The role of variation and plasticity in parental care during the adaptive radiation of three-spine sticklebacks

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Phenotypic plasticity might influence evolutionary processes such as adaptive radiations. Plasticity in parental care might be especially effective in facilitating adaptive radiations if it allows populations to persist in novel environments. Here, we test the hypothesis that behavioral plasticity by parents in response to predation risk facilitated the adaptive radiation of three-spine sticklebacks. We compared the behavior of fathers across multiple ancestral (marine) and derived (freshwater) stickleback populations that differ in time since establishment. We measured behavioral plasticity in fathers in response to a predator found only in freshwater environments, simulating conditions marine males experience when colonizing freshwater. The antipredator behavior of males from newly established freshwater populations was intermediate between marine populations and well-established freshwater populations. In contrast to our predictions, on average, there was greater behavioral plasticity in derived freshwater populations than in ancestral marine populations. However, we found greater individual variation in behavioral reaction norms in marine populations compared to well-established freshwater populations, with newly established freshwater populations intermediate. This suggests that standing variation in behavioral reaction norms within ancestral populations might provide different evolutionary trajectories, and illustrates how plasticity can contribute to adaptive radiations.

KEY WORDS: Adaptive radiation, behavior, paternal care, phenotypic plasticity, stickleback.

Phenotypic plasticity, or the ability of a single genotype to produce multiple phenotypes in response to the environment, is ubiquitous across organisms (Schlichting & Pigliucci 1998). Theory suggests that plasticity can influence adaptive evolutionary patterns (West-Eberhard 2003; Pigliucci et al. 2006; Ghalambor et al. 2007; Levis & Pfennig 2016). 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). Alternatively, phenotypic plasticity might hinder adaptation to novel environments, as multiple phenotypes resulting from a single genotype might allow populations

to attain fitness optima while shielding genotypes from selection (Schlichting 2004).

Phenotypic plasticity may play a role in colonization of new habitats if plasticity in the colonizing population—the "ancestral" population—triggers changes in phenotypes that allow the population to survive under novel conditions (West-Eberhard 2003; Levis & Pfennig 2016). As the ancestral "stem" species is exceptionally plastic, multiple colonization into different environments provide trajectories on which selection can then act, resulting in parallel evolution and/or radiation and diversification in derived populations. Models have suggested that plastic responses might become genetically accommodated or assimilated in derived populations (i.e., the original environmental stimulus is no longer

required to produce the phenotype) (Crispo 2007; Lande 2009). These models have received some empirical support. For example, in tiger snakes, head size is more plastic in the mainland population than in derived island populations (Aubret & Shine, 2009), and diet-induced plasticity in feeding regimes in spadefoot toad tadpoles may have become genetically assimilated as these tadpoles colonized new niches (Levis et al. 2018).

Plasticity in parental behavior might be an especially potent type of plasticity that can facilitate adaptation to new environments if parents experiencing a new environment can buffer their offspring from the environment and/or prepare their offspring for living there (West-Eberhard 2003; Dybala et al. 2013). Parental behaviors that affect the fitness of the next generation can provide a rapid mechanism for "integrating" a plastic phenotype such that the initial cue is no longer necessary to produce that phenotype (Badyaev & Uller 2009). Indeed, recent empirical evidence supports longstanding theory that maternal effects can affect the speed of directional evolution (Kirkpatrick & Lande 1989): In Western bluebirds, maternal effects facilitated the colonization of novel environments and cycles of species replacement (Duckworth et al. 2015).

Adaptive transgenerational plasticity has frequently been reported in the context of predation risk and has been documented in several taxa (daphnia: Agrawal et al. 1999; crickets: Storm & Lima 2010; lizards: Shine & Downes 1999). If parents can detect cues about the predators their offspring are likely to encounter, and somehow transmit that information to their offspring, their offspring might have a better chance of surviving in the face of novel predators (Uller 2008). For example, female crickets exposed to cues of predatory spiders produced offspring with altered antipredator behavior and improved survival in the face of predation risk by spiders (Storm & Lima 2010). In many cases, parental programing appears to occur via changes in parental behavior (Ghalambor & Martin 2002; Hale et al. 2003; Eggers et al. 2005; Lissaker & Kvarnemo 2006; Cooke et al. 2008; Chalfoun & Martin 2010; Stein & Bell 2012; Stein & Bell 2014). Therefore behavioral plasticity by parents in response to predation risk and its transgenerational consequences might allow derived populations to persist in new environments.

Sticklebacks are suitable subjects for testing plasticity's role in evolutionary patterns because the marine ancestral form is extant and has remained relatively unchanged (Bell & Foster 1994; Hohenlohe et al. 2010). 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 & McPhail 2000). Previous studies in three-spine sticklebacks compared plasticity in between ancestral (marine) and freshwater (derived) populations and found support for plasticity's role in colonization of new environments:

Patterns of divergence among freshwater populations reflected ancestral plasticity (Shaw et al. 2007; Wund et al. 2008). Here, we adopt a similar strategy in sticklebacks but add an additional comparison: We compare marine populations to well-established, naturally colonized freshwater populations ("well-established") and to populations that were experimentally introduced into freshwater for <30 generations ("newly established"), which allows us to investigate the rate at which plasticity can affect evolution.

We investigate plasticity in paternal care, a behavior essential for offspring survival that could be important during the early stages of establishment in a new environment. Previous studies in this system showed that male sticklebacks that were exposed to predation risk while they were providing care changed their paternal behavior (Stein & Bell 2012; Stein & Bell 2015) and produced offspring that had phenotypes associated with high predation pressure (Stein & Bell 2014). In this study, we induced behavioral plasticity in fathers by exposing them to a live Odonate naiad, an important predator on stickleback eggs and fry that exists only in freshwater, while they were providing care (Reimchen 1980; Reimchen 1994; Marchinko 2009). This strategy simulated conditions experienced by marine sticklebacks as they colonize freshwater and encounter a predator unique to freshwater for the first time. 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). Specifically, we measured the total amount of time the male spent fanning the eggs. Fanning is a paternal behavior that oxygenates the eggs (Wootton 1984), is important for offspring development (Wootton 1984), and consistently varies among fathers (Stein & Bell 2012; Stein & Bell 2015). We posit that behavioral plasticity in response to immediate predation threat has allowed populations to adapt to novel conditions, such as the presence of a novel predator (Levis & Pfennig 2016; West-Eberhard 2003). We also investigate the possibility that the outcome of the flexible stem process is phenotypic accommodation, such that derived populations are less plastic than marine populations (Lande 2009; Lande 2015). By comparing well-established freshwater populations and marine populations to newly established freshwater populations, we can gain insights into the rate at which changes in parental care in response to predation risk might facilitate adaptation to novel environments.

Methods
STICKLEBACK COLLECTION AND POPULATIONS
Juvenile three-spine sticklebacks (approximately 3 months old) 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 newly established freshwater populations and

four well-established freshwater populations derived from naturally occurring colonization events (Table S1; Fig. S1). Two of the newly established populations (Cheney Lake, Scout Lake) were experimentally seeded from marine populations in 2009 and 2011, respectively, and one of the newly established populations (Loberg Lake) was recolonized between 1983 and 1988; therefore at the time of collection, all three populations had been living in freshwater for less than 30 generations (Bell et al. 2004; Bell et al. 2016). Previous studies have found little population genetic subdivision ($F_{ST} = 0.0076$) between the two marine populations used in this study (Hohenlohe et al. 2010); however, the freshwater populations are genetically isolated (Bell & Foster 1994; Hohenlohe et al. 2010; Bell et al. 2016). Fish were shipped to the author's home institution and kept in freshwater 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 3 months. For the duration of the experiment (May–July 2013), fish were kept at 14°C on a summer light cycle (20L:4D).

QUANTIFYING UNDISTURBED PATERNAL BEHAVIOR AND BEHAVIORAL PLASTICITY

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 (13 × 13 × 3 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 postspawning. Opaque dividers were placed on all sides of the tank to reduce stress and encourage undisturbed parenting behavior. Undisturbed 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 to establish baseline parenting behavior and examine differences in parenting among populations (Figs. S2 & S3).

Three days postfertilization, we presented a subset of males from each population with a live dragonfly naiad (*Aeshna spp.*; Niles Biological Inc., Sacramento, CA). *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. Importantly, all of the experiments were carried out in freshwater, thereby simulating the conditions that marine animals encounter upon colonizing freshwater habitats.

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' parenting behaviors for 5 minutes prior to the introduction of the predator, and antipredator and parenting behaviors in the presence of the naiad for 5 minutes via a video recorder after the naiad entered the tank (JVC Everio), after which the naiad was removed. Males were then allowed to complete parenting until fry were independent (5 days after hatching in these populations). 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. We measured orientation to the predator (when the male turned his body to face the predator, Supplementary Video 1) and total time fanning. Parental behavior continued to be measured every day for 10 minutes for the remainder of the nesting cycle to establish how exposure to the dragonfly naiad influenced the entire period when males provided care (Fig. S3). We additionally measured undisturbed parenting behavior in the same way for a subset of individuals that did not experience the dragonfly naiad to compare population level differences in parenting and examine how naiad exposure affected parenting behavior (Fig. S2). An initial total of $N = 5$ males were used per population; due to deaths and unsuccessful nests, final sample sizes varied (see Results).

It is important to consider that the males that were used as fathers in this study were collected in the wild as juveniles, and reared in the lab for approximately 8 months, until they were sexually mature. Therefore it is possible that some of the behavioral variation observed in this study reflects enduring environmental effects of the males' early experience (or their fathers' experience) in the field. Although it is possible and commonplace to artificially fertilize stickleback eggs and incubate embryos in the lab without paternal care, rearing sticklebacks without paternal care results in a number of behavioral, neurological, and cognitive deficits that make it difficult to infer the adaptive significance of their behavior (e.g., McGhee & Bell 2014), therefore we elected to use wild-caught animals in this study. Investigating the genetic basis of variation in parental care and plasticity is an obvious topic for future work.

ETHICAL NOTE

All males resumed parenting behavior following measurements and observations, and care was taken to minimize stress. Naiads were placed on a tether too short to allow them to access the male's nest, and were too small to injure the adult males. All naiads resumed normal behavior upon being placed back in their home tanks. This study was conducted under Alaska Fish and Game Permit SF2012-130 and approved by the Institutional Animal Care and Use Committee at the author's home institution.

DATA ANALYSIS

We used general linear models to test effects on total time orienting to the predator (antipredator behavior) and percent change in proportion time fanning 1 hour prior to and during the predator introduction (plasticity). We estimated the percent change in proportion time fanning because populations differed in

undisturbed fanning levels over the nesting cycle (see Table S2, Fig. S2). Time orienting to the predator fitted a Gaussian distribution, while change in proportion time fanning was arcsine transformed prior to analysis. Models included population type (marine, new, and established) and population nested within population type as fixed effects. We do not have the statistical power to quantify I × E (individual × environment interactions; Nussey et al. 2007) in this study (Martin et al. 2011), therefore we assessed whether there was individual variation in plasticity within populations by computing the percentage of males within each type of population that decreased fanning while the predator was present. We compared the last 5 minutes of time spent fanning without the predator present (undisturbed) to 5 minutes of parenting with the predator present in the tank to obtain our reaction norms of how parenting changes when naiad predators are present. Length and egg mass were never significant covariates in any model and were removed from analysis. Additionally, we assessed the covariation between percent change in fanning and time orienting separately for the ancestral, "new," and derived populations, while accounting for length and egg mass using the MCMCglmm package (Hadfield 2010), which returns 95% credibility intervals (CIs). If the 95% CIs of covariance estimates did not overlap zero, we interpreted this as evidence that the estimates were statistically significant. We used noninformative 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 autocorrelation plots of five independent chains with 500,000 iterations, a 1000 burn-in period and thinning every 100 iterations for each model. All analyses were conducted in R v3.2.2 (R Core Team 2015).

Results

POPULATION-LEVEL DIFFERENCES IN ANTIPREDATOR BEHAVIOR

Males from marine populations responded less strongly to the Odonate naiad relative to their freshwater counterparts (Table 1; Figs. 1 & 2). Males from well-established freshwater populations spent more time orienting to the dragonfly naiad than males from marine populations. On average, males from newly established populations spent an intermediate amount of time orienting toward the Odonate, but their behavior was not significantly different from either marine or established freshwater populations (Fig. 1).

BEHAVIORAL PLASTICITY ACROSS POPULATIONS

Exposure to the predator caused males from freshwater populations to decrease fanning, with newly established freshwater populations intermediate between marine and well-established freshwater populations. In contrast, on average marine males did not change their parenting behavior in response to a predatory dragonfly naiad (Table 1; Fig. 2). Percent change in proportion time fanning and time orienting to the predator did not covary in any population (Ancestral: 0.30 [–8.50, 10.15]; New: –0.03 [–3.92, 4.12]; Derived: –0.35[–3.33, 2.71]).

Although these data suggest that marine males do not change their behavior on average 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 qualitatively considerable variation among individual marine males in how they responded to the predator (Fig. 3). While some marine males increased fanning when the predator was present, others decreased. In stark contrast, individual males from freshwater populations almost uniformly decreased fanning in response to the predator. For example, four of seven (58%) marine males increased fanning in the presence of the predator, while only one of 12 (8%) of newly established freshwater and zero of 18 (0%) of well-established freshwater individuals increased fanning. Across all freshwater populations, males exposed to the dragonfly naiad on average showed reduced parental care for 1 day before converging on similar fanning behavior to control individuals that did not experience predation risk (Figs. S2 & S3), similar to previous studies in stickleback with piscivorous predators (Stein & Bell 2012; Stein & Bell 2015).

Discussion

In this study, we investigated the evolution of behavioral plasticity during colonization of a new habitat. We posited that the presence of plasticity in an ancestor could facilitate adaptation to new environments, and tested the prediction that phenotypic plasticity is eventually genetically assimilated (Lande 2009). Although there was population-level variation in average plasticity, the pattern at first glance is not consistent with genetic assimilation: on average, ancestral (marine) populations were less behaviorally plastic in response to the predator than well-established derived (freshwater) populations, with newly derived populations intermediate between the two.

However, closer inspection of the individual behavioral reaction norms suggests that consistent selection on plasticity in freshwater has refined reaction norms such that in contrast to the marine populations, which exhibited interindividual variation in behavioral reaction norms; males from established freshwater populations exhibited almost uniformly negatively sloping behavioral reaction norms (Fig. 3). In other words, qualitative individual variation in response to a novel predator was greatest in ancestral marine populations and smallest in established freshwater

Table 1. General linear model testing for the effect of population and population type (marine, new or established) on behavior.

	Time orienting		Percent change in time fanning	
Factor	F (df)	P-value	F (df)	P-value
Population type	**2.01 (2, 22)**	**0.05**	**3.24 (2, 22)**	**0.03**
Population (Population type)	0.51 (6, 22)	0.80	0.89 (6, 22)	0.52

Note: Time orienting refers to time orienting to the dragonfly naiad. 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.
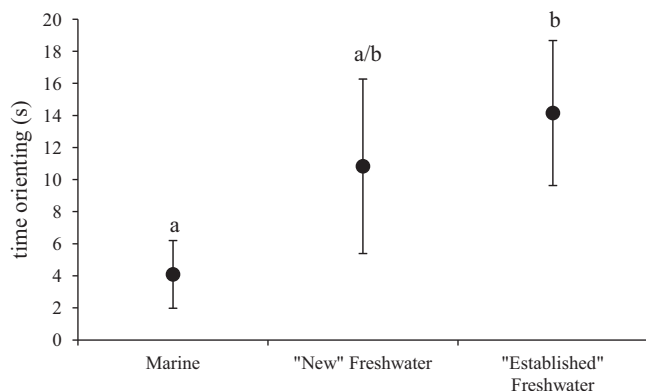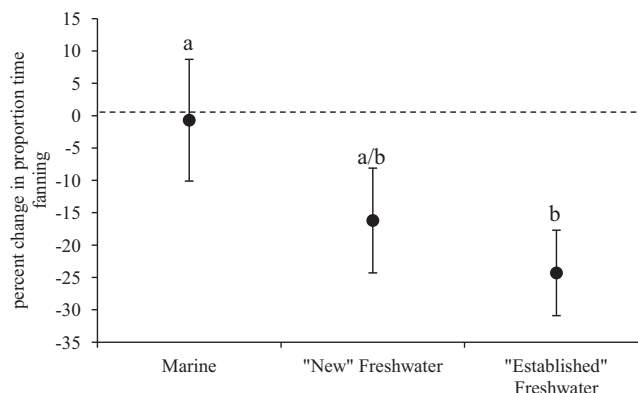

Figure 1. Males from marine populations spend less time orienting to the live predator than males from "established" freshwater populations. $N = 7$ marine, $N = 12$ "new" freshwater, and $N = 18$ "established" freshwater males. Error bars ± SE.
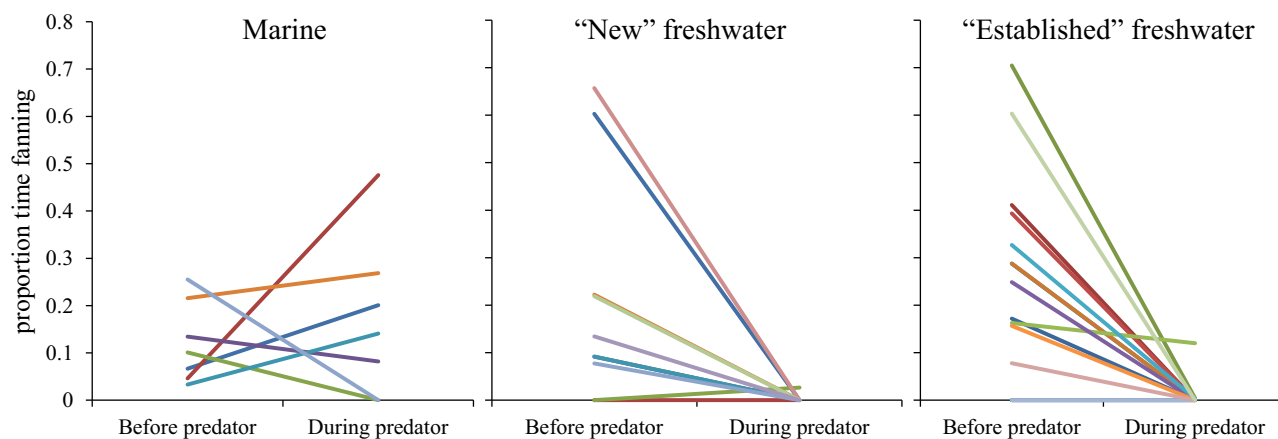

Figure 2. Males from "established" freshwater populations show a reduction in fanning following the introduction of a live predator, while males from marine populations on average do not change their behavior. Dotted line indicates no change in fanning. $N = 7$ marine, $N = 12$ "new" freshwater, and $N = 18$ "established" freshwater males. Error bars ± SE.

populations that have undergone many generations of selection with the freshwater predator (Fig. 3), which could reflect the process of genetic assimilation.

Extensive interindividual variation in reaction to stressors generally (Hofmann & Parsons 1991) and to novel predators in particular has been reported in other species (marmots: Blumstein et al. 2009, mosquitofish: Rehage et al. 2005). We presently do not know if the variation in behavioral reaction norms among marine males is adaptive and maintained by the greater diversity of predators in the ocean, or that the variation is neutral and reflects relaxed selection (Lahti et al. 2009). Alternatively, these variations in reaction norms could simply be random responses to a novel cue, and those responses that result in an increase in fitness (here, reduction in fanning and increase in nest protection) provide trajectories on which selection can then act.

Males from well-established populations, which experienced many generations of selection in freshwater, dramatically decreased parenting when the predator was present. This result is consistent with previous studies, which have shown that male sticklebacks from populations that experience high levels of predation by piscivorous fishes decrease parenting both in the presence of an immediately after predation risk (Stein & Bell 2012; Stein & Bell 2014; Stein & Bell 2015). It is likely that the reason

why males (on average) from marine populations did not change their behavior in response to the predator was because they did necessarily perceive the dragonfly—a novel predator to marine sticklebacks—as a threat. Indeed, unlike males from freshwater populations, males from marine populations did not orient to the dragonfly. It is possible that marine males cannot generalize across invertebrate predators such as those they might encounter in the ocean, for example, crabs. Odonate naiad are important predators on stickleback nests in freshwater (Marchinko 2009), therefore marine males that fail to recognize dragonfly in freshwater as a threat could suffer strong fitness consequences.

Because the animals in this study were wild-caught, we do not know if the variation among individuals and among populations reflects genetic variation, differences in which the males were raised (transgenerational plasticity/environmental effect), and/or their own experience with predators (developmental plasticity/environmental effect). If the pattern reflects genetic variation, and sons inherit their fathers' reaction norms, then those individuals that reduce fanning in response to Odonate naiad might have higher reproductive success because they produce offspring with predator-adapted phenotypes (Stein & Bell 2014;

Figure 3. Individual reaction norms showing proportion time fanning before and during a live predator introduction. Each line represents an individual male. $N = 7$ marine, $N = 12$ "new" freshwater, and $N = 18$ "established" freshwater males.

Stein & Bell 2018), eventually leading to the fixation of negatively sloping reaction norms in freshwater. This scenario is plausible because the heritability of parenting behavior in sticklebacks is very high, >0.9 (Bell et al. 2018). Alternatively, if the variation among reaction norms results from nongenetic mechanisms, then when marine males move into freshwater, those males that reduce fanning in response to dragonfly naiad could have higher fitness in freshwater because males spend more time guarding their nests (Stein & Bell 2015), resulting in higher survival rates of offspring. Another intriguing possibility is that the reduction in fanning might provide a cue to offspring about predation risk in their environment, resulting in offspring with predator-adapted phenotypes (Stein & Bell 2014; Stein et al. 2018). If sons learn from their fathers, then their male offspring will also reduce fanning. This process could lead to the fixation of negatively sloping behavioral reaction norms in freshwater so long as the population remains in freshwater. Over time, we might expect selection to further refine this plasticity via genetic accommodation (Lande 2009; West-Eberhard 2003).

Therefore, regardless of whether the variation reflects genetic or environmental causes, our results suggest that ancestral variation in behavioral reaction norms might have facilitated evolution in this system. Moreover, our results suggest that plasticity-mediated evolution in sticklebacks can happen quickly—young freshwater populations showed much less variation in the slopes of their behavioral reaction norms than marine populations, even after fewer than 30 generations. Our results provide empirical support that exposure to a novel predator has revealed cryptic variation in reaction norms in an ancestral population, providing trajectories for selection to act upon and a mechanism by which plasticity can influence evolutionary outcomes (Gibson & Dworkin 2004; Le Rouzic & Carlborg 2008; Schlichting 2008; McGuigan & Sgró 2009; McGuigan et al. 2011; Ledón-Rettig et al. 2014; Paaby & Rockman 2014). Here, we suggest that

both standing genetic variation and plasticity work in tandem to promote adaptive evolution: exposure to a novel predator exposed cryptic variation in reaction norms, providing trajectories on which selection can then act, as predicted by genetic assimilation hypotheses (Crispo 2007; Lande 2009). Altogether our results are consistent with the hypothesis that standing variation in reaction norms may have facilitated the adaptive radiation of three-spine sticklebacks via an especially intriguing trait: parental care.

AUTHOR CONTRIBUTIONS

L.R.S. and A.M.B. conceptualized and designed the experiment. L.R.S. collected stickleback, carried out the experiment, and performed data analysis. L.R.S. and A.M.B. wrote the manuscript.

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DATA ARCHIVING

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Population details. Number of generations is indicated at time of collection.

Table S2. Linear mixed model testing for the effect of population and population type (marine, new or established) on behavior.

Fig S1. Map showing locations where sticklebacks were collected.

Fig S2. Average total time fanning across the nesting cycle for marine, "new" freshwater, and "established" freshwater populations.

Fig S3. Average time fanning across the nesting cycle for marine, "new" freshwater, and "established" freshwater populations that experienced the live dragonfly naiad.

Supporting Information