and clutch 2. We did not detect any

differences in average behavior between the first and the second clutch ($P > 0.05$), but note that only eight males successfully spawned twice, so our power to detect differences was limited.

When the sculpin was introduced into a male's tank, males reacted by hiding, orienting to it and/or attacking the model. There were subtle, transient effects of exposure to predation risk on parental behaviors on the days immediately following exposure to predation risk (analysis restricted to first clutch only). For example, after being exposed to the sculpin with eggs in the nest, males spent less *Total time fanning* compared to control males (One day after exposure: $82.7 \pm 26s$, $n = 6$ vs $132 \pm 13s$, $n = 5$; Two days after exposure: $63.7 \pm 36s$, $n = 3$ vs $185.3 \pm 15s$, $n = 3$). The sample size is lower two days after exposure because some clutches hatched 4-5 days after fertilization. Another intriguing pattern was that individual variation among males exposed to predation risk was higher compared to control males. For example, the *Visits to nest* was not repeatable among males in the control group, but the repeatability of *Visits to nest* was 0.54 when males were exposed to predation risk (Table 2.2). The greater repeatability in the experimental group was due to an increase in between-individual variation (MS between vs MS within, Table 2.2). We detected the same pattern (repeatability of experimental males higher than repeatability of control males) for *Total time fanning*, and *Total squares moved*. In each case, the increase in repeatability within a clutch was due to greater variation among males when they experienced the experimental treatment, rather than smaller within-individual variation (Table 2.2).

DISCUSSION

Despite dramatic temporal changes in behavior over the course of the nesting cycle, fathers exhibited consistent individual differences in their parental behaviors both within and across breeding episodes (clutches). Parental behaviors varied substantially among males,

ranging from males that spent a large fraction of their time near the nest and fanning to males that were less 'attentive', and those differences were maintained even when males were transferred to a new environment (tank) and reproduced for a second time, with a different female. Moreover, these individual differences in parental behavior were not related to a male's body size or the mass of the eggs in his nest, suggesting that the individual differences do not reflect transient differences in 'state'. Altogether these results show that wild-caught threespined stickleback males show an individually distinct and consistent parenting style within and across clutches, even though their behavior changed dramatically over time.

The failure to find an influence of egg mass on parenting behaviors is unusual. Larger clutch size is typically associated with increased parental behaviors in many fish with paternal care (Sargent, 1988; Ridgway, 1989; Karino and Arai, 2006). Egg mass was highly variable during clutch 1 (range 0.1g – 0.7g) and so this result cannot be attributed to low variance. However, it is important to note that we used egg mass as a measure of clutch size; to minimize disturbance to the male we did not count the eggs in each nest. It has been suggested that parental investment in fish is driven primarily by total number of eggs rather than egg size (Kolm et al., 2006), and so if egg mass is not a reliable indicator of egg number we would not necessarily expect to see a strong effect of egg mass on parental behaviors.

It is likely that the temporal changes in behavior reflect a male's response to the changing needs of eggs or fry at different stages of development (van Iersel, 1953). For example, rates of fanning increased just prior to hatching, perhaps due to increased energetic needs of the eggs (Sevenster, 1961) Importantly, the differences among males in behaviors such as fanning do not reflect differences in territory quality or oxygen availability because males were all housed in identical tanks, and in a flow-through system with standardized conditions across tanks.

Males that were exposed to predation risk by a model sculpin altered their behavior on the days immediately following the threat by decreasing their amount of time fanning and increasing activity (*Total squares moved*). These results are consistent with the hypothesis that a threat to a male's territory causes him to reallocate activity toward territory defense at the expense of parental care. However, unlike previous studies in birds and other teleost fishes where exposure to predation risk resulted in long-term changes in parenting both within and across clutches (Eggers et al., 2005; Cooke et al., 2008; Chalfoun and Martin, 2010), the effect of predation risk on behavior in this experiment was relatively transient. This suggests that although males respond immediately to predation risk, they relatively quickly return to their behavioral type following a disruption. The other intriguing consequence of exposure to predation risk that we observed in this experiment is that males exposed to predation risk showed higher variation compared to control males. The increased between-individual variation under predation risk could reflect individual differences in strategies for coping with risk in the environment. This result is consistent with previous studies showing that exposure to predation risk increases behavioral consistency across contexts (Bell, 2005; Bell and Sih, 2007).

In a wide range of taxa, there are strong inter-individual differences in parental behavior, both among fathers and among mothers (Feuth-De Bruijn and Sevenster, 1982; Budaev et al., 1999; Champagne et al., 2001; Maestriperi, 2001; Meaney, 2001; Schwagmeyer and Mock, 2003; Nakagawa et al., 2007). Other studies have shown that there are consequences of fathering for offspring behavior in sticklebacks: in high-predation populations, offspring raised by their fathers show greater antipredator behavior than orphans (Tulley and Huntingford, 1987). Therefore, consistent individual differences in parental behavior in sticklebacks may influence the strength and direction of parental effects in this species.

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TABLES

A) Visits to nest

Source	df	MS	F-value	P-value
Male ID	10	17.74	3.61	0.001
Nesting Stage	11	9.07	1.84	0.06
Treatment	1	12.18	2.47	0.12
Error	81	4.92		

R ± SE: 0.22 ± 0.11

B) Total time fanning

Source	df	MS	F-value	P-value
Male ID	10	5819.77	4.33	< 0.0001
Nesting Stage	11	22953.1	17.07	< 0.0001
Treatment	1	36.67	0.027	0.869
Error	81	1345.06		

R ± SE: 0.27 ± 0.11

C) Total squares moved

Source	df	MS	F-value	P-value
Male ID	10	135.2	5.6	< 0.0001
Nesting Stage	11	67.92	2.81	0.004
Treatment	1	0.062	0.003	0.96
Error	81	24.163		

R ± SE: 0.33 ± 0.12

Table 2.1: ANOVAs testing for male ID, nesting stage, and treatment on A) visits to nest; B) total time fanning; C) total squares moved on behavior during clutch 1; n = 11. Repeatabilities were estimated from these ANOVA.

Behavior	Treatment	MS_{among}	MS_{within}	R ± SE
Visits to nest	C	9.24	5.66	0.10 ± 0.15
Visits to nest	E	27.62	3.4	0.54 ± 0.20
Total time fanning	C	5011.4	1333.3	0.33 ± 0.19
Total time fanning	E	10356.4	1768.6	0.45 ± 0.21
Total squares moved	C	31.5	27.6	0.025 ± 0.13
Total squares moved	E	182	31.5	0.44 ± 0.21

Table 2.2: Repeatability of behavior was greater for experimental males. Repeatabilities calculated from MS from ANOVA with male ID and nesting stage as fixed effects (first clutch only). Control (C): n = 6; Experimental (E): n = 5.

FIGURES

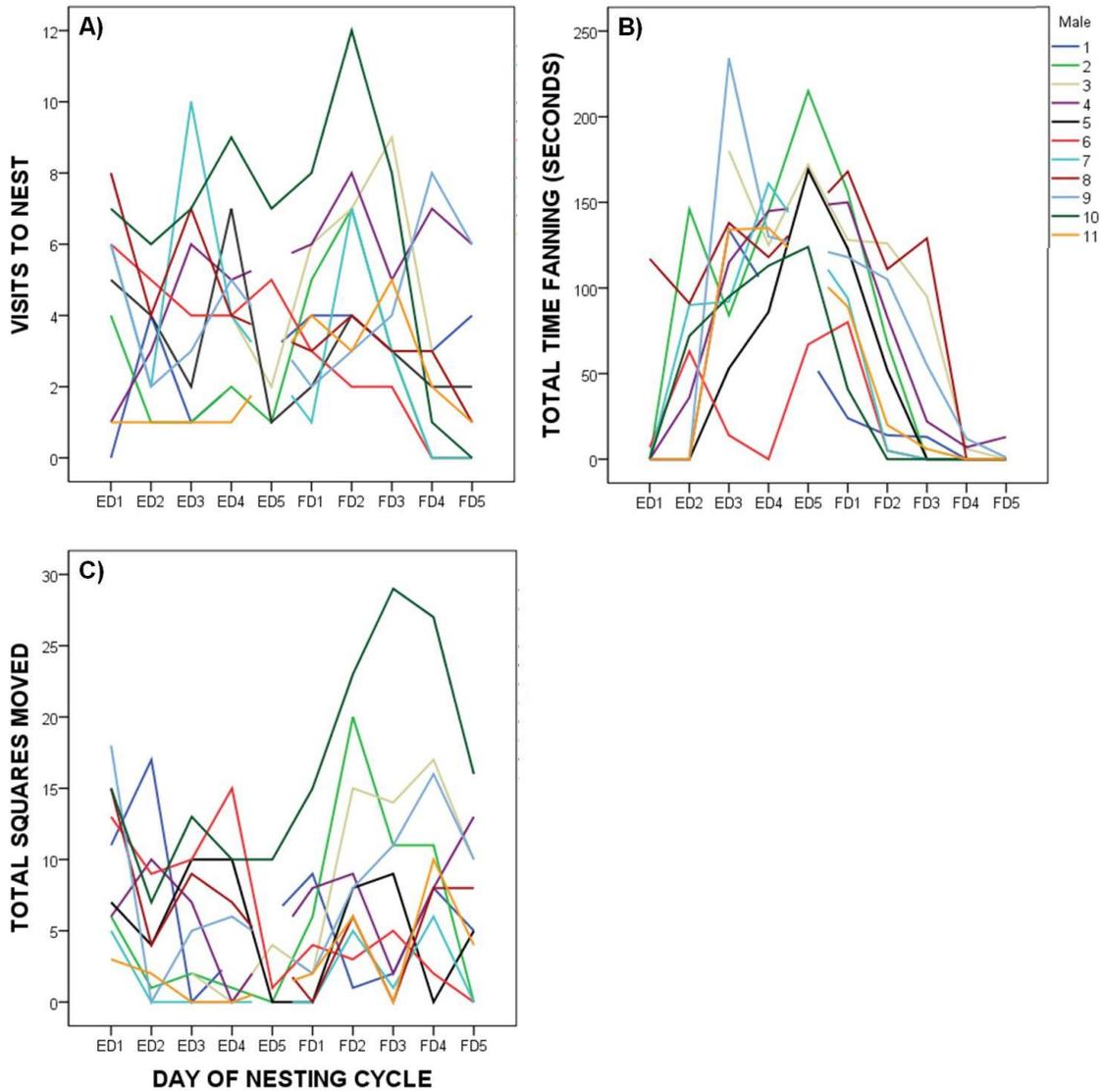


Figure 2.1: Individual differences in parental behaviors across days within a nesting cycle (clutch 1). ED1 = Eggs day #1 after fertilization; ED2 = Eggs day #2 after fertilization, etc. FD1 = Fry day #1 after hatch, etc. Each individual male is represented by a different color, with separate panels for each behavior; n = 11. A) Visits to nest; B) Total time fanning; C) Total squares moved.

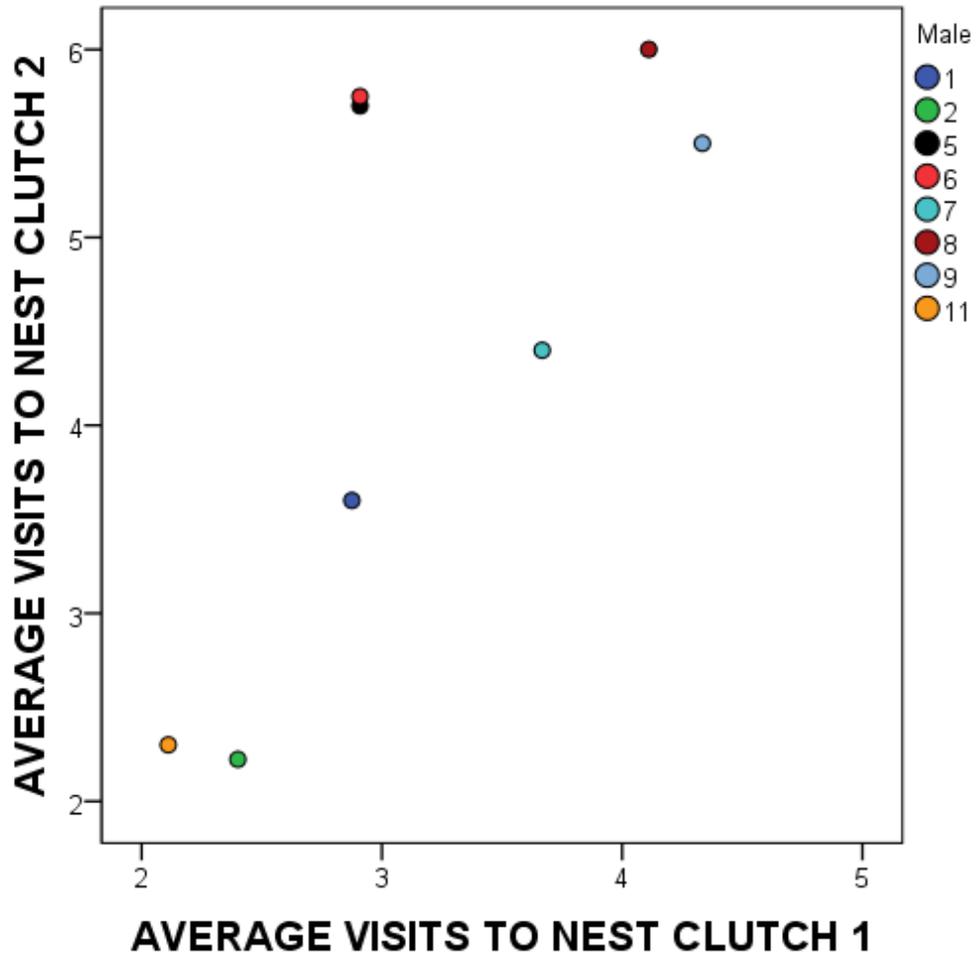


Figure 2.2: Individual differences in parental behaviors across two nesting cycles (clutches). Each point represents the average *Number of visits to nest* of a male within a clutch ($r = 0.717$, $P = 0.045$, $n = 8$)

CHAPTER 3: CONSISTENT INDIVIDUAL DIFFERENCES IN PATERNAL BEHAVIOR: A FIELD STUDY OF THREE-SPINED STICKLEBACK²

ABSTRACT

Consistent individual differences in parenting are widespread; however, we know little about why there is variation in parenting behavior among individuals within species. One possible explanation for consistent individual differences in parenting is that individuals invest in different aspects of parental care, such as provisioning or defense. In this field study we measured consistent individual differences in parenting behavior and evaluated correlations between parenting and other behaviors in threespine stickleback (*Gasterosteus aculeatus*). We repeatedly measured male parenting behavior and male behavior in the presence of three different types of live intruders: a female, a conspecific male, and a predator, meant to provoke courtship, aggressive and antipredator behavior, respectively. While males plastically adjusted their reactions to different types of intruders, we found consistent individual differences in behavior (behavioral types) both within and across contexts, even after accounting for variation in body size and nest characteristics. Males that performed more parenting behavior responded faster to all types of intruders. These results suggest that in nature, individual male stickleback exhibit robust parental behavioral types, and highly parental males are more attentive to their surroundings. Future studies are needed to examine the potential causes of individual variation in parental behavior in the field.

² This chapter appeared in its entirety in the journal Behavioral Ecology and Sociobiology and is referred to later in this dissertation as “Stein and Bell 2015”. Stein, LR and Bell, AM. 2015. Consistent individual differences in paternal behavior: a field study of three-spined stickleback. **69**, 227-236. This article is reprinted with permission of the publisher and is available from <http://www.springerlink.com/> and using DOI: 10.1007/s00265-014-1835-3

INTRODUCTION

Consistent individual differences in mothering are widespread (Meaney 2001; MacColl and Hatchwell 2003; Schwagmeyer and Mock 2003; Nakagawa et al. 2007; Westneat et al. 2011). There is also evidence that individual fathers differ in how they behave as parents. For example, male sticklebacks consistently differ in rates of fanning, a direct form of parental care (Stein and Bell 2012). Individual parents also consistently differ in indirect forms of parental care, such as offspring/nest defense (Kontiainen et al. 2009; Burtka and Grindstaff 2013).

However, we know little about why there is variation in parenting behavior among individuals within species. One possibility is that parents differ because they “program” their offspring for different types of environments, and variations in parenting act as cues to offspring about current conditions (Marshall and Uller 2007; Stein and Bell 2014). Another possibility is that variation in parenting reflects an individual’s physiology – highly aggressive individuals, for example, might be less parental due to a proximate constraint such as high levels of androgens (Ketterson and Nolan 1999; Wingfield et al. 1990). There might also be tradeoffs between direct and indirect forms of care such that highly attentive parents, for example, might trade off direct care with nest defense (Rangeley and Godin 1992; Lissaker and Kvarnemo 2006; Mutzel et al. 2013). Studies in birds have also provided evidence for positive correlations between direct offspring care (e.g. provisioning) and indirect forms of care (e.g. nest defense), suggesting that the most aggressive parents are also the most attentive to their offspring (Rytönen et al. 1995; Betini and Norris 2013; Wetzel and Westneat 2014). We might also expect courtship and parenting behaviors to be positively correlated if behavior during courtship provides an indication of future parenting behavior (Stiver and Alonzo 2009).

The majority of studies of parental care variation have been performed in the lab, where resources are abundant and predation non-existent. Fewer studies have been conducted in the field (but see Duckworth 2006; Patrick and Browning 2011; Barnett et al. 2012; Kazama et al. 2012; Mutzel et al. 2013; Cole et al. 2014; Wetzel and Westneat 2014), where there are more time and energy constraints due to few or patchy resources and predation risk. Therefore field studies might uncover constraints on the amount of care a parent can provide that are undetected in the lab. Here, we investigate male behavior on the breeding grounds in a natural population of threespine sticklebacks (*Gasterosteus aculeatus*).

Threespine sticklebacks are teleost fish in which the father is the sole provider of parental care, and paternal care is necessary for offspring survival (Wootton 1984). During the breeding season, male sticklebacks establish territories, build nests, and court females, while at the same time actively defending their nest from predators. Certain individual and territory qualities increase reproductive success, including large body size and nesting in deep water (Kraak et al. 1999a). After a female spawns, she leaves the territory and the male provides all of the parental care; males continue to court females to obtain more eggs for up to three days after the first spawning (Kraak et al. 1999b). During the incubation period (approximately 6 days in the population studied here), males “fan” the eggs with the pectoral fins, providing oxygen and clearing carbon dioxide (Wootton 1984) and remove rotten eggs and debris. Previous studies have shown that males that spend more time fanning their nest enjoy higher rates of hatching success (von Hippel 2000), and males are consistent in their fanning behavior both within and across clutches in the lab (Stein and Bell 2012). However, it is unknown whether males demonstrate consistent individual differences in parenting behavior in the field, where resources

are limited and parenting is not immune from competing demands on males' time and energy, such as territory defense and courtship opportunities.

Therefore, the goals of this field study were to 1) assess whether males consistently differed from one another in behavior, and 2) examine relationships between individual differences in parenting behavior and behaviors in other contexts. We observed undisturbed parenting males in the field to obtain a "baseline" measure of parenting behavior. We then recorded each male's behavior in the courtship, aggression and antipredator contexts by presenting the male with a gravid female, rival male stickleback, and a predator, respectively. The behavior of each male was repeatedly observed in each context for three days. After testing whether males consistently differ in behavior, we used these data to examine correlations between behaviors within and across contexts to determine whether parenting behavior was part of a larger suite of correlated behaviors.

METHODS

Study area and study system

This field study was performed in the South Fork of the Navarro River (Philo, Mendocino County, CA). The Navarro River is an undammed freshwater river running northwest along the California coast. Adult sticklebacks in this population experience predation by avian and fish predators (Feliciano 2004; LRS pers. obs.). While nests and fry in this population are preyed upon by a number of predacious insects and fish, predatory fish such as coastrange sculpin (*Cottus aleuticus*) and prickly sculpin (*C. asper*) additionally pose a threat to adults (Moodie 1972; Pressley 1981). A behavioral syndrome between aggressiveness and

boldness has been documented in this population (Bell 2005), opening the possibility that parental behavior may also be part of a larger suite of correlated behaviors.

Observations were conducted from 8 June – 1 July 2011. Individuals were observed between 1000 and 1700 PST every day. Only parenting males with eggs in the nest were used in this study. When a male stickleback was found guarding a nest, we observed the individual for up to 10 minutes for evidence of fanning, indicating the presence of eggs. We then tagged the nest using flagging tape tied on foliage or to a stick 30 cm from the nest. Flagging tape was at least 50 cm above the surface of the water to avoid attracting fish predators to the nest. There was no observed increase in avian predators to the study site after flagging tape was introduced. Parenting behavior in the absence of an intruder (“undisturbed” context) was then observed (see *Behavioral Assays* below). Following the observation of undisturbed parenting behavior, we gently removed the male from his nest using a dip net and placed him in a 19 liter bucket with fresh river water. We visually determined whether there were eggs in the nest and their stage of development (eyed or uneyed). In this population, fertilized eggs are uneyed for approximately three days, followed by three days in the eyed stage before hatching. Parenting behavior changes during the course of the nesting cycle (Stein and Bell 2012) and in this study we focus on the uneyed stage as males are still receptive to females during this time, allowing us to assess males’ courtship behavior. None of the clutches hatched during the three-day observation period, which suggests that nests were similar in age thereby reducing the possibility of offspring age-related parental investment. We then covered the nest with a wire cage to prevent depredation while the male was away from his nest. We quickly measured the male’s standard length and the depth of his nest in the water. Previous studies have demonstrated that larger males can fan the nest more efficiently (Kraak et al. 1999a; Künzler and Bakker 2000), and nest depth has been correlated

with greater reproductive success (Kraak et al. 1999a). We did not include coloration as a measure of male parental ability here because male coloration changes over the parenting cycle and may not be a reliable indicator of male quality in every population (Candolin 2007; Boughman 2007; Sparkes et al. 2008). The male was then returned to the nest and observed until he resumed parenting. Handling time of the males took less than 10 minutes (range 4 min – 9.5 min). All males resumed parenting and there was no indication that our activities caused males to abandon their nests.

Behavioral assays

Each male was observed in four contexts (undisturbed, courtship, aggression, and antipredator) in a fixed order every day for three consecutive days (three repeats per context per male). We used a fixed order because we were primarily interested in rank order consistency between individuals (Dingemanse et al. 2007; Bell 2013). To guard against carryovers across contexts, we tested individuals with the predator last, as we expected this intruder to have the most potential for carryover due to a slower stress recovery when male stickleback encounter a predator vs a conspecific (Bell et al. 2007; Bell 2013). Every day, animals used as intruders were captured using minnow traps baited with dog biscuits approximately eight kilometers upriver from the study site and were transported in opaque buckets. Intruders were presented to the focal male inside a 10x10x10 cm wire cage with 0.64 cm openings allowing visual and olfactory cues to reach the focal male. Preliminary observations suggested that an intruder elicited the maximum behavioral response when the cage was placed on the ground 3 cm in front of the nest opening, and there was no effect of an empty cage on fanning behavior (mean empty cage \pm SE: 22.09 \pm 8.49 s; mean no cage \pm SE: 25.73 \pm 7.78 s; paired t-test: $t_{10} = 0.39$, $P = 0.70$).

The cage was attached by string to the end of a rod and lowered remotely from the bank of the river. Individual intruders were used up to three times per day, once per male, and were returned to their point of capture at the end of the day. In total, $N = 114$ live gravid females (mean size \pm SE: 4.5 ± 0.3 cm), $N = 108$ live reproductive males (mean size \pm SE: 4.3 ± 0.3 cm), and $N = 98$ live sculpin (mean size \pm SE: 8.2 ± 1.8 cm) were used as intruders throughout the study.

In total, we observed the behavior of 30 parenting males. Complete datasets (daily observations of behavior in all four contexts for three days, $N = 12$ observations per male) are available for 25 males. Due to disruptions at the field site, some males ($N = 4$) were measured on two days, and $N = 1$ male was measured on one day.

We first observed males for 10 minutes without an intruder to obtain an “undisturbed” measure of parenting behavior. The observer stood three feet away from the nest facing the nest opening and recorded time fanning (moving the pectoral fins over the nest). We then introduced the intruders in a fixed order with an hour in between each observation. We measured behavior (described below) for two minutes after the first orient to the intruder. Then, the cage was removed. If the focal male did not orient within ten minutes after the cage was placed at the nest, the male was recorded as “not responding” and the cage was removed ($N = 6$ observations in the courtship context; $N = 5$ observations in the aggression context; $N = 6$ observations in the antipredator context). Non-responding males were assigned a latency score of 301 (one second greater than the maximum latency score) and remained in the analysis of latency to orient. These individuals were not used for analysis of fanning or intruder-directed behaviors (see below).

We recorded the following behaviors that were elicited by all three types of intruders: latency to orient, the number of bites directed towards the intruder, and the time spent fanning. Males can be aggressive toward females because females often attack nests and eat fertilized

eggs (Foster 1988). When the female intruder was presented, we recorded the number of zig-zags, a conspicuous courtship display (Wootton 1984). We measured two antipredator behaviors in response to the sculpin (*Cottus* spp): the number of times the male froze (head not moving for more than two seconds) and the number of jerky swims the male performed (quickly “darting” in one direction *sensu* McGhee et al. 2012).

Data analysis

In order to ease interpretation of our data, we first inverted latency to orient measures. We subtracted each individual’s latency from 302, one second higher than the maximum latency score (301 s). Therefore, we interpret high latency (inverted) scores as highly attentive behavior (i.e. the male quickly oriented to the intruder). The resulting scores, along with bites at the intruder and intruder-specific behaviors, were non-Gaussian distributed and best approximated a Poisson error distribution with additive overdispersion which we used for all further analyses unless otherwise stated.

We used generalized linear mixed models (GLMM) with Markov chain Monte Carlo (MCMC) estimation for both within- and across-context analyses. MCMC is a Bayesian statistical method that is powerful for fitting non-Gaussian distributions and partitioning variance among random effects (Hadfield 2010; Dingemanse and Dochtermann 2013). We used MCMCglmm (Hadfield 2010) in R v. 3.0.1, which returns 95% credibility intervals for random effects. Throughout, we used non-informative priors (Hadfield 2010) appropriate for the relative error distributions and preliminary analyses indicated that our results were not sensitive to changes in prior settings (data not shown). We ensured convergence and adequate chain mixing

by comparing the posterior distributions and auto-correlation plots of five independent chains with 500 000 iterations, a 1000 burn-in period and thinning every 100 iterations for each model.

We analyzed behaviors (time fanning, latency to orient, and bites at intruders) separately. As fanning was observed for ten minutes without an intruder and for two minutes with intruders, we examined the proportion of time spent fanning. Proportion time fanning was best approximated by a binomial distribution which was used for its models. Context (undisturbed, courtship, aggression, and antipredator) and day of observation were included as fixed effects, individual and trial number were included as random effects, and male standard length and nest depth were included as covariates in all models.

To determine whether males in the field exhibit consistent individual differences in behavior, we used variance components to estimate repeatability of each behavior within and across contexts as the proportion of total variation attributable to among-individual variation using variance components extracted from the univariate (across contexts) and multivariate (within contexts) GLMMs described below. We corrected all repeatability estimates as appropriate for the behavior's distribution (Poisson with additive overdispersion for intruder-directed behaviors; binomial for proportion total time fanning (Nakagawa and Schielzeth 2010)). We determined whether there were consistent individual differences by visually inspecting the posterior distribution of the repeatability estimate: if the estimate (and its 95% CI) was not pressed against zero, we interpreted this as evidence of consistent individual differences.

A goal of this study was to examine whether there was evidence of correlations in behavior within or across contexts. To examine this question *within* each context, we first ran a multivariate model including all behaviors measured within the context (courtship: fanning, bites, zig zags; aggression: fanning, bites; antipredator: fanning, bites, jerky swims, freezes). We

included day of observation, male standard length, and nest depth as fixed effects, and individual ID and trial number as random effects. We were particularly interested in correlations between fanning (our measure of direct parenting behavior) and the other variables. We partitioned the covariation into among- and within-individual variation components and converted covariance into correlations, per Dingemanse and Dochtermann (2013). For the correlations, if the 95% CIs did not overlap zero, we interpreted this as evidence that the correlations were statistically significant.

We took a different approach to examine evidence for correlations between undisturbed parenting behavior and behavior in other contexts. Because behaviors in different contexts were measured at different times, we were only able to partition among-individual variance (Dingemanse and Dochtermann 2013). Specifically, we ran a multivariate model with proportion time fanning, latency to orient, and bites at the intruder across all contexts, with context, day of observation, male standard length, and nest depth included as fixed effects, and individual ID and trial number included as random effects. This model produced covariances, which we then converted into correlations.

RESULTS

Consistent individual differences

Within each context, individual differences in behavior were consistent across all three days of observations, as evidenced by the statistically significant estimates of repeatability (Table 3.1; Fig. 3.1). For example, relative to other males, males that spent more time fanning when an intruding male was present on the first day continued to fan often when an intruding male was present on subsequent days. Indeed, fanning was especially repeatable compared to

other behaviors, with repeatability of fanning exceeding 0.5 in all contexts except in the courtship context (Table 3.1; Fig. 3.1).

Males also exhibited consistent individual differences in behavior across contexts, i.e. even in the presence of different ecologically relevant stimuli. The repeatability (R [95% CI]) of proportion of time fanning across all four contexts was $R = 0.18$ [0.10, 0.34]. There were also consistent individual differences in latency to orient ($R = 0.24$ [0.11, 0.41]) and number of bites at the intruder ($R = 0.16$ [0.08, 0.34]) across contexts.

Average differences in behavior across contexts

As expected, different types of intruders provoked different behavioral reactions (Table 3.2; Fig. 3.2). Males fanned less when an intruder was present (proportion time fanning, mean \pm SE: undisturbed: 0.09 ± 0.01 ; courtship: 0.02 ± 0.005 ; aggression: 0.01 ± 0.004 ; antipredator: 0.02 ± 0.006), and at comparable levels in the presence of different types of intruders (Fig. 3.2a). Males oriented faster to some intruders compared to others (Fig. 3.2b); for example, males oriented especially quickly to the predator (inverted latency to orient, mean \pm SE: courtship: 218.98 ± 11.30 s; aggression: 229.27 ± 12.67 s; antipredator: 249.02 ± 9.69 s). Different types of intruders elicited variable levels of aggression, with the predator provoking the fewest bites (mean \pm SE: courtship: 4.46 ± 0.86 bites; aggression: 6.01 ± 1.10 bites; antipredator: 1.05 ± 0.32 bites). We also found that males with nests in deeper water spent a greater proportion of time fanning and were quicker to orient to intruders (Table 3.2).

Correlations between parenting behavior and reactions to intruders

Although parenting behavior decreased in the presence of intruders, indicating a time or energy constraint (Fig. 3.2a), we did not find evidence for within-context correlations at either the among- or within-individual level between parenting behavior and reaction to intruders (Table 3.3). For example, if a male started to perform more fanning behavior when a female was present, it didn't come at the expense of courtship behavior. Similarly, males that fanned less in the presence of a female didn't necessarily perform more zig-zags.

We detected some evidence for behavioral correlations across contexts: males that provided more care (undisturbed fanning) oriented faster to all intruder types (Table 3.4, Fig. 3.3). Surprisingly, fanning was not related to other behaviors, and direct (fanning) and indirect (biting at the intruder) forms of parental care were unrelated both within and across contexts (Table 3.3; Table 3.4).

DISCUSSION

Male sticklebacks showed consistent individual differences in parenting, courtship, aggressive, and antipredator behaviors in the field, as they do in the lab (Stein and Bell 2012), suggesting wild stickleback exhibit robust behavioral types. We found little evidence that males exhibited correlations between parental and other behaviors within contexts, suggesting that over short timescales, males are capable of managing competing demands without sacrificing parental care. Between contexts, we found evidence that parental care was positively correlated with attentiveness toward intruders, suggesting a behavioral syndrome encompassing attention to offspring and attention to the surrounding environment.

Males showed consistent individual differences in behavior within and across contexts

There were consistent individual differences in parenting behavior. In general, males performing high levels of fanning on the first day of behavioral observations also showed high levels of fanning relative to other males on the second and third days. Although all males decreased fanning in the presence of an intruder, individuals that fanned the most when an intruder was absent also fanned the most when an intruder was present. Altogether, these results suggest a strong parental behavioral type in wild threespine sticklebacks that is robust to different ecological challenges.

Male size and nest depth have previously been shown to affect reproductive success (Kraak 1999a) and fanning behavior (Künzler and Bakker 2000) in sticklebacks. Our statistical results suggest that males with nests in deeper water fanned more often (Table 3.2). As oxygen concentrations decrease with water depths, males in deeper water might compensate for low oxygen availability by fanning. Males in deeper water also were quicker to orient to all types of intruders. The causal relationship between territory attributes and behavioral type is not straightforward. Having a nest in deeper water, for example, allow an individual to have a highly parental behavioral type because they are safer from bird predators. Alternatively, other aspects of the male's behavioral type (e.g. being more aggressive) may allow an individual to secure a more resource-rich territory. These relationships should be examined in future studies.

Different intruders provoked different behavioral reactions

Each intruder represented a different ecological challenge, and males adjusted their behavior accordingly. Given that intruders were presented in a fixed order, average differences in behavior across contexts might reflect a carryover effect such as habituation (Bell 2013). However, there was not a systematic change in average behavior over time, as one would

predict for a carryover effect, and the patterns were biologically reasonable. For example, males showed high levels of aggression toward intruding males, consistent with other studies (Huntingford 1976; Wootton 1984), and with the hypothesis that males are a serious threat because they might steal eggs or a territory. In addition, males oriented especially quickly to the predator compared to the other intruders, which is consistent with the observation that parenting males are particularly vulnerable to predators (Candolin 1998). Interestingly, males did not increase fanning during the courtship context, even though fanning can be a courtship display (Tinbergen and van Iersel 1947). Males in this study performed zig-zags, a behavior only observed during courtship, indicating the males in this study were receptive to females. Overall rates of fanning were lower in the presence of an intruder. It is possible that males might compensate for a reduction in fanning during periods when they were not observed; however, males do not compensate for a reduction in fanning in the lab (Stein and Bell 2012).

Little evidence for behavioral syndromes within contexts

We predicted that individual differences in parenting behavior reflect part of a behavioral syndrome, and that trade-offs between behaviors within contexts might explain variation in parenting behavior. For example, males that fan the nest the most might be relatively unaggressive (Lissaker and Kvarnemo 2006), or relatively timid around predators (Budaev et al. 1999). Alternatively, nest defense and direct parental care might be positively correlated (Betini and Norris 2013; Wetzel and Westneat 2014). This may be especially evident within contexts, where time budget tradeoffs are more pronounced.

We found little evidence in support for these ideas within contexts, although it is possible that high-parenting behavioral types might experience trade-offs with behavioral traits that were

not measured in this study, such as those having to do with immunity (Sabat 1994). A lack of evidence for correlations within contexts suggests that males are able to juggle multiple competing demands over very short timescales (here, a two minute intrusion occurring three times a day) without sacrificing direct or indirect care. Over longer timescales, or over multiple intrusions, time budget tradeoffs may become more evident. Additionally, with a small sample size in this study, the possibility of type II errors are inflated (Bell 2005), and it is possible that with a larger dataset correlations between these behaviors may be revealed.

It is interesting to note that within-individual correlations within the antipredator context were marginally significant (i.e. half of the model runs produced 95% CIs that did not overlap zero) (Table 3). Within-individual correlations can reveal tradeoffs that may not be detectable at the population (among-individual) level (van Noordwijk and de Jong 1986; Reznick et al. 2000). As a male increased fanning, he decreased nest defense (biting at the predator) and jerky swimming and freezing (antipredator behavior). This suggests that parents in this population may face a tradeoff between survival and reproduction (Cole et al. 2014).

Parenting-attentiveness behavioral syndrome across contexts

Males performing high levels of direct care appear to be more attentive to intruders into their territory. For example, males that fanned the nest more often oriented more quickly to a rival male stickleback, a female, and a predator. These across context correlations may reflect a larger “attentive” behavioral type: individuals that are overall more sensitive to cues from their environment may also be more sensitive to cues from their eggs or offspring.

Within-context correlations are likely strongly influenced by time budgets: if a male is currently fanning, he cannot attack an intruder. Across-context correlations, on the other hand,

can reveal whether behaviors in one context are correlated to behaviors in an unrelated context, without the constraint of time. Such across-context correlations may be important in uncovering ecological or evolutionary constraints (Bell 2005; Dochtermann and Dingemanse 2013). A boldness-aggressive syndrome has been previously established in this population (Bell 2005). The discovery of a parenting-attentiveness syndrome suggests a potential new personality axis in this population, and future studies should examine whether these behaviors are correlated in other populations and systems.

ETHICAL NOTE

All focal males resumed parenting behavior following measurements and observations. Intruders were placed in a wire cage with openings too small for either the focal male or the intruder to injure each other. All intruder individuals were held for less than five hours and released at their point of capture following observations; all intruders resumed normal behavior upon release. This study was conducted under California Fish and Game Permit SC-11131 and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign (protocol #09204).

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TABLES

Context	R [95% CI]
<i>Undisturbed</i>	
Fanning	0.55 [0.24, 0.67]
<i>Courtship</i>	
Fanning	0.11 [0.05, 0.35]
Orient	0.18 [0.04, 0.39]
Bites	0.15 [0.05, 0.40]
Zig-Zags	0.33 [0.14, 0.57]
<i>Aggression</i>	
Fanning	0.60 [0.36, 0.75]
Orient	0.23 [0.07, 0.45]
Bites	0.60 [0.35, 0.75]
<i>Antipredator</i>	
Fanning	0.50 [0.25, 0.67]
Orient	0.21 [0.08, 0.46]
Bites	0.14 [0.05, 0.32]
Jerky swims	0.19 [0.06, 0.41]
Freezes	0.21 [0.07, 0.47]

Table 3.1: Repeatability estimates (R) for behaviors within each context. Male behavior was measured in every context once per day for three days. Estimates include Day, Standard Length, and Nest Depth as fixed effects. All models include Trial and Individual ID as random effects. Numbers in brackets indicate 95% credibility intervals

Factor	Proportion time fanning	Latency to orient	Number of bites
Context	3.41 [2.38, 4.67]	0.12 [0.02, 0.20]	1.76 [-2.54, -1.02]
Day	-0.23 [-1.21, 0.84]	-0.31 [-0.55, -0.04]	-0.22 [-0.82, 0.46]
Length	0.36 [-0.10, 0.83]	0.07 [-0.12, 0.26]	0.05 [-0.24, 0.32]
Depth	0.18 [0.07, 0.30]	0.07 [0.22, 1.22]	0.03 [-0.76, 1.80]

Table 3.2: GLMM table for fixed effects. Numbers in brackets indicate 95% credibility intervals. Bolded estimates are significant; * indicates marginal significance

Context	Correlations [95% CI]	
	Among	Within
<i>Courtship</i>		
Fanning*Orient	0.09 [-0.16, 0.30]	0.11 [-0.12, 0.33]
Fanning*Bites	0.006 [-0.25, 0.25]	-0.13 [-0.35, 0.10]
Fanning*Zig zags	0.11 [-0.11, 0.44]	0.11 [-0.08, 0.34] *
<i>Aggression</i>		
Fanning*Orient	0.08 [-0.18, 0.45]	0.03 [-0.12, 0.19]
Fanning*Bites	-0.07 [-0.40, 0.29]	-0.06 [-0.12, 0.17]
<i>Antipredator</i>		
Fanning * Orient	0.06 [-0.27, 0.35]	0.05 [-0.12, 0.23]
Fanning * Bites	0.07 [-0.18, 0.40]	-0.09 [-0.31, 0.07] *
Fanning * Jerky swims	-0.01 [-0.32, 0.26]	-0.15 [-0.35, 0.03] *
Fanning * Freezes	0.01 [-0.36, 0.28]	-0.10 [-0.32, 0.06] *

Table 3.3: Correlations (among- and within-individual) between fanning within the context and other behaviors measured within the same context. Estimates include Day, Standard Length, and Nest Depth as fixed effects. All models include Trial and Individual ID as random effects. “Among” indicates among-individual correlations; “Within” indicates within-individual correlations. Numbers in brackets indicate 95% credibility intervals. Bolded correlations are significant; * indicates marginal significance

Context	Correlations [95% CI]
<i>Courtship</i>	
Fanning*Orient	0.10 [0.01, 0.52]*
Fanning*Bites	0.07 [-0.60, 0.81]
Fanning*Zig zags	0.18 [-0.68, 0.73]
<i>Aggression</i>	
Fanning*Orient	0.69 [0.07, 0.91]
Fanning*Bites	0.52 [-0.32, 0.86]
<i>Antipredator</i>	
Fanning * Orient	0.23 [0.10, 0.45]
Fanning * Bites	0.26 [-0.75, 0.85]
Fanning * Jerky swims	-0.30 [-0.75, 0.68]
Fanning * Freezes	-0.36 [-0.72, 0.64]

Table 3.4: Correlations (among-individual) between undisturbed parenting behavior (fanning) and behavior in different contexts. Estimates include Day, Standard Length, and Nest Depth as fixed effects. All models include Trial and Individual ID as random effects. Numbers in brackets indicate 95% credibility intervals. Statistically significant correlations are indicated in bold, * indicates marginally significant correlations.

FIGURES

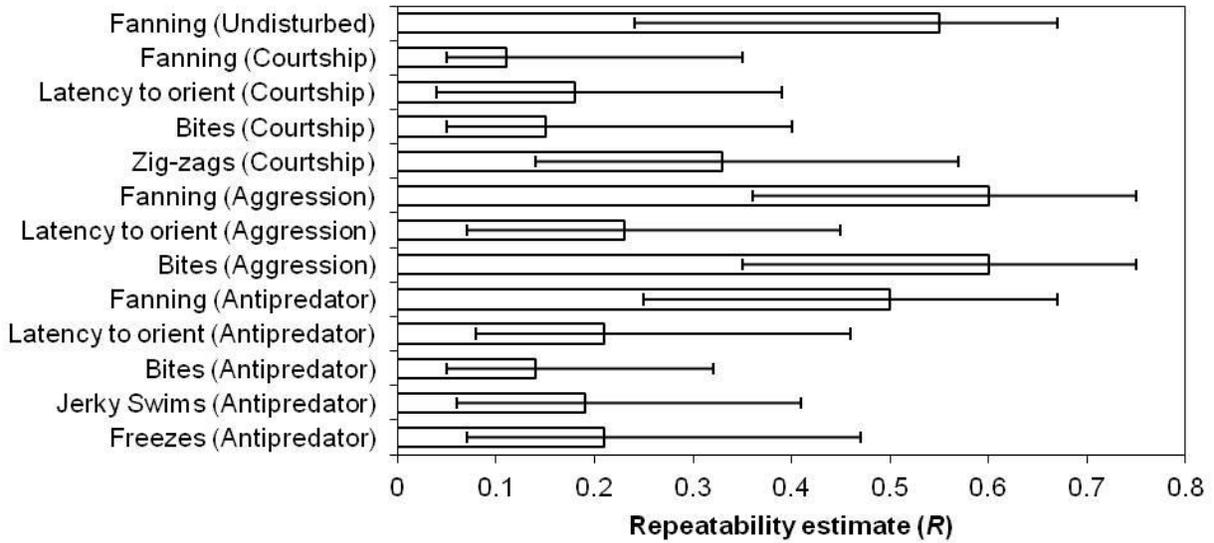


Figure 3.1: All behaviors measured were repeatable, indicating consistent individual differences in behavior the field. If the estimate (and its 95% credibility interval) is not pressed against zero, we interpreted this as evidence of consistent individual differences. Error bars are 95% credibility intervals.

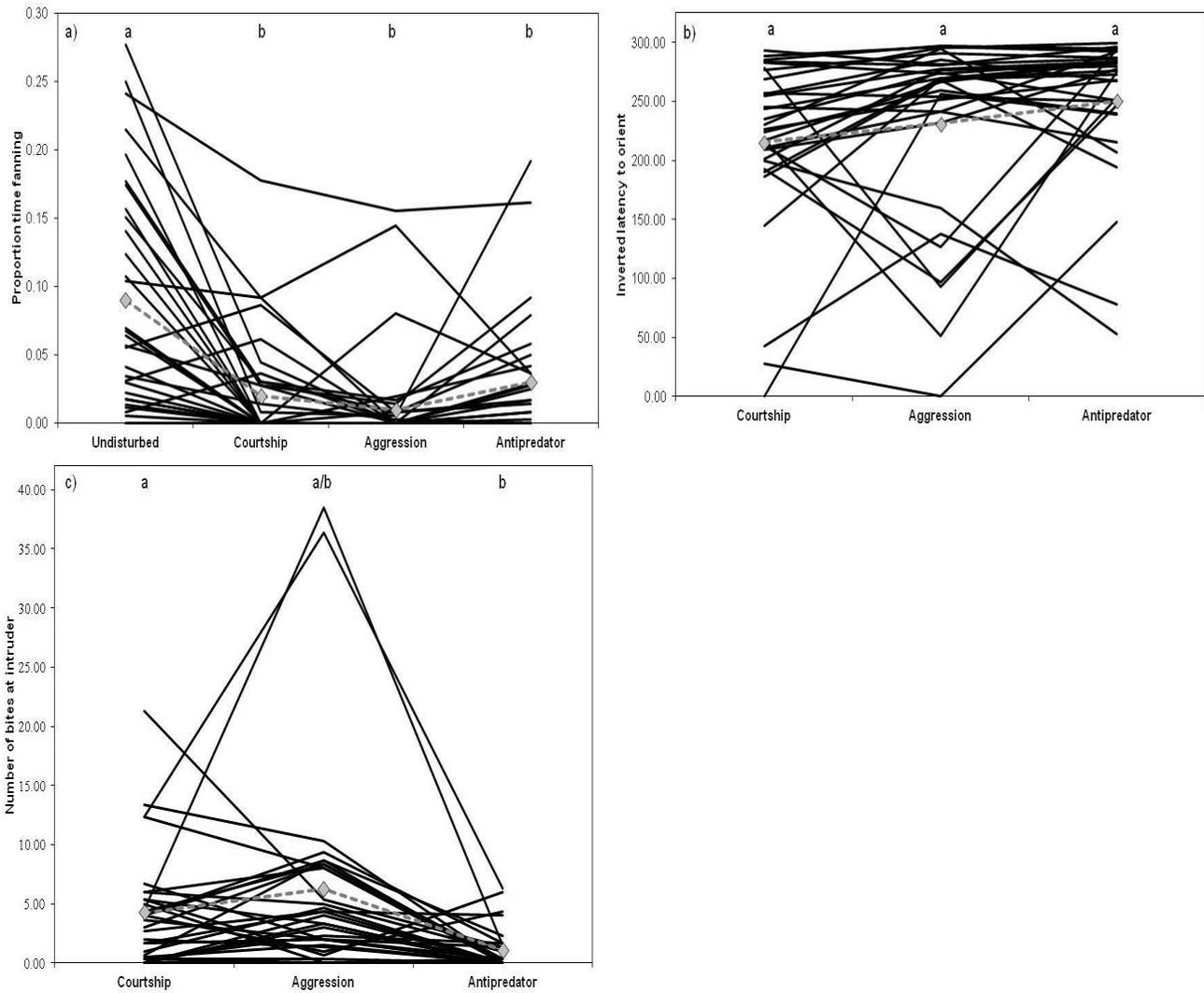


Figure 3.2: Males adjusted their behavior across contexts. Each line represents an individual male; gray dotted line and diamonds indicate average levels of behavior. $N = 30$. Letters indicate significant differences between contexts

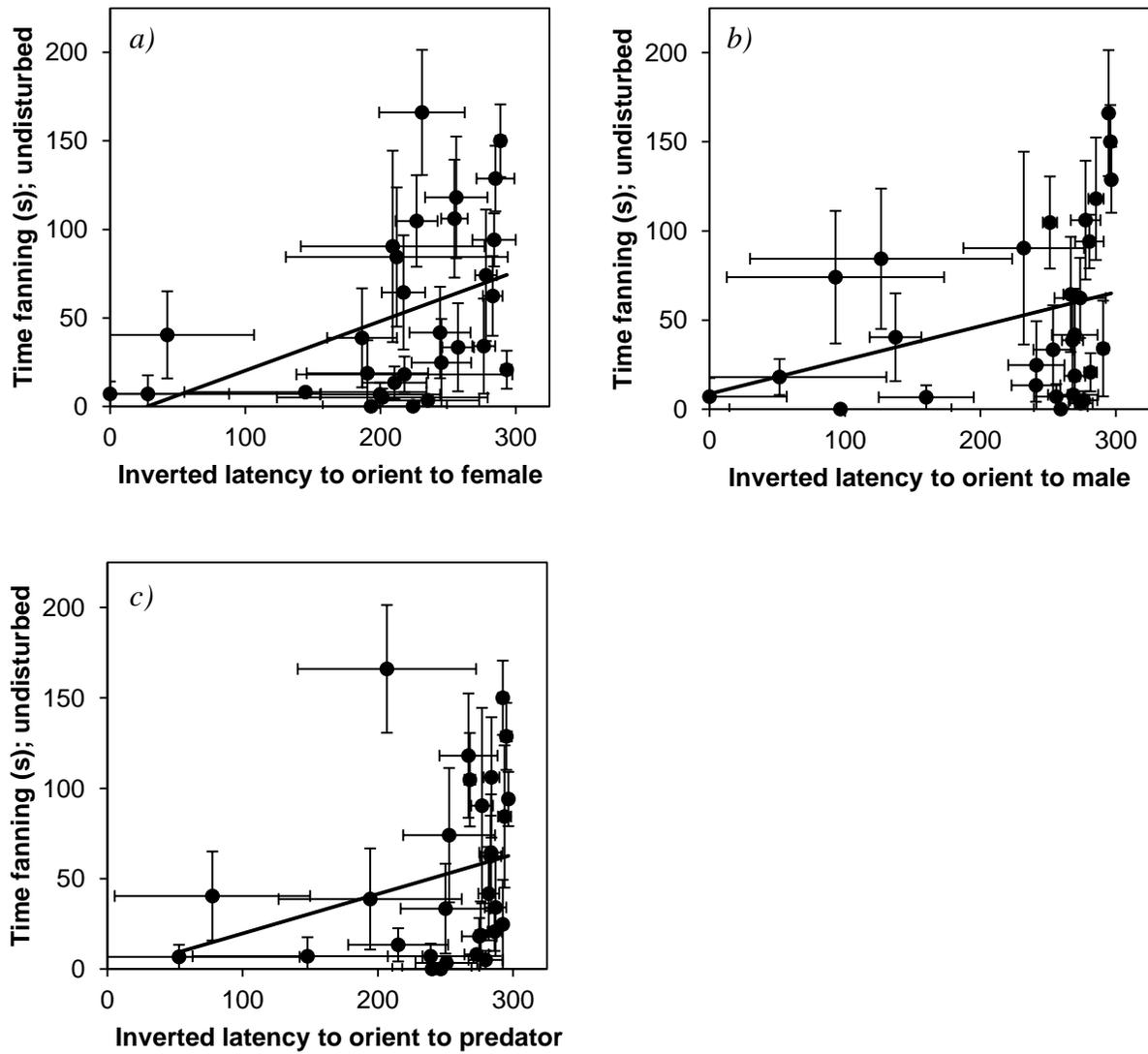


Figure 3.3: Correlations between undisturbed parenting behavior (fanning) and inverted latency to orient to an intruder. Males that spent more time fanning without an intruder were quicker to orient to all intruders. $N = 30$ for (a) courtship and (b) aggression contexts; $N = 28$ for the (c) antipredator context. Error bars are ± 1 SE

CHAPTER 4: PATERNAL PROGRAMMING IN STICKLEBACKS³

ABSTRACT

In a wide range of organisms, including humans, mothers can influence offspring via the care they provide. Comparatively little is known about the effects of *fathering* on offspring. Here, we test the hypothesis that fathers are capable of programming their offspring for the type of environment they are likely to encounter. Male threespine stickleback (*Gasterosteus aculeatus*) were either exposed to predation risk while fathering or not. Fathers altered their paternal behavior when exposed to predation risk, and consequently produced adult offspring with phenotypes associated with strong predation pressure (smaller size, reduced body condition, reduced behavioral activity). Moreover, more attentive fathers produced offspring that exhibited stronger antipredator responses. These results are consistent with behaviorally-mediated paternal programming: fathers can alter offspring phenotypes to match their future environment and influence offspring traits well into adulthood.

INTRODUCTION

In a wide range of organisms, including humans, mothers' experiences can affect offspring (Mousseau & Fox 1998; Uller 2008). For example, maternal exposure to predation risk alters offspring morphology (Agrawal et al. 1999; Weisser et al. 1999), physiology (Sheriff et al. 2010), and behavior (Storm & Lima 2010). There is also an emerging literature showing that the way mothers behave toward their offspring can have a long-lasting influence on their offspring

³ This chapter appeared in its entirety in the journal *Animal Behaviour* and is referred to later in this dissertation as "Stein and Bell 2014". Stein, LR and Bell, AM. *Paternal programming in sticklebacks*. **95**, 165-171. This article is reprinted with permission of the publisher and is available from <http://www.elsevier.com/> and using DOI: 10.1016/j.anbehav.2014.07.010

(Champagne 2008).

Comparatively less is known about the significance of *fathers'* experiences and behavior for offspring. At first glance it might appear that there is little opportunity for fathers' experiences to become embedded in offspring because there is rarely intimate contact between fathers and developing embryos. However, a growing number of studies is showing that fathers' experiences prior to fertilization can influence offspring via, for example, changes in sperm morphology or seminal fluid (beetles: Sirot et al. 2007), the sperm epigenome (mammals: Curley et al. 2011), and sperm microRNAs (rats: Rodgers et al. 2013). A relatively unexplored possibility is that fathers adjust their parenting in response to stressors, and adjustments in care have long-term consequences for offspring (as has been shown for mothers (McLeod et al. 2007)).

Predation is a strong selective pressure that shapes many traits (Endler 1995; Abrams & Rowe 1996). In a predator-rich environment, anti-predator defenses are key for reproductive success and offspring survival, and predation risk often alters phenotypes in predictable ways. For example, prey in high-predation environments tend to be smaller, less active, and have faster life history trajectories than prey from low-predation environments (guppies: Endler 1995; tadpoles: Relyea 2004; lizards: Vervust et al. 2007). If parents can respond to cues that future predation risk is likely to be high, and if they can prepare offspring for living in a predator-rich environment, then this transgenerational plasticity could be adaptive.

Here, we investigate the effects of paternal experience with predation risk on offspring morphological, behavioral, and physiological traits in threespine sticklebacks (*Gasterosteus aculeatus*). Sticklebacks are teleost fish in which the father is the sole provider of parental care that is necessary for offspring survival. Therefore, there is no opportunity for differential

allocation or compensation by the mother (Curley et al 2011). During the approximately five day incubation period, male sticklebacks “fan” the eggs with pectoral fins, providing oxygen and clearing carbon dioxide, and remove rotten eggs and debris (Wootton 1984). Once the eggs hatch, fathers continue to defend their offspring and retrieve young fry that stray too far from the nest. Previous studies suggested that offspring learn appropriate antipredator behavior from their father after hatching (Feuth-De Bruijn & Sevenster 1983; Tulley & Huntingford 1987).

We used a within-subjects breeding design to test the hypothesis that paternal exposure to predation risk *during* parenting influences offspring traits. Specifically, males experienced predation risk during one parenting episode (“predator-exposed”), and were not exposed to predation risk during the other parenting episode (“unexposed”). We evaluated the effect of paternal predator exposure on the morphology, behavior and physiology of reproductively mature adult offspring. We further examined correlations between paternal behavior and offspring behavior to test the hypothesis that paternal effects on offspring are mediated by paternal behavior.

METHODS

Study population and breeding

Adult threespine stickleback were collected from Putah Creek, a dammed, regulated freshwater stream in northern California, in April 2010. Sculpin (*Cottus spp*), a fish predator known to prey on stickleback eggs, fry, and adults (Moodie 1972; Pressley 1981) are present at this site. Fish were shipped to the University of Illinois at Urbana-Champaign, and males were introduced into separate 9.5L (36 x 21 x 18 cm) tanks with a refuge (plastic “plant”), an open plastic box (13 x 13 x 3 cm) filled with fine sand, and filamentous algae for nest building.

Following nest completion, males were presented with a gravid female and allowed to spawn. Each male spawned with a unique female. After spawning, the female was removed. Fish were kept at 20 degrees Celsius on a summer (16L:8D) photoperiod. Water was cleaned via a recirculating flow-through system that consists of a series of particulate, biological, and UV filters (Aquanearing, San Diego, USA). 10% of the water volume in the tanks was replaced each day. Fish were fed a mixed diet consisting of frozen bloodworm, brine shrimp and Mysis shrimp in excess each day.

Exposing fathers to predation risk and recording paternal behavior

Males were randomly assigned to either the “unexposed” or “predator-exposed” treatment for their first clutch. On the third day after males spawned (when the embryos were three days old), males in the “predator-exposed” treatment were chased with a 10 cm rubber model sculpin (Jewel Bait Company) for two minutes to simulate a nest predation attempt. A predator of this size is a threat to the eggs and fry, but not to the adult males (Moodie 1972; Pressley 1981). Previous research has shown that stickleback show relevant anti-predator behaviors when confronted with a realistically painted model (Grobis et al. 2013). At this developmental stage, the optic cups of the embryo are still developing (Swarup 1958) and the eggs were covered by nesting material, making it unlikely the embryos were exposed to visual cues of the model predator. For males in the “unexposed” treatment, we removed the top of the tank and gently splashed the water when the eggs were three days old to simulate the water disturbance caused when the model predator entered the tank. Males were only exposed to the predator once.

After spawning, paternal behavior was observed every day for five minutes between 1000 and 1300 CST from one day after spawning through five days after the eggs hatched (when fry from this population naturally disperse in the wild). We measured the total time the male was within one body length of the nest (total time at nest) and the total amount of time the male spent fanning his eggs, a paternal behavior that oxygenates the eggs (Wootton 1984), is important for proper offspring development (von Hippel 2000), and consistently varies among fathers (Stein & Bell 2012). The simulated predation threat (or water splashing) occurred after the daily observation of paternal behavior. There were subtle but detectable effects of predator exposure on paternal behavior. For example, “predator-exposed” fathers decreased total time fanning relative to control males for two days after they were exposed to the model predator, but afterwards resumed normal activity (Stein & Bell 2012). More details on parental behavior are presented in Stein & Bell (2012).

Five days after the eggs hatched, males were placed in new tanks and allowed to construct second nests and the entire process, including daily behavioral observations, was repeated for the second clutch. Males that had been in the “predator-exposed” treatment in the first clutch were assigned to the “unexposed” treatment for the second clutch and vice versa.

$N = 10$ males completed at least one clutch; of these, $N = 8$ completed two. Initial treatment did not affect whether males completed a second clutch (of those that completed two clutches, $N = 4$ were “unexposed” and $N = 4$ were “predator-exposed” in their first clutch), and parental behavior in the first clutch did not predict whether a male completed a second clutch (Stein & Bell 2012). We did not detect a difference in parental behavior between the first and second clutches or an effect of the order in which a male experienced a model predator (i.e., a

male's experience with parenting or with a predator did not influence his behavior in his second clutch).

Offspring morphology and anti-predator behavior

Once fry were approximately one cm in length (approx. one month of age), each full sibling family was split across at least two tanks at a density of six fish per tank. Offspring were fed newly-hatched *Artemia nauplii* shrimp in excess each day until they reached three cm in length, at which time they were fed the adult slurry of frozen food. Offspring were kept this way for one year; during this time they experienced a simulated winter (8L:16D) photoperiod from November 2010 – March 2011.

At one year of age, when the offspring were reproductively mature, we measured their morphology, behavior and cortisol response to predation risk. Specifically, we measured standard length and weight and scored color (males only) using a ranking method (Boughman 2007). Throat redness was measured as the sum of throat red area and red intensity scores (each a score ranging from 0-3). Body brightness ranged from 0-5, with 5 being very bright. Throat hue and body brightness were measured on the side of the fish.

For behavioral testing of predator responses, fish were transferred individually to an observation tank in an opaque cylinder (10 cm in height, 10 cm diameter) plugged with a cork. The observation tank (53 x 33 x 24 cm) had a 5x2 grid drawn on the front, a gravel bottom and two plastic plants for refuge, one on each side of the tank. After a 15-minute acclimation period, we removed the cork remotely and after emerging from the cylinder, the fish acclimated to the observation tank for one hour.

We recorded behavior with a high-definition JVC Everio camcorder from behind a blind. Behavior was recorded (see below for details) for three minutes without a stimulus in order to obtain a baseline level of behavior (“Before”). After three minutes, we introduced a 15-cm clay sculpin (model predator) painted with natural markings to the tank to measure anti-predator behavior. A predator this size is a threat to the adult stickleback (Moodie 1972; Pressley 1981). The model predator was attached with fishing wire to a rod that could be manipulated from behind the blind. We introduced the model to the right side of the tank, and moved it in a clockwise direction around the tank for one minute. We then placed the model on the gravel for two minutes, simulating the sit-and-wait predation style of sculpin and recorded behavior for the three total minutes the predator was in the tank (“During”). After two minutes, we removed the sculpin model and recorded behavior for an additional three minutes in order to determine whether behavior differed from baseline after offspring observed a predator (“After”). We recorded total number of squares moved (a measure of activity), total time freezing (an anti-predator behavior), and total number of jerky swims (a quick “burst” of speed, a conspicuous behavior) using JWatcher (UCLA and Macquarie University). The three observers recording behaviors were blind to offspring family and father treatment, and we did not detect an effect of observer on behavior.

Behavioral observations were carried out between 1100 and 1700 CST from August – September 2011. Only one fish per rearing tank was tested each day; we recorded the behavior of 12 fish per day. There were a total of $N = 18$ full-sibling families, with $N = 8$ pairs of half-sibling families. $N = 9$ clutches were from predator-exposed fathers, while $N = 9$ clutches were from unexposed fathers. In total, there were $N = 91$ offspring from fathers in the predator-exposed treatment, and 66 offspring from fathers in the unexposed treatment (total $N = 157$ offspring).

Measuring plasma cortisol in offspring

We measured circulating plasma cortisol after the behavioral assay as a means of detecting offspring cortisol response to predation risk. Previous studies in threespine stickleback showed that circulating plasma cortisol peaks 15 minutes after a predator encounter (Mommer & Bell 2013). Therefore, 15 minutes following the introduction of the predator to the observation tank, we quickly netted the stickleback and immediately euthanized it with an overdose (>0.2mM) of MS-222. We then removed the caudal peduncle just before the cloaca with sharp scissors. We collected blood from the caudal vein using 75mm heparinized microhematocrit tubes (StatSpin, Westwood, MA). Tubes were centrifuged on a microhematocrit rotor (StatSpin, Westwood, MA) to pellet circulating cells. Plasma supernatant was aspirated and kept at -20C until enzyme-linked immunosorbent assay (ELISA) could be performed. At this time we also removed the caudal fin and stored it in 70% ethanol for later determination of genetic sex using a male-specific genetic marker (Peichel et al. 2004).

To confirm that exposure to predation risk during the behavioral assays elicited a cortisol stress response, a subset of 20 offspring ($N = 10$ from predator-exposed fathers and $N = 10$ from unexposed fathers) were not put through the behavior assay. Instead, these fish were randomly selected from their home tanks and immediately euthanized with an overdose of MS-222 (“Control” treatment). We then measured standard length, weight, scored coloration, collected the caudal fin, and extracted plasma as above. These control measurements also allowed us to determine whether offspring from predator-exposed fathers had higher “baseline” levels of cortisol than offspring from unexposed fathers.

Plasma samples were thawed and their cortisol concentration measured in duplicate by competitive ELISA according to the manufacturer's protocol (Enzo Life Sciences, Plymouth Meeting, PA). To extend the manufacturer's recommended standard curve for the ELISA an 8th standard of cortisol (78 pg/mL) was included on each plate. Plasma samples across both levels of paternal treatment (predator-exposed and unexposed) and behaviorally assayed and control offspring were represented on each of the four plates. The inter-assay coefficient of variation (CV) between all ELISA plates ($N = 4$ plates) was 6.65%; intra-assay CVs averaged $6.77 \pm 6.20\%$ (mean \pm standard deviation). 35 individuals either gave very little blood or the plasma was lost from the capillary tube during isolation, therefore the final sample size for cortisol analysis was 123 offspring exposed to the model predator and 19 control individuals.

Statistical Analysis

To determine whether paternal exposure to predation risk influences offspring, we compared offspring between paternal treatments ("predator-exposed" or "unexposed" fathers). We constructed separate models to test for effect of paternal treatment on offspring i) body size, ii) body condition (calculated as the residuals of length by weight regression), iii) male nuptial coloration, iv) activity, v) time freezing, vi) jerky swims, and vii) circulating plasma cortisol concentration. All models included paternal treatment (predator-exposed, unexposed) and offspring sex as fixed effects, and father ID, clutch nested within father ID, offspring home tank nested within clutch, and order in which the father saw a predator as random effects. We did not detect any differences between gravid and nongravid female offspring (results not shown). Offspring activity (total squares moved) and time freezing were measured at three time points: before, during, and after predator exposure. Therefore for these variables we also included stage

(Before, During, and After predator exposure) as a fixed effect and individual ID as a random effect to account for multiple measurements of the same individual. To determine whether exposure to a model predator triggered a cortisol stress response in offspring, we used a t-test comparing offspring that had or had not been exposed to the model predator.

We wished to control for the effect of offspring body size on behavior and cortisol, but paternal treatment affected offspring length (see results). Therefore we regressed each behavior and cortisol concentration on length and analyzed the length-corrected residuals. Squares moved and total time freezing were natural log-transformed (trait + 1 to account for zeroes) prior to regression to obtain normality; number of jerky swims was square-root (+1) transformed.

To test the hypothesis that paternal behavior influenced offspring traits, we examined correlations between father behavior (averaged within a clutch) and offspring behavior (regressed on length and averaged within a clutch) using Pearson correlations. This analysis was performed at the clutch level rather than at the father level because a given male behaved differently during his two clutches, so each of a male's clutches experienced a different rearing environment. We used sequential Bonferroni correction to account for multiple correlation tests (Rice et al. 2008).

All statistical analyses were conducted with R version 2.15.2 (R Core Development Team 2012). Linear mixed models (LMMs) were performed using the lmer function from the 'lme4' (Bates et al. 2012) and 'lmerTest' (Kuznetsova et al. 2013) packages. We used REML estimation and a diagonal covariance structure for our models, with Satterthwaite approximation for degrees of freedom. We determined whether levels of fixed factors differed from one another using Tukey's HSD test. We calculated effect sizes (Cohen's D) from means and standard deviations and interpret 0.5 as a large effect, 0.3 as a medium effect, and 0.1 as a small effect (Cohen 1992).

ETHICAL NOTE

We took measures to maximize animal welfare by minimizing the duration of exposure to simulated predation risk and by providing refuges (plants and gravel) in the sticklebacks' tanks. Animals were sacrificed via overdose of anesthetic in order to minimize suffering. The experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign (Protocol number 09204, approved on 1 September 2009).

RESULTS

Paternal effects on offspring morphological traits

Whether or not a father experienced predation risk influenced the body size of his offspring (Table 4.1; Figure 4.1a; Cohen's $D=0.35$). When fathers were exposed to predation risk, they produced offspring that were significantly smaller (38.09 ± 4.20 mm) than when they were not exposed to predation risk (39.48 ± 3.84 mm). Paternal treatment also influenced body condition: when fathers were exposed to predation risk, they produced offspring in worse condition (-0.35 ± 1.64) than when they were not exposed to predation risk (0.48 ± 1.46 , Figure 4.1b; Cohen's $D=0.54$). Male offspring of fathers in the predator-exposed treatment had lower color scores (2.49 ± 1.52) than male offspring of fathers in the unexposed treatment (3.12 ± 2.22 ; Cohen's $D=0.33$). However, this effect was driven by the negative effect of paternal predator exposure on body size: when male offspring nuptial coloration was corrected for offspring length, there was no difference between paternal treatments (effect of paternal treatment; $F_{1,41.15}=0.12$, $P = 0.73$).

Paternal effects on offspring anti-predator behavior

Offspring reacted to the model predator during the behavioral assay (Table 4.2, effect of stage). For example, when the predator was present, offspring reduced activity (During: 7.7 ± 6.9 squares) compared to before the predator was introduced (Before: 8.6 ± 9.9 squares). Offspring maintained relatively low levels of activity after the predator was removed (After: 6.09 ± 8.06 squares). Offspring increased the amount of time spent frozen when the predator was present (Before: 70.6 ± 67.3 seconds, During: 96.1 ± 62.3 seconds), and then returned to ‘before’ levels after the predator was removed (After: 76.7 ± 69.8 seconds). Offspring only performed the jerky swimming behavior when the predator was present. Altogether these behavioral data indicate that offspring reacted to the predator as if it were a threat.

Paternal predator exposure influenced offspring activity (Table 4.2, effect of paternal treatment; Cohen’s D before predator: 0.28; Cohen’s D during predator: 0.23; Cohen’s D after predator: 0.32). During all stages of the behavioral assay (Before, During, and After predator exposure), offspring of predator-exposed fathers were less active compared to offspring of unexposed fathers (Figure 4.2a). We did not detect an effect of paternal predator exposure on freezing behavior (predator-exposed father: 83.6 ± 67.5 seconds; unexposed father: 77.7 ± 66.9 seconds; Figure 4.2b), or jerky swimming (predator-exposed father: 1.45 ± 2.87 ; unexposed father: 1.85 ± 2.87 ; Figure 4.2c).

Paternal effects on offspring cortisol stress response

We did not find an effect of paternal treatment on circulating cortisol ($F_{1,114.4}$, $P = 0.69$; Cohen’s D = -0.06). However, the LMM suggested that the effect of paternal treatment on offspring cortisol response might depend on sex (effect of paternal treatment*sex: $F_{1,114.3} = 2.77$,

$P = 0.09$). Therefore we ran separate LMMS for the two paternal treatments, testing for the effects of sex as a fixed factor and father ID, clutch nested within father, home tank nested within clutch, order in which father saw a predator, and ELISA plate as random factors. While there was no sex difference in offspring of unexposed fathers (effect of sex: $F_{1,45.9} = 0.14$, $P = 0.70$), female offspring had higher circulating cortisol than male offspring of predator-exposed fathers (effect of sex: $F_{1,68.9} = 9.71$, $P = 0.003$; Figure 4.3).

We also collected plasma from a baseline, ‘control’ group of offspring that were sacrificed directly from their home tanks. These data verify that offspring reacted to the model predator as a stressor: control offspring had lower cortisol than offspring that were exposed to the predator during the behavioral assay (t-test: $t_{21.4} = 6.05$, $P < 0.0001$). We did not detect a difference in levels of baseline circulating cortisol between paternal treatments ($F_{1,15} = 0.065$, $P = 0.80$) or sexes ($F_{1,15} = 0.007$, $P = 0.93$).

Relationships between fathers’ behavior and offspring traits

Previous analyses (Stein & Bell 2012) revealed subtle but detectable effects of predator exposure on fathers’ behavior. In particular, when fathers were predator-exposed, they performed less parental behavior than when they were unexposed. Therefore we hypothesized that changes in fathers’ behavior might be driving some of the differences between offspring of predator-exposed versus unexposed fathers. Consistent with this hypothesis, fathers that made more visits to the nest produced offspring that performed more jerky swims ($r = 0.57$, $N = 16$, $P = 0.016$, Figure 4.4, a result that passes the sequential Bonferroni test). We did not find any significant correlations between fathers’ behavior, body size, body condition, nuptial coloration, cortisol, total squares moved during predator exposure, or total time freezing during predator exposure.

DISCUSSION

Exposing fathers to predation risk caused them to produce offspring with phenotypes associated with living under high predation risk. In particular, offspring of predator-exposed fathers were smaller, in poorer condition, and less active than offspring of unexposed fathers, and paternal exposure to predation risk induced a sex-specific difference in offspring cortisol stress response to predation. A growing literature suggests that mothering can influence offspring via nongenetic mechanisms (review: Champagne 2008), our results suggest that fathers are capable of something similar. Studies on fathering to date have focused on the effects of fathers' experience *prior* to parenting on offspring (Sirot et al. 2006; Curley et al. 2011; Crean et al. 2013; Rodgers et al. 2013; Dias & Ressler 2014) and have suggested that paternal effects can be of similar magnitude to maternal effects (Head et al. 2012). Our results show that a fathers' experience *during* parenting can influence offspring phenotype in much the same way that mothers can. Our data also suggest that long-term differences between offspring of predator exposed and unexposed fathers can be attributed to short-term adjustments in paternal behavior in response to predation risk. Altogether, these results suggest that fathers from natural populations can transmit information about the current environment to their offspring, and that the way that fathers behave toward their offspring can shape their developmental trajectories into adulthood.

One of the advantages of the stickleback system is that there is an extensive literature on morphology and behavior in the field and lab that provides a framework for interpreting some of the paternal effects observed in this study. For example, predation pressure on sticklebacks (and other species; see Endler 1995; Relyea 2004; Vervust et al. 2007) is associated with smaller body size at sexual maturity (Bell et al. 2011), lower body condition (Frommen et al. 2011), reduced

male nuptial coloration (Candolin et al. 1998), and reduced activity (Lacasse & Aubin-Horth 2012). In this study, offspring of predator-exposed fathers had traits associated with predator-rich conditions: they were smaller, in poorer body condition, had reduced nuptial coloration in males, and were less active than offspring of unexposed fathers. These findings suggest that the nongenetic paternal effects found here could reflect transgenerational plasticity for coping with a high-predation environment. Remarkably, our results also suggest that fathers are capable of flexibly ‘programming’ their offspring for the environment that fathers experience while parenting, or adaptive anticipatory paternal effects. Fathers made short term adjustments to their parenting behavior in response to predation risk, but a father’s experience with predation risk during one breeding episode did not carry over to influence his behavior or offspring in a subsequent breeding episode (Stein & Bell 2012), consistent with paternal programming. Offspring of predator-exposed fathers were less active than offspring of unexposed fathers. Lower activity in the presence of predation risk can improve stickleback survival, presumably because it makes them less conspicuous to predators (McGhee et al. 2012). Offspring of predator-exposed fathers were less active at all stages of the behavioral assay (before, during, and after the predator was present). Interestingly, we did not detect a predator-induced paternal effect on offspring freezing behavior or jerky swimming, two behaviors which can be effective strategies to avoid capture (McGhee et al. 2012). The fact that paternal predator exposure influenced offspring activity generally rather than influencing behaviors involved in immediately avoiding predation suggests that fathers might provide their offspring with general skills for the environment they are likely to encounter rather than providing offspring with specific tools for avoiding capture. An alternative explanation for the patterns observed in this study is that perturbations while parenting caused males to reduce parenting behavior, which caused males to

produce offspring in worse condition, and poor condition caused offspring to be inactive. However, we did not detect a relationship between offspring condition and activity. Therefore, effects on behavior are unlikely to have been driven by effects on condition. An obvious task for future studies on paternal programming is to determine whether offspring of predator-exposed fathers have higher fitness in the face of predation risk.

We did not find an overall difference in offspring cortisol response to predation risk based on paternal experience. However, we detected a trend suggesting a sex-specific paternal effect on cortisol concentration. Specifically, female offspring of predator-exposed fathers showed a higher cortisol response to predation risk than male offspring of predator-exposed fathers (similar to Zohar & Weinstock 2011 [rats]). Other studies have also found sex differences in hormonal and behavioral stress responses of offspring based on maternal experience with a stressor (review: Brunton 2013), but the proximate mechanisms that contribute to them and their ecological consequences are not well understood.

There are multiple mechanisms by which the paternal effects observed in this study might have occurred. We found that fathers that visited their nest more frequently in the presence of a model predator produced offspring that performed more antipredator behavior (jerky swimming), which is consistent with the hypothesis that the paternal effects are behaviorally-mediated. Fathers decreased fanning behavior following predator exposure, which is also consistent with behavioral mediation (Stein & Bell 2012). As fanning is an important predictor of reproductive success and provides oxygen to developing embryos (von Hippel 2000), it is possible a reduction in oxygenation might have affected offspring development. It is also possible that olfactory cues from the father might be involved. In stickleback, paternal odor is an important cue for imprinting (Kozak et al. 2011). Spiggin, the protein produced by male kidneys and used to glue

the nest together, may also contain cues for offspring about the fathers' state (Kozak et al. 2011) and is continually added to the nest throughout parenting. The paternal effects observed here might also have been hormonally-mediated: fish release steroid hormones, including cortisol, into the water via the gills (Scott & Ellis 2007), fathers are in close contact with their nests, and fish embryos can take up cortisol from their surroundings (McCormick 1999). Therefore it is possible that offspring were exposed to paternal hormones, potentially even paternal cortisol, which could have had organizational effects on the development of the HPI axis, body size, and behavior.

That being said, we can rule out some alternative explanations for the differences between offspring of predator-exposed and unexposed fathers that were observed in this study. For example, fathers were exposed to predation risk after the eggs were already fertilized so the paternal effects cannot be due to changes in sperm (Curley et al. 2011; Dias & Ressler 2014) or seminal fluid (Sirot et al. 2006; Simmons 2011; Rodgers et al. 2013). It is also unlikely that the effects of paternal treatment can be attributed to the direct exposure of offspring to visual cues of the predator (Darmaillacq et al. 2008). Fathers in the 'predator exposed' treatment were exposed to predation risk by a model sculpin when their offspring were three days old (post-fertilization, pre-hatching), when the optic cups of the embryo are still developing (Swarup 1958) and the eggs were covered by nesting material. Additionally, because fathers were exposed to a rubber model predator rather than a live predator, there was no opportunity for olfactory cues from a predator to reach the eggs (Ferrari & Chivers 2010; Nelson et al. 2013).

While maternal effects on offspring have been examined in natural systems, paternal effects on offspring and their ecological and evolutionary implications have remained relatively understudied. Our results provide some of the first evidence for behavioral transmission of the

paternal environment to offspring. Fathers made short-term adjustments in response to predation risk that influenced both the morphology and behavior of their offspring, but these effects were reversible within fathers, i.e. a male's subsequent clutches were not influenced by his previous experience. In a system such as sticklebacks where fathers breed more than once during the breeding season (Wootton 1984), this suggests that fathers' experiences and behavior from one clutch to another can produce offspring with potentially different fitness outcomes based on the environment in which they occur. Whether this is a true anticipatory parental effect, such that offspring show greater fitness when raised in the environment their father experienced, remains to be explored.

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TABLES

Factor	Length		Body condition	
	<i>F</i> (df)	<i>P</i> – value	<i>F</i> (df)	<i>P</i> – value
Paternal treatment	4.78 (1,60.1)	0.03	10.82 (1,62.4)	0.002
Sex	0.63 (1,152.9)	0.43	0.27 (1,152.5)	0.60
Paternal treatment*Sex	0.02 (1,152.2)	0.89	1.86 (1,152.1)	0.18

Table 4.1: Linear mixed model results for offspring morphological traits.

Factor	Squares moved		Time freezing		Jerky swims	
	<i>F</i> (df)	<i>P</i>	<i>F</i> (df)	<i>P</i>	<i>F</i> (df)	<i>P</i>
Paternal treatment	4.22 (1,50.9)	0.045	1.42 (1,153)	0.24	0.78 (1,12.86)	0.40
Stage	7.18 (2,306)	0.0009	13.62 (2,306)	<0.0001	————	
Sex	0.35 (1,152.7)	0.55	0.35 (1,153)	0.56	0.74 (1,152.8)	0.39
Paternal treatment*Sex	0.40 (1,152.4)	0.53	0.20 (1,153)	0.66	1.82 (1,152.8)	0.18
Paternal treatment*Stage	0.78 (2,306)	0.46	0.63 (2,306)	0.54	————	
Stage*Sex	1.08 (2,306)	0.34	0.67 (2,306)	0.51	————	
Paternal treatment*Stage*Sex	1.28 (2,306)	0.28	0.46 (2,306)	0.63	————	

Table 4.2: Linear mixed model results for offspring anti-predator behaviors. LMMs were run on length-corrected residuals of behaviors.

FIGURES

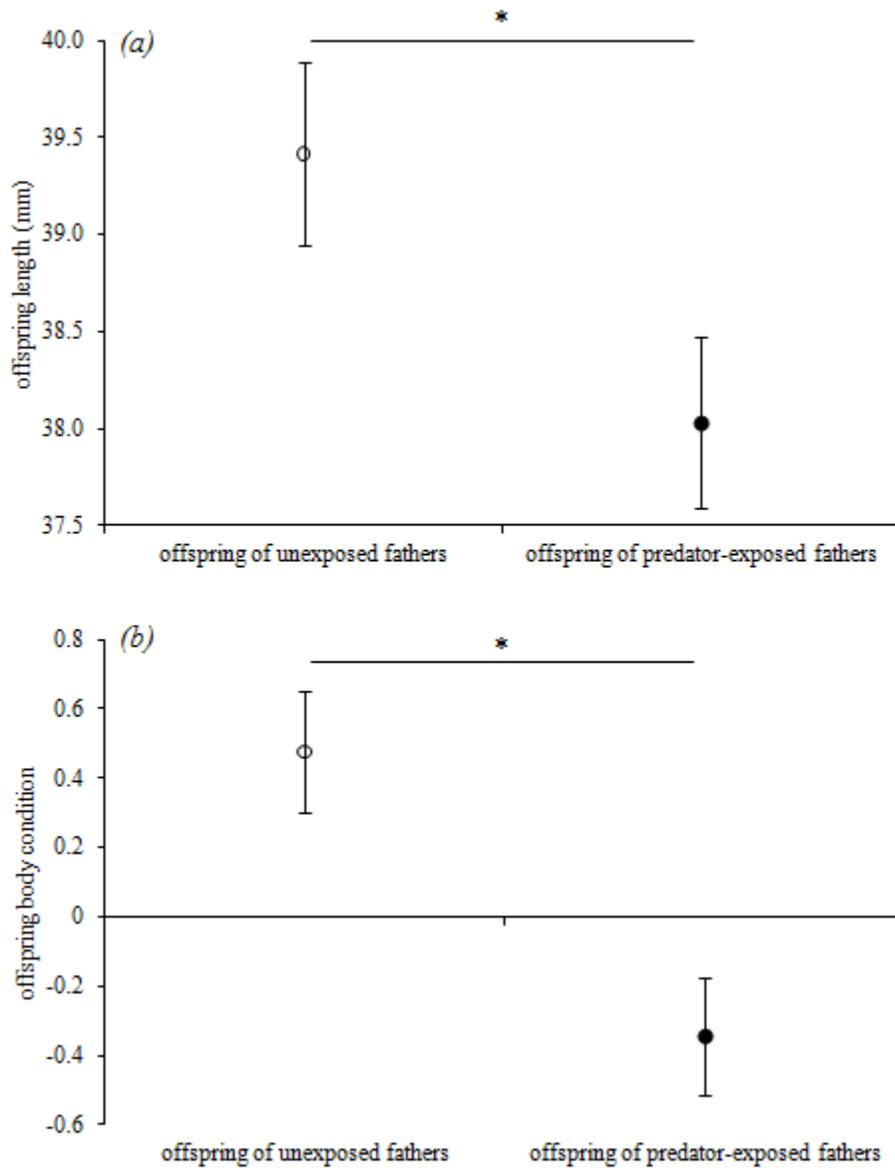


Figure 4.1: Paternal treatment influenced offspring morphology. Compared to offspring of unexposed fathers (open circle), adult offspring of predator-exposed fathers (closed circle) were A) smaller and B) in lower body condition. Figure shows raw data (uncorrected for length). Error bars ± 1 S.E.

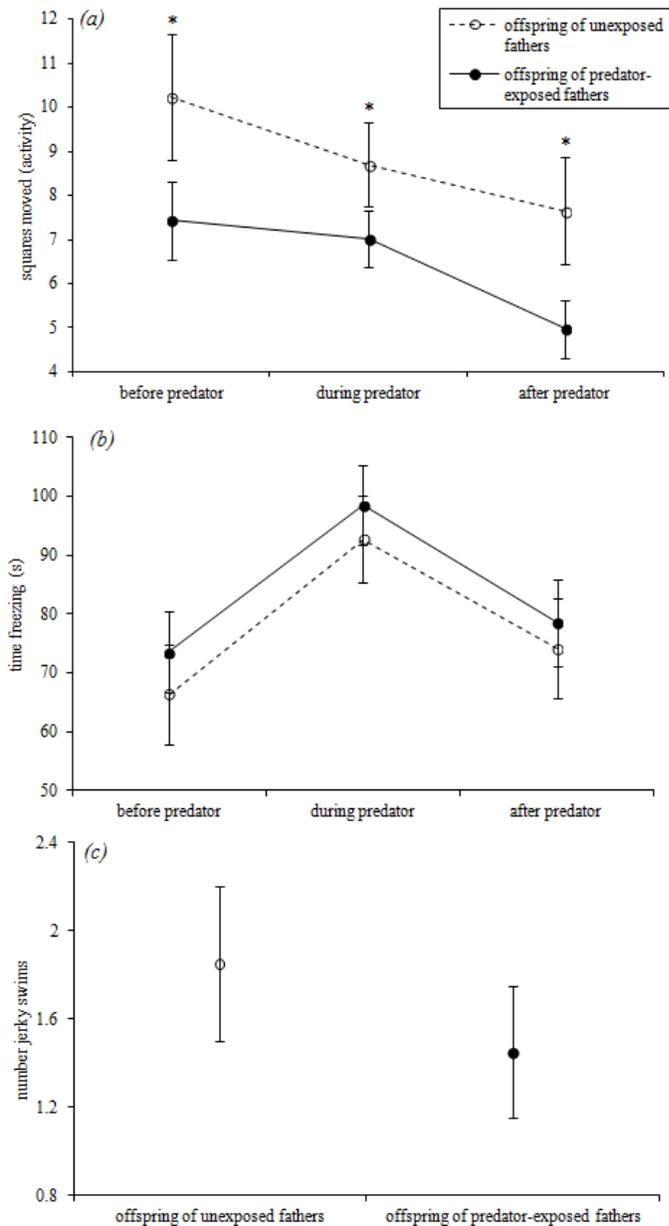


Figure 4.2: Offspring reacted to the model predator, and paternal treatment influenced offspring behavior. A) Offspring reduced activity in the presence of a predator, and activity remained low following removal of the predator. At all stages, offspring of predator-exposed fathers (closed circles, solid line) were less active than offspring of unexposed fathers (open circles, dotted line). B) Offspring spent more time freezing in the presence of a predator, and returned to prior levels of freezing behavior after predator removal. There was no observed effect of paternal treatment on time freezing. C) There was no effect of paternal treatment on number of jerky swims (only performed while the predator was present). Figure shows raw data (uncorrected for length). Error bars ± 1 S.E.

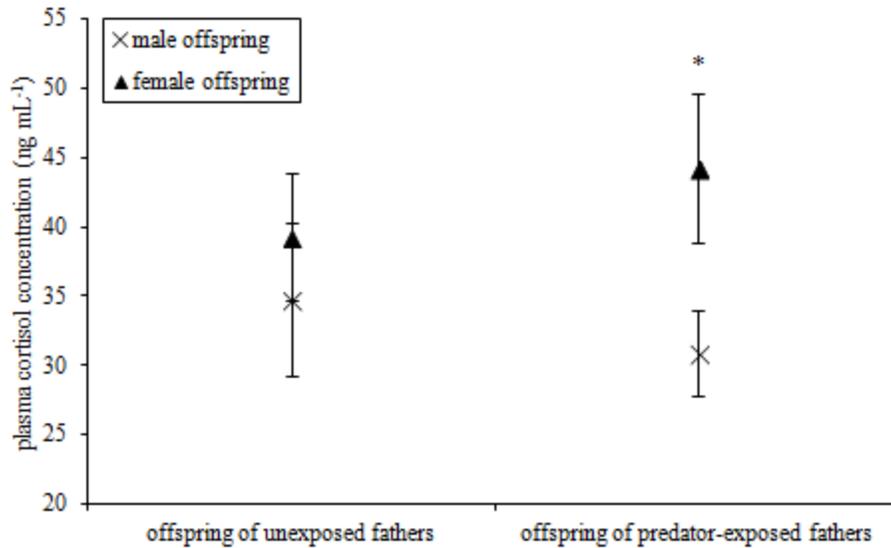


Figure 4.3: Paternal treatment induced a sex-specific difference in circulating cortisol in response to predation risk. There was not a detectable sex difference in circulating cortisol concentration in offspring of unexposed fathers (females: $N = 28$; males: $N = 20$), whereas female offspring of predator-exposed fathers ($N = 45$) showed greater levels of circulating cortisol than male offspring of predator-exposed fathers ($N = 29$) following exposure to the predator model. Figure shows raw data (uncorrected for length). Error bars ± 1 S.E.

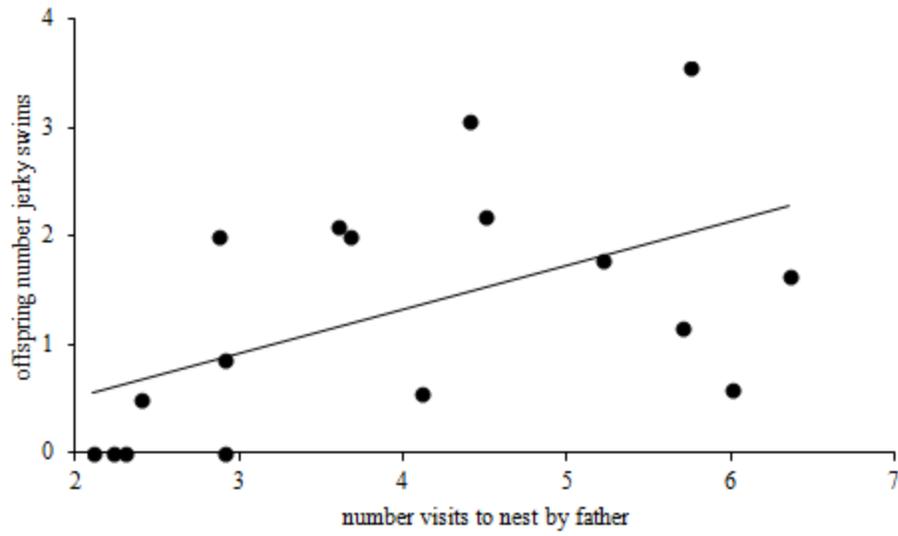


Figure 4.4: Males that visited the nest more often produced offspring that performed more jerky swims in the presence of a predator. The regression line is shown for illustrative purposes only. Figure shows raw data (uncorrected for length).

CHAPTER 5: A VARIABLY FLEXIBLE STEM FACILITATES ADAPTIVE EVOLUTION
IN THREESPINE STICKLEBACK

ABSTRACT

The flexible stem model of adaptive radiations states that plasticity in an ancestral population can facilitate rapid, adaptive evolution in derived populations. A key prediction of this model is that ancestral populations will show greater plastic responses than derived populations. Plasticity in parental care may be an important mechanism in adaptive radiations by allowing populations to persist in novel environments. Here, we test the hypothesis that behavioral plasticity by fathers in response to predation risk might have facilitated the adaptive radiation of threespined sticklebacks. The threespine stickleback radiation provides an excellent opportunity to test the flexible stem model in relation to parental behavior because the marine ancestor is extant, has repeatedly given rise to many locally adapted freshwater populations, and sticklebacks have extensive parental care that is necessary for offspring survival. We compared the behavior of parenting males across multiple ancestral (marine) and derived (freshwater) stickleback populations that differ in time since establishment. We measured behavioral plasticity in parenting males in response to a predator found only in freshwater environments (dragonfly larvae), simulating conditions marine males might experience when colonizing freshwater. Contrary to the prediction of the flexible stem hypothesis, we found greater behavioral plasticity in freshwater than marine populations. However, we found greater variation in behavioral reaction norms in marine populations compared to freshwater populations. Altogether, our results suggest that, rather than ancestral populations showing greater overall levels of plasticity than derived populations, ancestral populations instead show greater standing variation in behavioral reaction norms, potentially providing different

trajectories on which selection can then act. These results offer an intriguing twist on the flexible stem model of adaptive radiations.

INTRODUCTION

Phenotypic plasticity, or the ability of a single genotype to produce multiple phenotypes in response to the environment, is ubiquitous across organisms (Schlichting and Pigliucci 1998). Theory suggests that plasticity can influence adaptive evolutionary patterns (West-Eberhard 2003; Pigliucci et al. 2006; Ghalambor et al. 2007). For example, plasticity may accelerate adaptation as modeled by the flexible stem hypothesis, which states that when an exceptionally plastic stem species encounters a novel environment, plasticity in ancestral populations provides potential pathways that allow for many different evolutionary trajectories (West-Eberhard 2003).

The flexible stem hypothesis predicts that ancestral populations are more plastic than derived populations (West-Eberhard 2003). As the stem species is exceptionally plastic, multiple colonizations into different environments have the potential to result in parallel evolution and/or radiation and diversification in derived populations. Consequently, plastic responses might become genetically accommodated in derived populations (i.e., the original environmental stimulus is no longer required to produce the phenotype) (Crispo 2007). The flexible stem hypothesis has received some empirical support. For example, both courtship and feeding morphology in the threespined stickleback is more plastic in ancestral than derived populations (Shaw et al. 2007; Wund et al. 2008). In tiger snakes, head size is more plastic in the mainland population than the derived island populations (Aubret and Shine 2010).

Parental care and transgenerational plasticity might be particularly likely to facilitate adaptive evolution because parental care can be a buffering mechanism that allows animals to

persist in novel habitats (West-Eberhard 2003; Dybala et al. 2013). Moreover, parental behaviors that affect the fitness of subsequent generations can provide a potential mechanism for “solidifying” or “integrating” the originally plastic phenotype (Badyaev and Uller 2009). For example, imagine that a group of individuals moves in to a new environment, with novel selection pressures. If parents can somehow prepare their offspring for living in this environment, then the population might be more likely to persist (Uller 2008). Transgenerational plasticity can allow offspring to avoid the costs of individuals having to learn on their own, and can occur on much shorter timescales than genetic change. Indeed, theory predicts that parental effects can accelerate the speed of directional evolution (Kirkpatrick and Lande 1989), and there is recent empirical evidence that transgenerational plasticity plays a key role in colonization of novel environments and cycles of species replacement in Western bluebirds (Duckworth et al. 2015).

There is evidence for adaptive transgenerational plasticity (‘parental programming’), specifically with respect to predation risk (daphnia: Agrawal et al. 1999; crickets: Storm and Lima 2010; lizards: Shine and Downes 1999). For example, stickleback fathers that were exposed to predation risk while they were providing care produced offspring that had phenotypes associated with high predation pressure (Stein and Bell 2014). In many cases the exact mechanism underlying transgenerational plasticity in response to predation risk is unknown, but it is likely that parental programming can occur via changes in parental behavior (Ghalambor and Martin 2002; Hale et al. 2003; Eggers et al. 2005; Lissaker and Kvarnemo 2006; Cooke et al. 2008; Chalfoun and Martin 2010; Stein and Bell 2012,2014). Together, behavioral plasticity by parents in response to predation risk and its transgenerational consequences might allow derived

populations to persist in environments with novel predators, as predicted by the flexible stem hypothesis.

Here, we test the hypothesis that behavioral plasticity by fathers in response to predation risk might have facilitated the adaptive radiation of threespined sticklebacks. In stickleback, the marine ancestral form is extant and has remained relatively unchanged (Bell and Foster 1994). Multiple independently-derived freshwater populations have repeatedly diverged from the ancestral marine form, resulting in numerous replicates of derived freshwater populations, many of which are locally adapted, but which vary in time since establishment (Taylor and McPhail 2000). In freshwater, major predators on stickleback fry and juveniles are Odonate (dragonfly) larvae, a predator that is not present in marine populations (Reimchen 1980; Reimchen 1994), which exerts an important selective pressure (Marchinko 2009).

In stickleback, the male is the sole provider of parental care, and paternal care is necessary for offspring survival (Wootton 1984). Importantly, there is evidence for behavioral and transgenerational plasticity in this species. Males confronted with predation risk plastically adjusted their parental care (Stein and Bell 2012). These short-term adjustments by fathers had long-term consequences for their offspring, causing offspring of predator-exposed fathers to resemble sticklebacks that live in high-predation environments (Stein and Bell 2014). Therefore, it is possible that fathers that show greater plasticity in parental care can adaptively prepare their offspring for survival with particular predators. This system provides the opportunity to observe how males from ancestral populations respond to novel freshwater predators, simulating conditions experienced by ancestral populations colonizing freshwater, and compare behavioral plasticity among ancestral, newly-established and well-established derived populations.

We induced behavioral plasticity in fathers by exposing them to live Odonate larvae, an important predator on stickleback eggs and fry that occurs only in freshwater. The parental behavior of individual males was observed both in the presence and absence of predation risk, allowing us to estimate individual ‘behavioral reaction norms’ (Dingemanse et al. 2010). Importantly, all of the behavioral experiments were carried out in freshwater, therefore simulating the conditions that marine animals encounter upon entering new freshwater habitats. The flexible stem hypothesis predicts that behavioral plasticity in response to immediate predation threat is greatest in ancestral marine populations and smallest in established freshwater populations that have undergone many generations of selection with the freshwater predator and thus be more canalized in their responses. We compared established freshwater populations and marine populations to newly established freshwater populations in order to gain insight into the pace at which behavior and behavioral plasticity evolves.

METHODS

Stickleback collection and populations

Juvenile threespine stickleback were collected via minnow traps from nine populations along the Kenai Peninsula and the Matanuska-Susitna Valley in Alaska in the summer of 2012. We collected fish from two marine (ancestral) populations, three young (“new”) freshwater populations, each experimentally seeded from marine populations and living in freshwater for less than 30 generations, and four “established” freshwater populations (Fig. 5.1). Marine populations are considered panmictic and there is likely gene flow between the two marine populations here (Hohenlohe et al. 2010); however, the freshwater populations are genetically isolated (Bell and Foster 1994; M. Bell unpublished data). Fish were shipped to the University

of Illinois Urbana-Champaign and kept in mixed-sex groups within their home population until the breeding season. To stimulate breeding, fish were kept at 10°C on a winter light cycle (4L:20D) for three months. For the duration of the experiment (May-July 2013), fish were kept at 14°C on a summer light cycle (20L:4D). All populations were kept under freshwater conditions in order to simulate marine fish invading freshwater environments.

Paternal behavior and predator assays

Once males began showing nuptial coloration in spring 2013, they were measured for length and weight and transferred into individual tanks with a refuge (plastic ‘plant’), an open plastic box (13x13x3 cm) filled with fine sand, and filamentous algae for nest building. Following nest completion, males were presented with a gravid female from their home population and allowed to spawn. Each male spawned with a unique female. Females were patted dry and weighed prior to and after spawning; egg mass was obtained as the weight difference pre- and post-spawning. Opaque dividers were placed on all sides of the tank to reduce stress and encourage undisturbed parenting behavior. Parental behavior (time fanning) was scored for 10 minutes every day of the nesting cycle via a mirror placed over the top of the tank ($N = 10$ per population). Due to mortality and nest failures, final sample sizes for baseline parenting behavior were $N = 15$ marine, $N = 22$ “new” freshwater, and $N = 35$ “established” freshwater.

Three days post-fertilization, we presented males with a live dragonfly naiad (*Aeshna* spp.; Niles Biological Inc., Sacramento, CA) ($N = 5$ per population). *Aeshna* spp. are only present in freshwater, prey on stickleback fry and juveniles, and are present at all freshwater

populations in this study (Lescak et al. 2012). Therefore, this predator is novel to the marine populations.

The naiad was tethered to the side of the tank such that it could move and interact with the adult male, but not access the nest and eggs. We recorded fathers' anti-predator and parenting behaviors in the presence of the naiad for five minutes via a video recorder (JVC Everio), after which the naiad was removed. Males were then allowed to complete their nests normally. Final sample sizes for males exposed to the predator were $N = 7$ marine, $N = 12$ "new" freshwater, and $N = 18$ "established" freshwater. Anti-predator and parenting behaviors were later coded using the JWatcher program (UCLA). Individuals were coded such that the observer was blind to their population of origin.

Data analysis

We used linear mixed models to test effects on undisturbed time fanning ("baseline" parenting behavior), total time orienting to the predator (anti-predator behavior) and percent change in proportion time fanning prior to and during the predator introduction (plasticity). Percent change in proportion time fanning allows for standardization of plastic responses across populations that differ in baseline fanning levels. Models included population type (marine, new, established) and population nested within population type as fixed effects. For undisturbed time fanning, individual ID was included as a random effect to account for multiple measurements ($N = 16$ observations per individual). We do not have the statistical power to quantify IxE (individual x environment interactions, Nussey et al. 2007) in this study (Martin et al. 2011). Length and egg mass were never significant covariates in any model and were removed from analysis.

RESULTS

Population-level differences in parenting and anti-predator behavior

On average, males from marine populations spent more time fanning than males from both new and established freshwater populations (Table 5.1; Fig 5.2a). Males from all populations showed the same general pattern of fanning across the nesting cycle (fanning increased as eggs age and peaked right before hatching, then decreased rapidly).

Population type had a significant effect on time orienting to the predator (Table 5.1). Visually, the trend in the data is for time orienting to the predator to increase with familiarity with this predator (that occurs only in freshwater). Indeed, post-hoc tests showed males from marine populations spent significantly less time orienting to the predator than males from both freshwater populations (Fig 5.2b).

Behavioral plasticity across populations

Exposure to the predator caused males from freshwater populations to decrease fanning, with 'new' freshwater populations intermediate between marine and established freshwater populations. In contrast, marine males did not change their parenting behavior on average in the presence of a predatory dragonfly larva (Table 5.1; Fig 5.3).

Although these data suggest that marine males do not change their behavior in response to the predator, the averages are misleading. Closer examination of the behavioral reaction norms, which describe how individual males changed their behavior in response to the predator, suggests that there was considerable variation among individual marine males in how they responded to the predator. While some males increased fanning while the predator was present,

others decreased. In stark contrast, individual males from marine populations were much more likely to uniformly decrease fanning in response to the predator. Indeed, 4/7 (58%) marine males increased fanning in the presence of the predator, while only 1/12 (8%) of “new” freshwater and 0/18 (0%) of “established” freshwater individuals increased fanning (Fig 5.4).

DISCUSSION

The flexible stem hypothesis states that ancestral plasticity can fuel adaptation and predicts that there is more plasticity in ancestral compared to derived populations. We observed the opposite: although ancestral (marine) populations performed more parental behavior compared to derived (freshwater) populations, ancestral populations were *less* behaviorally plastic in response to the predator than established derived freshwater populations, with newly-derived populations intermediate between the two. Importantly, the predator used in these assays, Odonate larvae, is only present in freshwater environments and is thus novel to males from ancestral populations. Males from ancestral populations did not appear to interpret the freshwater predator as a threat; they did not spend as much time orienting as freshwater populations. Odonate larvae are important predators on stickleback nests (Marchinko 2009), therefore failing to recognize Odonate larvae as a threat may have strong fitness consequences. It is perhaps not surprising that males from well-established populations, which have experienced many generations of selection in freshwater, dramatically decreased parenting when the predator was present.

While these results are not strictly consistent with the flexible stem hypothesis, they prompt a more nuanced one because closer inspection of behavioral reaction norms revealed greater variation among individuals in behavioral plasticity in ancestral populations than derived

populations. Standing variation in behavioral reaction norms could potentially provide different trajectories on which selection can act, therefore we hypothesize that ancestral variation in reaction norms can provide the raw material for adaptation to new environments. Specifically, we suspect that those marine males that reduce parenting in response to predation risk enjoy greater reproductive success in freshwater, perhaps via adaptive parental effects (Stein and Bell 2014). Selection may then act to further refine this plasticity, resulting in the dramatic reduction in parenting that we observed in both new and established freshwater population types; note that freshwater males show steeper slopes than those observed in marine males. Our results also suggest that this process happens quickly – young freshwater populations show much less variation in slopes than marine populations, even after only 2 generations.

Assuming that there is a heritable basis to variation in reaction norms (GxE), genotypes that decrease fanning in response to Odonate larvae will be more likely to produce well-adapted offspring, and will increase in frequency in the population. Although we presently do not know for certain if there is a heritable basis of parental behavior and parental plasticity, the repeatability (which sets an upper bound on heritability) of parental behavior in this species, is high (Stein and Bell 2012; Stein and Bell 2015), and most quantitative behavioral traits have a heritable component (Stirling et al. 2002). Moreover, the animals in this study were collected in the field as juveniles but otherwise reared in a common garden in the lab. Therefore it is likely that some of the variation observed in this study reflects heritable variation.

This hypothesis builds upon well-established ideas from two literatures. First, there is growing appreciation of the importance of standing genetic variation as raw material for adaptation (Barrett and Schluter 2008). Indeed, studies of sticklebacks have made an important contribution to this literature: marine populations harbor allelic variation in loci that influence

armor plating, and this variation has been repeatedly drawn upon during the radiation of sticklebacks (Colosimo et al 2005). Our results suggest that ancestral variation in reaction norms, in addition to ancestral genetic variation in non-plastic traits, could facilitate adaptation and diversification.

Second, there is a rich literature on the existence (Newman 1994; Stinchcombe et al. 2004) and importance of genetic variation in reaction norms (GxE) as fuel for the evolution of plasticity (Scheiner 1993; Schlichting and Pigliucci 1998; Tufto 2000; Pfennig et al. 2010). Our results suggest that variation in reaction norms is not only important for the evolution of plasticity, but can facilitate adaptive radiations: standing variation in reaction norms could provide the opportunity for selection to produce different evolutionary outcomes.

These results have broad implications for the general debate about the relative importance of standing variation versus plasticity in fueling adaptive radiations (Ghalambor et al. 2007). Our data suggest that both views are correct, but with a twist: selection acts on variation in plasticity (behavioral reaction norms) within ancestral populations, with particular reaction norms favored in derived populations. Previous studies in this species have found that fathers from natural populations can transmit information about the current environment to their offspring via short-term adjustments in paternal behavior, and that this shapes offspring's developmental trajectories into adulthood in potentially adaptive way (Stein and Bell 2014). Altogether, our results suggest that plasticity in parental behavior might have facilitated the radiation of threespine sticklebacks by allowing offspring to persist in novel environments, and offer a different perspective for thinking about how plasticity can influence evolution, and adaptive radiations in particular.

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TABLES

Factor	Time fanning		Time orienting		Percent change in time fanning	
	<i>F</i> (df)	<i>p</i> -value	<i>F</i> (df)	<i>p</i> -value	<i>F</i> (df)	<i>p</i> -value
Population type	3.64 (2,93.4)	0.03	2.01(2,22)	0.05	3.24 (2,22)	0.03
Population(Population type)	1.63 (6,94.2)	0.15	0.51 (6,22)	0.80	0.89 (6,22)	0.52

Table 5.1: Linear mixed model testing for the effect of population and population type (marine, new or established) on behavior. Time fanning (10 minutes per day every day of the nesting cycle) included individual as a random effect to account for repeated measurements on the same individuals. Time orienting refers to time orienting to the dragonfly larva. Percent change in time fanning was calculated as the percent change in proportion time fanning immediately prior to and during the introduction of the dragonfly larva). Significant values ($p \leq 0.05$) are shown in bold.

FIGURES

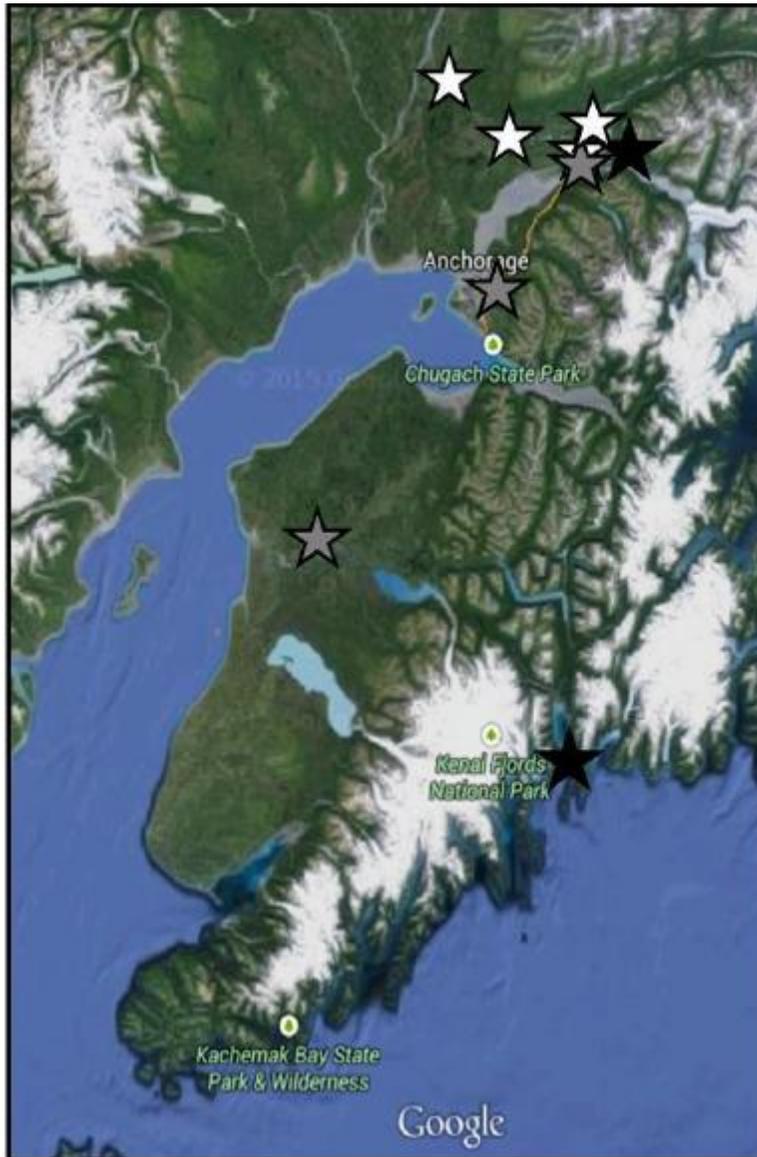


Figure 5.1: Population locations. Black stars represent marine populations ($N = 2$), grey stars represent “new” freshwater populations ($N = 3$), and white stars represent “established” freshwater populations ($N = 4$). Map © Google Earth 2015.

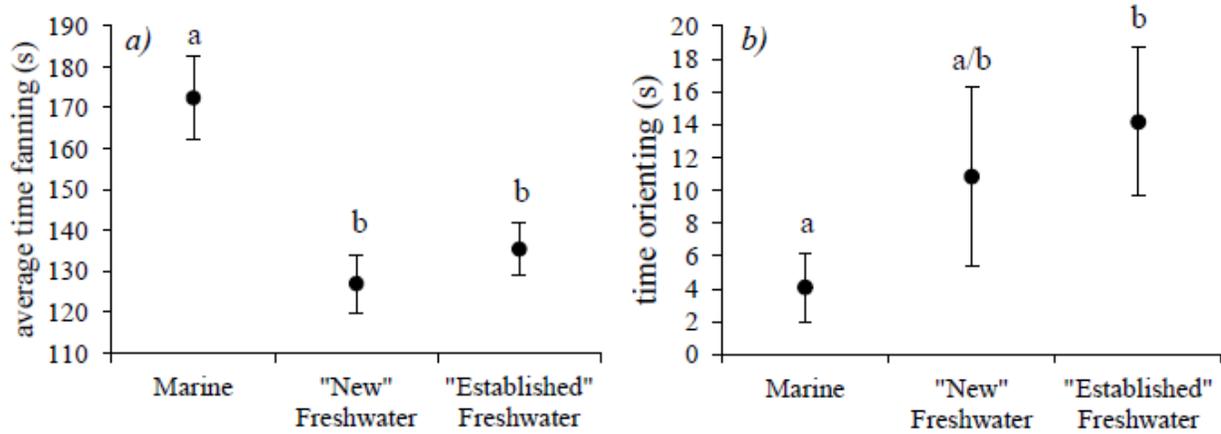


Figure 5.2: Marine (ancestral) populations differed from freshwater populations. A) Marine populations fanned more overall than either young or established freshwater populations. B) Marine populations spent less time orienting toward the predator than young and established freshwater populations. Error bars ± 1 S.E.

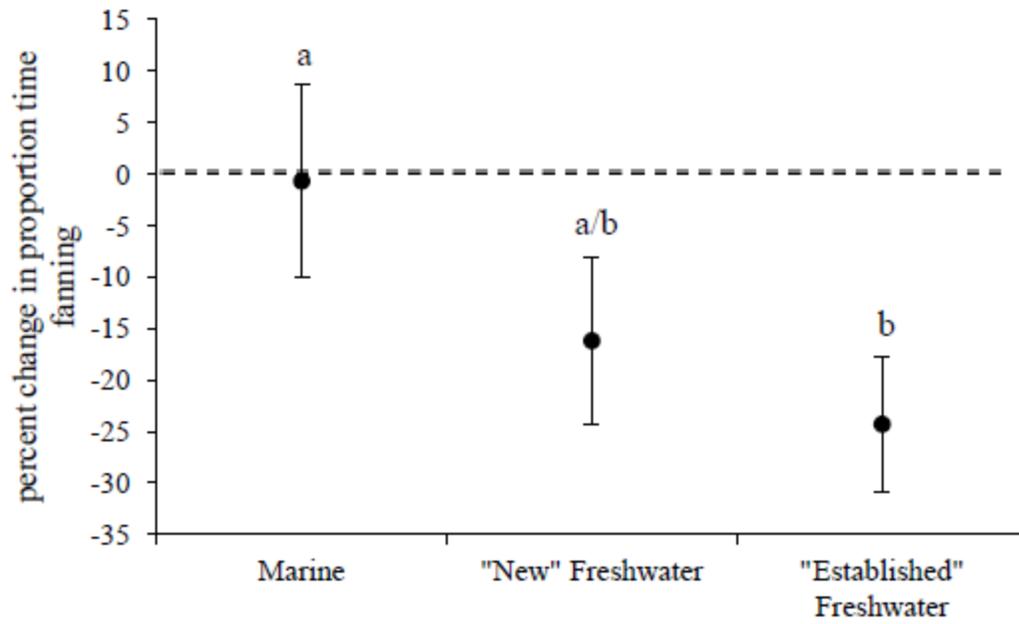


Figure 5.3: Established freshwater populations altered their parenting behavior significantly more in the presence of a predator than marine (ancestral) populations. Dashed line indicates no change in fanning, points below the line indicate a decrease in fanning. Error bars ± 1 S.E.

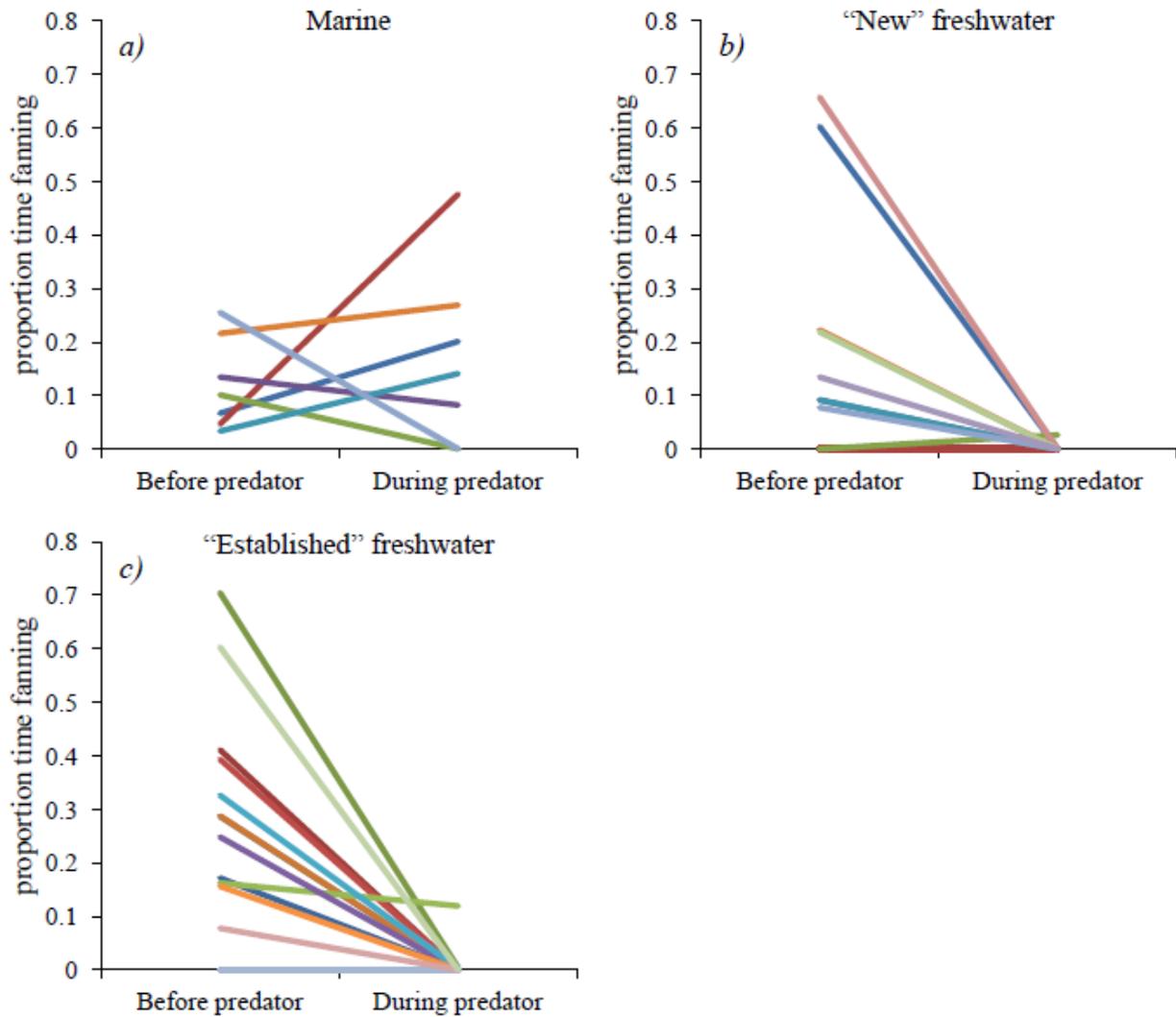


Figure 5.4: Populations differed in individual variation in plasticity. Each line represents an individual male. Individuals in marine populations (A) varied in whether they increased or decreased fanning in the presence of a predator, while only one male increased fanning in “new” populations (B). No males increased fanning in the presence of a predator in “established” populations (C).