

Plastic responses to parents and predators lead to divergent shoaling behaviour in sticklebacks

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Abstract

Population divergence in antipredator defence and behaviour occurs rapidly and repeatedly. Genetic differences, phenotypic plasticity or parental effects may all contribute to divergence, but the relative importance of each of these mechanisms remains unknown. We exposed juveniles to parents and predators to measure how induced changes contribute to shoaling behaviour differences between two threespine stickleback species (benthics and limnetics: *Gasterosteus* spp). We found that limnetics increased shoaling in response to predator attacks, whereas benthics did not alter their behaviour. Care by limnetic fathers led to increased shoaling in both limnetic and benthic offspring. Shoaling helps limnetics avoid trout and avian predation; our results suggest that this adaptive behaviour is the result of a combination of paternal effects, predator-induced plasticity and genetic differences between species. These results suggest that plasticity substantially contributes to the rapid divergence in shoaling behaviour across the post-Pleistocene radiation of sticklebacks.

Introduction

In geographical widespread species, divergence in antipredator behaviour occurs rapidly between populations and tracks variation in primary predators (Huntingford & Wright 1994; Magurran *et al.*, 1995; Magurran, 1999). For example, in Trinidadian guppies, schooling and predator inspection behaviour have diverged between populations with different predation regimes in < 100 generations (Magurran *et al.*, 1992). Substantial previous work has documented that population differences in antipredator behaviour are genetically based (Seghers, 1974; Breden *et al.*, 1987; Magurran & Seghers, 1990). However, in some species, predator attacks can induce changes in prey behaviour indicating plasticity in response to predators (Magurran, 1990; Kelley & Magurran, 2003; Losos *et al.*, 2004). For instance, fish that experience a predator attack form schools more frequently (Magurran, 1990; Huizinga *et al.*, 2009).

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Additionally, the magnitude of these environmentally induced responses can differ between populations. In minnows, experiencing a predator attack enhances schooling differences: high predation minnows school even more; low predation minnows do not alter their behaviour (Magurran, 1990). Thus, genotype by environment interactions appear to underlie these population differences between high and low predation populations.

Therefore, evolved genetic differences between populations may only be one factor contributing to rapid divergence in antipredator behaviour. Phenotypic plasticity, the ability of a single genotype to produce different phenotypes depending on the environment, is likely to be another factor, because predator attacks frequently produce changes in morphology and behaviour (Schlichting & Pigliucci, 1998; Relyea, 2001; Bell *et al.*, 2011; Dennis *et al.*, 2011). Plastic changes in phenotype can also be induced specifically by parents. Maternal manipulation of offspring environment can produce changes in offspring phenotype through hormonal transfer to embryos or behavioural interactions with young (Mousseau & Fox, 1998; Rasanen & Kruuk, 2007; Uller, 2008; Duckworth, 2009; Ruuskanen & Laaksonen, 2010). In species where fathers contribute to parental care, father-offspring interactions can also induce phenotypic

changes (Tulley & Huntingford, 1987; Huntingford *et al.*, 1994; Frazier *et al.*, 2006). Parental effects and predator-induced plasticity could alter the evolution of anti-predator behaviour and the stability of predator-prey dynamics (Kirkpatrick & Lande, 1989; Paenke *et al.*, 2007; Yamamichi *et al.*, 2011). In particular, environmentally induced effects can potentially slow genetic divergence between populations if plastic responses alone can produce an optimal phenotype (Ancel, 2000; Paenke *et al.*, 2007).

However, recent work has proposed that plasticity can promote adaptation and population divergence, rather than hinder it. In particular, plasticity has been hypothesized to bring populations in proximity to a new adaptive peak rapidly when populations colonize new habitats (Borenstein *et al.*, 2006; Sutter & Kawecki, 2009). Thus, plasticity may allow persistence in the new environment long enough for genetic evolution to occur. By contributing to the phenotype, plasticity can also alter the selective pressures on genes, reducing the size of mutations needed to produce an optimal phenotype (Price *et al.*, 2003; Ghalambor *et al.*, 2007). The idea that plasticity promotes population divergence has received substantial theoretical support, but empirical support remains sparse (Ghalambor *et al.*, 2007; Pfennig *et al.*, 2010).

Therefore, to understand the relative contributions of predator-induced plasticity, parental effects and evolved genetic differences to population divergence in antipredator behaviour, we need studies that measure the contributions of all three to a given phenotype. Furthermore, we need to measure the effects of plasticity in populations that have been separated for different periods of time. Most of the work on phenotypic plasticity and divergence has been carried out on transplanted populations over relatively short time scales (Magurran *et al.*, 1992; Losos *et al.*, 2004). Plasticity may contribute early in divergence, but its effects on phenotype may be short-lived and genetic evolution may play a greater role over longer time scales. However, ancestral plasticity may be maintained if there is little cost to plasticity (DeWitt *et al.*, 1998; Auld *et al.*, 2010). In our study, we address these concerns by testing how predator-induced plasticity and parent-offspring interactions contribute to grouping behaviour differences between two stickleback species with different predation regimes that have diverged in the past 12 000–15 000 years.

Grouping tendency is a key component of antipredator behaviour (Bertram, 1978; Lima & Dill, 1990; Krause & Ruxton, 2002), because being in a group increases the likelihood of detecting predators (Treherne & Foster, 1980) and decreases the chance of attack on any one individual (Hamilton, 1971; Godin, 1986). Previous work on sticklebacks has established that grouping tendency often varies between populations, with some populations not forming groups, some forming loose schools referred to as shoals and some forming tight schools of fish (Doucette *et al.*, 2004; Wark *et al.*, 2011b). Recent work

has established that heritable differences in schooling exist between stickleback populations, suggesting that there is an appreciable genetic component to this behaviour (Wark *et al.*, 2011a). Although predator-induced effects on grouping tendency have been found in several other fish species (Magurran, 1990; Huizinga *et al.*, 2009), they have not been previously documented in sticklebacks. However, father-offspring interactions have been shown to produce differences in stickleback antipredator behaviours such as freezing and predator inspection (Tulley & Huntingford, 1987; Huntingford *et al.*, 1994). Maternal effects on grouping behaviour also occur in sticklebacks: offspring of females exposed to predators form tighter groups than offspring of unexposed females (Giesing *et al.*, 2011). Therefore, we chose to focus on how plasticity induced by predators and parental care contribute to grouping behaviour differences.

We studied grouping behaviour in benthic and limnetic threespine sticklebacks (*Gasterosteus* spp.), pairs of closely related sympatric stickleback species that inhabit six lakes in coastal British Columbia (McPhail, 1994). Genetic, physiological and geological data suggest that these benthic-limnetic pairs evolved from two separate colonizing waves of anadromous threespine sticklebacks (*Gasterosteus aculeatus*) (McPhail, 1984, 1992, 1993, 1994; Kassen *et al.*, 1995; Taylor & McPhail, 1999, 2000; Gow *et al.*, 2008). Benthics are hypothesized to be descended from the initial colonizing population and limnetics descended from later colonists (McPhail, 1993). The rapid evolution of these species appears to be driven by adaptation to the different habitats each species occupies within a lake. Benthics live and feed in the littoral zone of the lake in the cover of vegetation; benthic predators are mainly invertebrates that prey mostly on juvenile benthics (odonate nymphs, backswimmers, diving beetles: Reimchen, 1980; Vamosi, 2002; Marchinko, 2009). Limnetics live and feed in the open water of the pelagic zone where they experience high levels of predation from cutthroat trout and diving birds at all ages (Reimchen & Douglas, 1980; Reimchen, 1990, 1994; Vamosi, 2002; Vamosi & Schluter, 2002). Benthics and limnetics are genetically distinct and strongly reproductively isolated by discrimination against heterospecific mates and ecological selection against hybrid offspring (Schluter, 1995; Rundle *et al.*, 2000; Boughman, 2001; Boughman *et al.*, 2005; Gow *et al.*, 2007).

Corresponding to their predator differences, benthics and limnetics differ in many antipredator traits including body armour and their tendency to form groups. Limnetics have more substantial body armour than benthics, and this armour affects susceptibility to predatory attack (Hagen & Gilbertson, 1973; Reimchen, 1980; Reimchen & Douglas, 1980; McPhail, 1992; Peichel *et al.*, 2001; Marchinko, 2009). Limnetics form large groups, whereas benthics are more solitary (Larson, 1976; Vamosi, 2002). Previous work has documented divergence in many of the genes underlying armour plates

and spines in these species (Peichel *et al.*, 2001; Cresko *et al.*, 2004; Shapiro *et al.*, 2004; Colosimo *et al.*, 2005). This rapid evolutionary change in morphological traits related to predation suggests that there may be corresponding genetic change in behavioural traits. Thus, we can ask how the selective pressures imposed by predation affect the evolution of behaviour in addition to morphology.

Our previous research found that shoaling behaviour differences between benthics and limnetics were reduced in first-generation laboratory-raised fish, so some environmental component is likely to contribute to shoaling (Kozak & Boughman, 2008). Sticklebacks raised in the laboratory lack exposure to predators, and they also are commonly raised without parents. In the wild, stickleback females lay eggs in male nests and males subsequently provide care to embryos, moving oxygen-rich water over the nest and guarding the nest from egg predators (van Iersel, 1953). In the laboratory, embryos created via *in vitro* fertilization and then raised in high oxygen conditions lack this parental care (Hatfield & Schluter, 1999; Barber & Arnott, 2000; Kozak & Boughman, 2008).

We tested the effects of parents and predators on shoaling behaviour in both species. In one experiment, we tested how exposure to trout predators affected shoaling behaviour. Benthics and limnetics were raised in the laboratory without parents. Then, families were split into two groups: one group of siblings experienced simulated attacks by a trout predator over a period of 4 months (starting when fish were 5 months old) and one group of siblings did not. Trout chase their prey (Reimchen, 1990, 1994), and we had a model trout predator chase sticklebacks around their tank. We measured shoaling behaviour after this experience between siblings that had seen a trout predator and those that had not. We predicted that predator exposure would increase shoaling behaviour. If predator-induced plasticity was the primary reason the species differ in behaviour, benthics and limnetics exposed to trout would shoal more than those without exposure. However, if the species showed consistent differences in shoaling, this would suggest a strong genetic component to the behaviour. Furthermore, if siblings with different exposure to predators still showed similar shoaling tendencies, it would suggest a genetic basis, and if families differ from one another, it would suggest that there is a genetic variation for shoaling within species. If benthics and limnetics responded differently to predator exposure, then there may be genetic differences between the species in predator-induced plasticity (a genotype by environment interaction).

In a second experiment, we tested parental effects on shoaling by cross-fostering whole clutches across species. We raised benthics and limnetics without a father, with a limnetic father or with a benthic father. We compared shoaling when fish reached adulthood. We predicted that

those raised with fathers would shoal more than those raised without fathers. Differences between species in response to fathers would suggest that benthic and limnetic offspring respond differently to experience. Alternatively, limnetic fathers may have different effects on offspring behaviour than benthic fathers. By cross-fostering clutches to the other species, we could separate such species-specific paternal effects from maternal and genetic effects.

Materials and methods

Rearing conditions

Benthic and limnetic sticklebacks were bred and raised at the University of Wisconsin-Madison in 2007 and 2008 (IACUC protocol# L00317). All sticklebacks were derived from benthics and limnetics captured in Paxton Lake, British Columbia, in spring 2006, 2007 and 2008. Parents of experimental fish were either first-generation laboratory-raised fish or wild-caught fish that had been kept in the laboratory for at least 1 month (so all eggs were produced in the laboratory in a predator-free environment). All fish were raised in 2007 and 2008 in 109-L tanks that were visually and chemically isolated from other tanks. Fry hatched between April and September; shoaling tendency was tested over the following year. Fish were fed a mixture of brine shrimp (*Artemia* spp.) and bloodworms (*Chironomus* spp.). To mimic seasonal changes in daylight, in October, we began reducing the light cycle by one-half hour per week from 16 to 10 h of light. In March, we began increasing the light cycle back to 16 h of light. Increasing the light cycle stimulates reproduction in sticklebacks; however, we only used pre-reproductive adults in our shoaling tests (individuals without nuptial colour or developing eggs).

Experiment 1: predator exposure

All fish in the predator experiment were raised in 2008 without fathers. We artificially fertilized eggs stripped from females with male sperm in Petri dishes and then transferred them to egg cups with window screen bottoms over airstones (Hatfield & Schluter, 1999). When fry hatched, they fell to the bottom of their tanks. Fish were housed by family until 12 weeks of age. Between 12 and 14 weeks of age (mean \pm SE = 13.43 \pm 0.22 weeks), we took 20 individuals from each family and split them into two 109-L tanks (ten fish each), one that would experience a trout predator and one that would act as a control. We created 12 sets of predator and no predator tanks from 22 source families (ten benthic and 12 limnetic families). Ten individuals from a family of limnetics shared a tank with ten individuals from a family of benthics that were < 21 days different in age. Predator and no predator tanks had the same family composition (i.e. the same limnetic and

benthic family sharing each). Two large benthic families provided offspring for two separate sets, each paired with a different limnetic family; however, one of these additional benthic sets was removed from the final analysis (see below). Benthics and limnetics sharing a tank guaranteed that both species experienced the same predator stimulus, and we controlled for any social experience effects by giving all fish equal experience with each species (Kozak & Boughman, 2008). Benthics and limnetics within a tank were distinguished based on morphological differences in shape, colour and body armour (benthics lack a pelvic girdle) (McPhail, 1992; Shapiro *et al.*, 2004; Kozak & Boughman, 2008).

We tested initial shoaling tendency for two fish per family per tank 6.74 weeks after families were split (range = 3.71–11.14). Fish were still juveniles at this time; mean age of fish at initial testing was 20.17 ± 0.44 weeks. After initial social testing, fish experienced a trout predator or an innocuous object ten times over 15 weeks (12 tanks each; mean age at first exposure = 23.33 ± 0.23 weeks). We gave sticklebacks experience with a predator by presenting them with olfactory, visual and tactile cues associated with a trout attack in their home tank. We used a 25.4-cm-long model rainbow trout (Castaic Swimbaits: Platinum Naturals Trout Body Swimbait) with a double-jointed soft body that moved realistically when placed in flowing water. The head section of the model was coated with trout scent (Pro-Cure Super-Gel Rainbow Trout scent bait). The model trout was then attached to 26-cm-long wooden dowel and chased the sticklebacks around their home tank (making ten full revolutions of the tank per exposure). One of the three rainbow trout models was selected randomly for use, and a single person performed all chases in the experiment. In the wild, stickleback predators are cutthroat trout (*Oncorhynchus clarki*) rather than rainbow trout (*Oncorhynchus mykiss*). However, these two species are very closely related, morphologically similar (differing mostly in coloration and spotting pattern) and often hybridize (Behnke, 1992; Young *et al.*, 2001). Given the similarity between rainbow and cutthroat trout, we feel the use of the commercially available rainbow trout models and scent provided an accurate predator stimulus. For control exposures, we submerged a novel and nonthreatening object into the home tank and held it in a fixed position for 30 s. The control object was a 26-cm-long wooden dowel identical to the one used to guide the trout models. Exposure occurred every 2 weeks for the first five exposures and every week for the last five. Thus, in our experiment, predation rate increased with age. We do not know whether this increase matches the natural situation in benthics and limnetics; however, this does occur in other fish predator–prey systems (Wilbur, 1988). Final social testing occurred within 18 days of the final (10th) exposure to the predator (mean = 9.71 ± 0.97 days). Mean age of fish at final testing was 37.63 ± 0.28 weeks

(range = 36.14–41). We tested two fish per family per treatment (benthics: 11 families; limnetics: 12 families; one of benthic family was removed from the analysis as adults from one tank hid during the shoaling test).

Shoaling tendency test

Shoaling testing was carried out in a large tank that was divided into three compartments: two stimulus compartments (left and right) and a centre focal compartment (with two preference zones and a neutral zone). Window screen mounted on Plexiglas frames separated the compartments, allowing both visual and chemical cues to pass between the focal and stimulus fish. On a transparency taped to the front of the tank, we marked the shoaling preference zones: the area of the focal compartment within two body lengths of each stimulus compartment (6 cm for juveniles, 12 cm for adults), comparable to natural shoaling distances of 2–4 body lengths (Pitcher & Parrish, 1993). In one stimulus compartment, a group of ten individuals was placed; in the other, a single individual. The group of ten was randomly assigned to either the left or right compartment. Stimulus fish were conspecifics that were unrelated and unfamiliar to focal fish. Further details on the shoaling tendency test can be found in Appendix S1 and in the study by Kozak & Boughman (2008).

Focal individuals were captured individually from their home tanks. The focal fish was placed in an opaque cup suspended in the middle of the focal compartment, allowed 120 s to acclimate, then released into the focal compartment. Its movements in and out of each of the preference zones were measured for 600 s. We only used the last 300 s of tests; the first 300 s allowed fish time to explore the tank and fish visited both preference zones at least once in all tests. We calculated shoaling preference as the difference in time spent in the preference zone near the shoal minus the time spent in the zone near the single fish (out of 300 s). We tested two individuals per tank and calculated the mean time spent shoaling for each tank.

We rearranged the stimulus fish between the tests of the first and second focal individuals from each tank. A new stimulus single individual was selected from the stimulus group and placed in the single compartment, whereas the initial single individual was returned to the group; this rotation minimized the effects of the identity of the single fish.

We used the same stimulus fish for each family so both trout and control tanks saw the same stimulus fish. However, we altered the placement of the group of ten: if the group of ten was in the left compartment during control fish tests, trout fish saw the group in the right compartment (assignment of compartments was randomized across families). The placement of the group of ten remained the same between each tank's juvenile and adult tests: if juveniles saw the group of ten on the right,

adults did as well. This was carried out to minimize the effects of any side preferences on differences in shoaling before and after trout exposure. None of the fish in the predator experiment were used as stimulus fish, and no predator cues were present in the testing tank. All stimulus shoals were selected from families raised without fathers.

Sticklebacks prefer to shoal with individuals similar in size (Ranta *et al.*, 1992; Kozak & Boughman, 2008) and dense cohesive groups (Frommen *et al.*, 2009). Therefore, we statistically controlled for size differences by including as a covariate the absolute value of the difference between the mean length of individuals in the shoal and the focal individuals (the standard length of all individuals was measured with vernier callipers after testing). We also scored the cohesiveness of the stimulus shoal using a score that ranged from 1 (individuals as far apart as possible) to 5 (individuals all within one body length of one another) and used it as a covariate.

Statistical analysis

We tested for differences in shoaling after predator exposure using a mixed model ANCOVA with family nested within species as a random factor (using Satterthwaite approximation for degrees of freedom). As fixed factors, we included species (benthic, limnetic), predator (trout, none) and their interaction. Average absolute length difference from the shoal was included as a covariate. To ensure that any differences seen were the result of trout exposure and not random differences among fish, we also analysed initial juvenile shoaling tendencies using a similar model. This also allowed us to determine whether families initially differed in shoaling tendency, but these genetic differences were overwhelmed by predator experience. Shoal cohesiveness was also included as a covariate, but was nonsignificant in both models ($F_{1,39,3} < 0.56$, $P > 0.46$) and removed from the analysis. Statistical analyses were carried out in SAS v.9.1 2007 (SAS Institute Inc., Cary, NC, USA). We made comparisons among least-squared means, and we adjusted P -values for all comparisons using false discovery rate (Benjamini & Hochberg, 1995; Verhoeven *et al.*, 2005). For effect size of fixed effects, we calculated partial eta-squared (partial η^2) and 95% confidence intervals (Smithson, 2003).

Experiment 2: parent exposure

In the parent experiment, families were raised in the laboratory with no father, benthic fathers or limnetic fathers in 2007 and 2008; they received no exposure to predators. A clutch of eggs from one or more females were fertilized with the sperm of a single conspecific male. Within 22 h of fertilization, clutches were placed in a parental exposure treatment. Parental exposures were reared by a limnetic father, reared by a benthic father

and reared without a parent (in an eggcup over an airstone). Details of the fostering procedure are described elsewhere (Kozak *et al.*, 2011). We summarize them here. All foster fathers were males caught in the wild in the year they were used as fathers. Fathers had a female spawn (deposit her eggs) in their nest. If males parented their biological clutch, nothing further was done. In fostered treatments, we removed the nesting tray from the tank, removed the eggs from the male's nest using forceps, replaced the eggs with other recently fertilized eggs and immediately replaced the tray. Fathers interacted with their clutch for 18 days (matching the time period of care from the wild: Kynard, 1978). Eggs typically hatch after 10 days, so during the final 8–9 days offspring were free-swimming fry. After 18 days, we removed fathers from the tank. Our cross-fostering design allowed us to test for differences in paternal effects of benthic and limnetic fathers, although it could not separate genetic from maternal effects.

We tested shoaling of two fish per family from 68 families in total. We tested 11 benthic families raised without fathers, 14 with benthic fathers (eight foster and six biological) and ten with limnetic foster fathers. We tested ten limnetic families without fathers, nine with benthic foster fathers and 14 with limnetic fathers (nine foster and five biological). There were no differences in behaviour between those raised by biological or conspecific foster fathers (see Appendix S1). Fry were kept in their home tank until they were 20 weeks old. Then, density was adjusted to around 0.2 fish per litre. Adult shoaling tendency was tested when fish were 35.59 ± 0.51 weeks old (range = 25.7–44.1; February–April in 2008 and 2009).

Methods of testing shoaling tendency were identical to those described for the predator experiment. However, for parent exposure fish, the same stimulus fish in the same position were only used for one family per father exposure. In 2007, all stimulus shoals were selected from families raised by conspecific fathers owing to availability. In 2008, stimulus shoals were selected from families raised without fathers. Stimulus limnetics in 2007 did form more cohesive shoals than stimulus limnetics in 2008 (limnetics: 2007 = 4.08, 2008 = 2.99, $t_{78} = 5.79$, $P < 0.001$; benthics: 2007 = 3.5, 2008 = 3.35, $t_{78} = 0.99$, $P = 0.33$). Therefore, we included shoal cohesiveness as a covariate in our analyses for this experiment.

We analysed the mean shoaling of each family in an ANCOVA. We included offspring species (benthic, limnetic), father identity (benthic, limnetic, none) and their interaction. Length difference and shoal cohesiveness included were covariates. Because we were particularly interested in the ability of conspecific fathers to cause divergence in behaviour, we compared species differences (limnetic shoaling minus benthic shoaling) for fish raised by conspecific, heterospecific or no father using a similar model with these father classifications.

Results

Predators and shoaling behaviour

Predator exposure produced divergent shoaling behaviour in benthics and limnetics (Table 1). Prior to predator exposure, fish in the trout exposure treatment tended to shoal slightly less than control fish (trout vs. control: limnetics = -58.11 ± 34.41 , $t_{20.8} = 1.69$, $P = 0.11$, $P_{\text{FDR}} = 0.14$; benthics = -66.84 ± 35.96 , $t_{20.9} = 1.86$, $P = 0.077$, $P_{\text{FDR}} = 0.14$; Fig. 1a), and there were no differences between species in either treatment group (control limnetics vs. benthics: -63.73 ± 37.95 , $t_{40.4} = 1.68$, $P = 0.10$, $P_{\text{FDR}} = 0.14$; trout limnetics vs. benthics: -55.00 ± 37.99 , $t_{40.4} = 1.45$, $P = P_{\text{FDR}} = 0.16$). After exposure, trout-exposed limnetics shoaled significantly more than trout-exposed benthics (142.80 ± 47.18 , $t_{38.2} = 3.03$, $P = 0.004$, $P_{\text{FDR}} = 0.018$; Fig. 1b). Trout-exposed limnetics also shoaled more than their siblings that were not exposed to trout (109.66 ± 42.43 , $t_{20.9} = 2.58$, $P = 0.017$, $P_{\text{FDR}} = 0.035$). Family origin had little effect on shoaling tendency before or after testing (before: $\sigma^2 = 1160.61 \pm 1785.70$, Wald Z-test = 0.65, $P = 0.51$; after: $\sigma^2 = 96.70 \pm 2743.66$, $Z = 0.04$, $P = 0.97$). Furthermore, differences in shoaling tendencies cannot be attributed to differences in body size: there were no differences in length between individuals of the same species in the trout and control treatments (benthic adults: control = 55.36 mm, predator = 56.76 mm, control vs. trout $t_{20} = -0.93$, $P = 0.36$; limnetics: control = 44.59 mm, predator = 44.76 mm, control vs. trout $t_{22} = -0.11$, $P = 0.91$).

Table 1 Analyses of shoaling behaviour before and after predator exposure with effect sizes (η_p^2) and 95% confidence intervals (CI) lower and upper bounds shown for fixed effects.

	d.f.	F	P	η_p^2	95% CI
Before exposure					
Predator	1,20.9	6.30	0.020	0.23	0.00–0.48
(none, trout)					
Species	1,21.4	4.28	0.051	0.17	0.00–0.42
(benthic, limnetic)					
Predator × species	1,20.8	0.03	0.86	0.001	0.00–0.13
Length difference	1,37.7	0.34	0.56	0.009	0.00–0.14
from shoal					
Family	21,20	1.32	0.27		
After exposure					
Predator	1,19.5	1.31	0.27	0.06	0.00–0.31
(none, trout)					
Species	1,15.7	4.43	0.052	0.22	0.00–0.50
(benthic, limnetic)					
Predator × species	1,23.1	5.68	0.026	0.20	0.001–0.44
Length difference	1,23.3	1.62	0.22	0.07	0.00–0.30
from shoal					
Family	21,20	1.02	0.49		

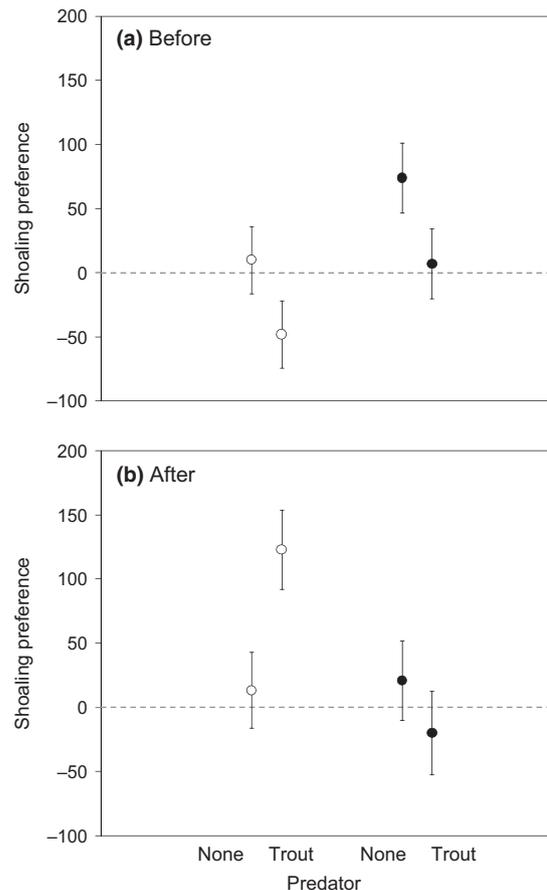


Fig. 1 Shoaling behaviour changes with predator exposure. Least-squared mean shoaling tendencies (\pm SE) are shown for benthics and limnetics in each predator exposure treatment. Benthic offspring are represented by filled symbols, and limnetics by open. (a) Before predator exposure; (b) after predator exposure.

Parents and shoaling behaviour

Only offspring raised by limnetic fathers showed a significant preference for the shoal (64.42 ± 24.39 , $t_{62} = 2.64$, $P = 0.01$) and shoaled significantly more than those offspring raised by benthic fathers (92.39 ± 35.04 , $t_{62} = 2.63$, $P = 0.011$, $P_{\text{FDR}} = 0.03$; Table 2; Fig. 2). Fish did not show any significant shoaling tendency when raised by benthic fathers (-27.96 ± 24.93 , $t_{62} = -1.12$, $P = 0.27$) or without fathers (23.29 ± 26.1 , $t_{62} = 0.89$, $P = 0.38$). The effect of fathers was similar for limnetic and benthic offspring (species by father interaction was nonsignificant and removed from the model: $F_{2,60} = 1.29$, $P = 0.28$). We compared shoaling patterns between the species when fish were raised with a conspecific father as opposed to without a father and found that conspecific fathers drastically altered shoaling (conspecific vs. no father = 176.04 ± 70.20 , $t_{60} = 2.51$, $P = 0.015$, $P_{\text{FDR}} = 0.03$). Thus, species-typical shoaling

Table 2 Analysis of parental effects on shoaling behaviour with effect sizes (η_p^2) and 95% confidence intervals (CI) lower and upper bounds shown.

	d.f.	<i>F</i>	<i>P</i>	η_p^2	95% CI
Father (none, benthic, limnetic)	2,62	3.49	0.037	0.10	0.00–0.23
Species (benthic, limnetic)	1,62	1.09	0.30	0.02	0.00–0.12
Length difference from shoal	1,62	1.54	0.22	0.02	0.00–0.13
Shoal cohesiveness	1,62	1.97	0.17	0.03	0.00–0.14

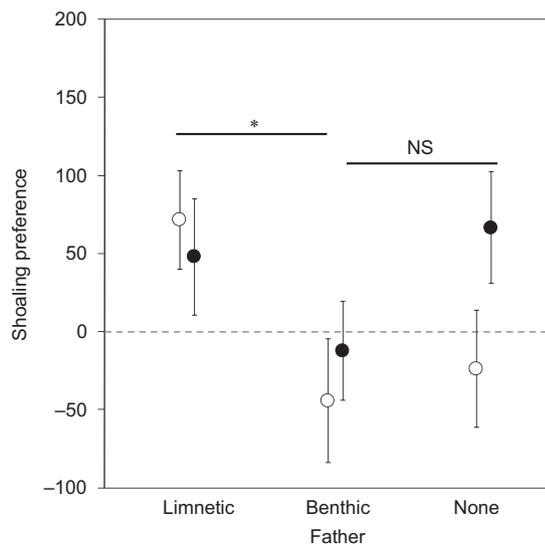


Fig. 2 Shoaling behaviour differs based on father. Least-squared mean shoaling tendencies (\pm SE) are shown for each father exposure and offspring species. Benthic offspring are represented by filled symbols, and limnetics by open. Differences in means from zero (no preference): limnetic offspring raised by limnetic father ($t_{60} = 2.27$, $P = 0.03$), benthic father ($t_{60} = -1.11$, $P = 0.27$), no father ($t_{60} = -0.63$, $P = 0.53$); benthic offspring raised by limnetic father ($t_{60} = 1.29$, $P = 0.20$), benthic father ($t_{60} = -0.38$, $P = 0.70$), no father ($t_{60} = 1.88$, $P = 0.07$). Significance of comparisons between father exposures is shown above (P -values adjusted using FDR). * $P < 0.05$, NS = nonsignificant.

behaviour is due in part to the divergent effects of limnetic and benthic fathers on their offspring.

Discussion

Our results show that antipredator behaviour differences in sticklebacks result from both paternal effects and predator-induced plasticity and that these plastic responses interact with genetic differences between species. Benthics and limnetics differed in their response to trout attacks. Trout predators dramatically increased shoaling in limnetics but had little effect on benthics. In

addition, benthic and limnetic fathers had different paternal effects on offspring shoaling behaviour. Limnetic fathers increased shoaling behaviour, whereas benthic fathers tended to decrease it, suggesting that there are important differences in how limnetic and benthic fathers care for fry and fathers can prime offspring to express the appropriate shoaling behaviour. In the wild, limnetics typically are raised by a conspecific father and experience trout predation: this may explain why wild-caught limnetics have higher shoaling tendencies than benthics (Vamosi, 2002).

Plasticity in antipredator behaviour may be of widespread importance in the post-Pleistocene freshwater radiation of sticklebacks. In conjunction with previous work on plasticity in predation inspection (Tulley & Huntingford, 1987; Huntingford *et al.*, 1994), predator-induced changes in courtship (Candolin, 1998; Shaw *et al.*, 2007) and predator-induced maternal effects (Giesing *et al.*, 2011), our results suggest that plasticity helps sticklebacks adjust their behaviour to the current predation regime. Predation in freshwater lakes and streams varies greatly over space and time (Reimchen, 1990, 1994). Therefore, predator and parent-induced plasticity in antipredator behaviour may have played a critical role in the ability of anadromous sticklebacks to invade and persist in novel freshwater habitats (Borenstein *et al.*, 2006). Although few studies have focused on plasticity in antipredator behaviour in anadromous sticklebacks, the existence of predator and parent-induced effects in multiple freshwater descendent populations (from the American Pacific coast to Northern Europe) strongly suggests that plasticity in antipredator behaviour exists in all anadromous threespine stickleback populations. The rapid pace of divergence between freshwater and anadromous populations of sticklebacks is thought to have been facilitated by both standing genetic variation and phenotypic plasticity in morphology (Day *et al.*, 1994; Cresko *et al.*, 2004; Colosimo *et al.*, 2005; Barrett *et al.*, 2008; Wund *et al.*, 2008); our results suggest that plasticity in antipredator behaviour may have contributed as well.

On the basis of theoretical and empirical work, it has been hypothesized that plasticity is most likely to facilitate adaptation in new environments when plastic responses move populations in the range of an adaptive peak, but not all the way to the optimum (Price *et al.*, 2003; Ghalambor *et al.*, 2007). Our finding that plasticity by itself cannot produce species-typical shoaling behaviour in benthics and limnetics (because benthics do not respond to trout predation by shoaling) is consistent with this hypothesis that plasticity may facilitate behavioural adaptation rather than shield populations from the effects of selection. For example, colonization of the benthic habitat by sticklebacks may have been accompanied by reduced shoaling behaviour because benthic vegetation provides substantial cover and lower encounter rates with trout (Vamosi & Schluter, 2002). Over time, benthic predators such as odonate nymphs (which are primarily

stalking ambush predators rather than chasing predators) may have exerted selection for further genetically based reductions in shoaling behaviour (Pritchard, 1965; Reimchen, 1980; Magurran & Seghers, 1990). In support of the idea that genetic differences contribute to reduced benthic shoaling behaviour, other recent work comparing benthics to anadromous sticklebacks found heritable differences in the latency to join a model school and amount of time spent with a school (Wark *et al.*, 2011a).

Previous work has supported the role of phenotypic plasticity in initial phenotypic divergence after introduction to a new habitat (Magurran *et al.*, 1992; Losos *et al.*, 2004), but our results suggest that plasticity can continue to contribute to divergence even after significant periods of time (more than 10 000 generations) and after considerable genetic differentiation between species. It has been hypothesized that the maintenance of plasticity could be costly and might be selected against over long time scales in relatively stable environments (van Tienderen, 1991; DeWitt *et al.*, 1998; Scheiner & Berrigan, 1998). However, fluctuations in the environment across generations, such as variation in predator densities, might make plasticity in antipredator behaviour more likely to be maintained (Via & Lande, 1985; Kelley & Magurran, 2003; Stomp *et al.*, 2008; Mery & Burns, 2010). Additionally, behavioural traits are more inherently labile than morphological ones and seem to be more likely to maintain a degree of plasticity (Ghalambor *et al.*, 2010). For example, in contrast to our results on antipredator behaviour, differences in morphological antipredator defences between stickleback populations appear primarily due to changes in allele frequencies of armour genes and mutations in regulatory genes (Peichel *et al.*, 2001; Cresko *et al.*, 2004; Shapiro *et al.*, 2004; Colosimo *et al.*, 2005; Barrett *et al.*, 2008; Marchinko, 2009).

Our finding that offspring raised by benthic fathers behaved differently than those raised by limnetics indicates paternal care differs between species. We did observe that limnetic fathers spent more time near fry than benthic fathers 8 days after hatching (benthic father time = 149 ± 26.4 s, limnetic father time = 242.14 ± 33.29 s, benthic vs. limnetic: $F_{1,26} = 4.74$, $P = 0.039$; see Appendix S1). However, further work is needed to identify the extent of species differences and whether these differences in care are heritable or are themselves plastic responses. For example, the predation environment which fathers experience could affect parental care behaviour, with fathers from a high predation environment behaving differently than those from low predation environments. However, other factors, such as the risk of egg cannibalism, could have led to changes in paternal behaviour. Limnetic fathers face a greater risk of cannibalism because they nest in close proximity to other males and are subject to raiding groups of benthic females that prey specifically on limnetic nests (Ridgway & McPhail, 1987, 1988; Foster, 1988; Foster & Baker, 1995). This hypothesis that the risk of cannibalism selects

for differences in care behaviour could be tested by comparing parental care and cannibalism rate differences across lakes.

Parental care differences and their subsequent effects on offspring may be an important factor contributing to behavioural divergence between populations. The effects of parental care on offspring behaviour are pervasive in vertebrates, altering grouping behaviour (this study), predator inspection (Tulley & Huntingford, 1987; Huntingford & Wright, 1994; Huntingford *et al.*, 1994), aggression (Frazier *et al.*, 2006; Arnold & Taborsky, 2010), stress response (Liu *et al.*, 1997) and maternal behaviour (Boccia & Pedersen, 2001). Therefore, even slight differences in parental behaviour between populations may have significant consequences for offspring behaviour. However, little attention has been paid to this link between parental and offspring behaviour in the context of divergence and speciation, despite a great deal of work on parental effects on divergence (Rasanen & Kruuk, 2007).

The potential for parental effects to interact with predator-induced plasticity later in life suggests that plasticity may play a greater and potentially more complex role in antipredator behaviour divergence than previously thought. We found that experience with a father early in life and with predators throughout development altered adult shoaling behaviour. Maternal transmission of hormonal substances to embryos is a further way in which parents can affect offspring antipredator behaviour. Although we did not examine maternal effects in our study, in other populations of sticklebacks, maternal exposure to predators induces changes in the level of cortisol in eggs and increases fry shoaling behaviour (Giesing *et al.*, 2011). Therefore, grouping behaviour appears to depend not only on the predator and parental environment the offspring experience but also on the predator environment which the parents experience. Such multigenerational environmental effects may frequently interact to create behavioural divergence between populations in the wild; however, they may also create complex evolutionary dynamics, such as time lags in adaptation (Kirkpatrick & Lande, 1989). Currently, we lack an integrated understanding of the consequences when trait induction depends on parental hormonal transmission, parental care behaviour and predator exposure. Such complex environmental determination of traits may alter the extent of adaptation, population divergence and evolutionary change.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Materials and methods.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

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