TITLE: The evolution of anti-predator behaviour following relaxed and reversed selection in Alaskan threespine stickleback fish

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1 Changing environments, whether through natural or anthropogenic causes, can lead to the loss
2 of some selective pressures (“relaxed selection”) and possibly even the reinstatement of
3 selective agents not encountered for many generations (“reversed selection”). We examined
4 the outcome of relaxed and reversed selection in the adaptive radiation of the threespine
5 stickleback fish (*Gasterostues aculeatus* L.), in which isolated populations encounter a variety of
6 predation regimes. Oceanic stickleback, which represent the ancestral founders of the
7 freshwater radiation, encounter many piscivorous fish. Derived, freshwater populations, on the
8 other hand, vary with respect to the presence of predators. Some populations encounter native
9 salmonids, whereas others have not experienced predation by large fish in thousands of
10 generations (relax-selected populations). Some relax-selected populations have had sport fish,
11 including rainbow trout (*Oncorhynchus mykiss*), introduced within the past several decades
12 (reverse-selected). We examined the behavioural responses of stickleback from three
13 populations of each type to simulated attacks by trout and birds to determine whether relaxed
14 and reversed selection has led to divergence in behaviour, and whether this divergence was
15 predator-specific. Fish from trout-free populations exhibit weak responses to trout, as predicted,
16 but these responses are similar to those of oceanic (ancestral) populations. Fish from
17 populations that co-occur with trout, whether native or introduced, show elevated anti-predator
18 responses, indicating that in freshwater, trout predation selects for enhanced anti-predator
19 responses, which can evolve extremely rapidly. Comparison of laboratory-reared and wild-
20 caught individuals suggests a combination of learned and genetic components to this variation.
21 Responses to a model bird fly-over were weakly linked to predation environment, indicating that
22 the loss of predation by trout may partially influence the evolution of responses to birds. Our
23 results reject the hypothesis that the consistent presence of predatory birds has been sufficient
24 to maintain responses to piscivorous fish under periods of relaxed selection.

25 *Keywords*

26 anti-predator behaviour, *Gasterosteus*, relaxed selection, reversed selection, stickleback, trout

27 Changing environments, whether due to natural processes or anthropogenic activity, are a
28 ubiquitous challenge for natural populations. Environmental modification can introduce new
29 selective agents, and also can result in the reduction or loss of others, leading to “relaxed
30 selection” on traits associated with a former selective agent (Lahti et al., 2009). Whether or not
31 traits persist, become vestigial, or completely degrade during episodes of relaxed selection,
32 depends upon a complex set of factors. These include, but are not limited to, the maintenance
33 of alternative selection pressures favoring trait persistence, correlations with other traits
34 maintained by their own selective agents, the presence and nature of costs associated with trait
35 development and maintenance, the degree to which trait expression is plastic versus
36 constitutive and the rate of mutational decay (reviewed in Fong, Kane, & Culver, 1995; Lahti et
37 al., 2009). Evaluating the outcome of relaxed selection not only allows us to gain insight into the
38 nature of the adaptive process, but also helps us predict how a population will respond to future
39 changes. The degree to which an adaptation has decayed following relaxed selection has
40 important consequences should the original selection pressure be restored (“reversed
41 selection”), or should a similar selective agent arise (Lahti et al., 2009).

42 The loss or reduction of predation is one of the most common contexts in which the
43 consequences of relaxed selection has been evaluated (Berger, Swenson, & Persson, 2001;
44 Bleakley, Martell, & Brodie, 2006; Blumstein & Daniel, 2002; Blumstein, Daniel, Griffin, & Evans,
45 2000; Fullard & Ratcliffe, 2006; Fullard, Ratcliffe, & Soutar, 2004; Runemark, Brydegaard, &
46 Svensson, 2014). Variation in predation among prey populations is relatively easy to identify
47 (particularly at the level of presence or absence of predators; but see Thomas E. Reimchen,
48 1994), as are adaptations whose primary function is to avoid predation. Some examples in
49 which anti-predator adaptations have been reduced or lost following relaxed selection by a
50 subset of predators include anti-predator responses in island-dwelling wallabies (Blumstein &
51 Daniel, 2002; Blumstein et al., 2000), reduction in tail autotomy in lizards living in the absence of
52 vipers (Pafilis, Foufopoulos, Poulakakis, Lymberakis, & Valakos, 2009), and the decline of the

acoustic startle response in noctuid moths no longer subject to bat predation (Fullard & Ratcliffe, 2006; Fullard et al., 2004).

When the costs of trait production and maintenance are high, relaxed selection is generally expected to lead to trait degradation. However, behavioural defenses, like any plastic trait, might only incur costs in the context of an inducing stimulus; when the predator is not encountered the behaviour is not expressed, and thus the costs of responding are not incurred. This reduction in realized costs might cause behavioural adaptations to persist longer under relaxed selection than morphological adaptations (Edelaar, Piersma, & Postma, 2005; Lahti et al., 2009). The outcome of relaxed selection on behaviour becomes even more difficult to predict when the possibility of learning is taken into account. When a predator is removed and prey no longer exhibit a predator-specific response, does this loss represent the evolutionary (genetic) loss of the capacity to produce the behaviour, the lack of opportunity to learn to produce the behaviour, or some combination of learned and genetic mechanisms?

A number of studies have investigated variation in anti-predator responses of fish that encounter either high or low levels of predation by larger, piscivorous fish. Guppies (*Poecilia reticulata*) in Trinidad are a model system in this regard. Guppies that live upstream of waterfalls experience fewer predatory fish than those populations that live downstream, which can be accessed by predators such as the pike cichlid (*Crenicichla alta*). Upstream, low predation populations typically show reduced anti-predator responses relative to their high predation, downstream relatives (Huizinga, Ghalambor, & Reznick, 2009; Kelley & Magurran, 2003a; Seghers, 1974). Experimental transplants from high to low predation sites demonstrate significant evolutionary declines in anti-predator behaviour over the course of only a few decades (Magurran, Seghers, Carvalho, & Shaw, 1992). When the behaviour of laboratory-bred (and thus predator-naïve) individuals has been evaluated, some of the differences observed among wild-caught individuals are maintained, indicating a genetic response to

78 relaxed selection (Bleakley et al., 2006), but learning is clearly important as well (Kelley &
79 Magurran, 2003a).

80 Threespine stickleback fish (*Gasterosteus aculeatus*) are also a valuable model for
81 evaluating the causes of variation in anti-predator behaviour, particularly with respect to the role
82 of relaxed selection from predators (reviewed in F. Huntingford & Coyle, 2007; F. A.
83 Huntingford, Wright, & Tierney, 1994). Similar to guppies, Scottish freshwater populations from
84 low predation sites exhibit reduced anti-predator responses relative to stickleback from a high
85 predation site (Giles & Huntingford, 1984; F. A. Huntingford et al., 1994). (Bell & Foster, 1994)
86 These differences in anti-predator responses extend more generally to boldness-
87 aggressiveness behaviour. For example, wild-caught, juvenile stickleback from high predation
88 populations exhibit stronger correlations among boldness and aggressive behaviours than do
89 stickleback from low predation populations (Dingemanse et al., 2007). Of course, differences
90 among wild caught fish might be due to experience rather than or in addition to genetic
91 divergence, and comparisons among populations reared under controlled conditions are
92 especially helpful in disentangling the relative roles of learning and genetic divergence (Kelley &
93 Magurran, 2003b). However, even the effects of learning can diverge among populations—for
94 example, stickleback from one high predation population in the United Kingdom require early
95 experience with their fathers in order to fully develop their anti-predator responses, whereas
96 parental care has no such effect on the development of anti-predator behaviour in one low
97 predation population (F. A. Huntingford et al., 1994; Tulley & Huntingford, 1987).

98 In this study, we investigate the outcomes of both relaxed and reverse selection in
99 response to altered predation environments in Alaskan freshwater populations of threespine
100 stickleback fish. Many freshwater populations in Alaska lack native predatory fish, and thus
101 experience relaxed selection compared to the ancestral condition; in a subset of these
102 populations, predatory fish have been reintroduced, providing the opportunity to study reverse
103 selection within this system as well. We specifically addressed four questions. First, does

relaxed selection from piscivorous fish lead to reductions in anti-predator behaviour? Second, does the re-imposition of these predators due to game fish stocking lead to the re-emergence of ancestral anti-predator responses? Third, do changes in behaviour associated with the presence or absence of predatory fish influence responses to other predators, such as birds, which are ubiquitous? Fourth, are differences in behaviour observed among populations of wild-caught stickleback maintained in laboratory-reared fish, indicating genetic divergence, or instead does a lack of experience lead to a general reduction in anti-predator behaviour regardless of natural predation pressure?

This experiment is an extension of our previous work, in which we demonstrated that relaxed selection by predatory fish was associated with moderately weaker anti-predator responses, and that reverse selection following the introduction of rainbow trout (*Oncorhynchus mykiss*) was associated with much greater anti-predator responses than those exhibited by stickleback in either the relax-selected or marine (ancestral) population (Messler, Wund, Baker, & Foster, 2007). The present study expands upon (2007) this work in three important ways. First, we consider the behaviour of both wild-caught and laboratory-reared individuals to evaluate the possibility that differences in experience lead to differences in behaviour. Second, we evaluate the responses of stickleback from freshwater lakes where trout are native in order to determine how the behaviour of freshwater stickleback might differ from that of their oceanic ancestors even when selection by predatory fish has been maintained. Finally, in the present study, we examine three replicate populations within each predation environment to draw more robust conclusions.

129 METHODS

130 *Field Collection and Populations of Study*

131 Threespine stickleback were collected using unbaited 3- and 6-mm mesh minnow traps
132 each June from 2005 – 2010. Fish were collected in 12 locations in south-central Alaska (the
133 Matanuska-Susitna River valley and Kenai Peninsula): three widely separated sites where
134 oceanic (marine or anadromous) stickleback were breeding, three lakes where piscivorous fish
135 are native, three lakes where piscivorous fish are absent, and three lakes where piscivorous fish
136 are naturally absent, but to which rainbow trout (*Oncorhynchus mykiss*) have been routinely
137 stocked by the Alaska Department of Fish and Game for the past several decades (Table 1).
138 Rainbow trout are native to the region, however, they do not naturally occur in the many kettle
139 lakes in the area that lack inlet or outlet streams. Minnow traps were set for 2 to 24 hours at
140 each site. Live fish were transported to the University of Alaska Anchorage in 45 L coolers (~ 30
141 L H₂O) equipped with air stones, where they were maintained in an outdoor fish husbandry
142 facility in large, shaded wading pools or cattle tanks filled with dechlorinated tap water with 3ppt
143 Instant Ocean® sea salt. Branches were placed in the holding tanks to provide habitat
144 enrichment. Populations were maintained in separate pools. Data for the 2005 wild-caught
145 populations are reported elsewhere (Messler et al., 2007), but those data are included here as
146 part of this larger study.

147

148 *Production of Population Crosses and Captive Maintenance*

149 Wild-caught fish were used for one of two purposes: either directly as subjects in the
150 behavioural experiments (see below), or to produce offspring that would be reared in the
151 laboratory and used in behavioural experiments the following spring. Thus, both wild-caught and
152 laboratory-reared fish from each population were used in all experiments. In all cases, non-
153 reproductive individuals were used in experiments to avoid any effects that motivation to breed

might have on anti-predator responses. Reproductive status was judged by a lack of nuptial colouration in males and a lack of abdominal distension in females.

Crosses for laboratory rearing were made using our standard procedures (Wund, Valena, Wood, & Baker, 2012 for details). Males were euthanized in an overdose of buffered MS-222, their testes were removed and macerated. Eggs were gently extruded from females, who were then euthanized in MS-222. Sperm from the macerated testes was spread over the eggs and fertilization took place over the next two minutes. Clutches of embryos were then repeatedly rinsed in distilled water with 6 ppt Instant Ocean®, and then maintained in that solution in Petri dishes. Each day, dead embryos were removed and the water was changed. Within 2-3 days post-fertilization, clutches were rinsed in a 1% iodine solution, and within 5 days post-fertilization, they were shipped overnight to Clark University in coolers containing ice packs. Once at Clark University, embryos, and subsequently fry, were reared first in Petri dishes and then in 1 L jars, with regular water changes. They were maintained on a diet of brine shrimp nauplii (platinum grade®, Argent Chemical Laboratories). At about 30 days post hatching, fry were transferred to 37-L aquaria in a recirculating husbandry system filled with dechlorinated tap water maintained at 3 ppt sea salt (1 full-sib family per aquarium, eventually attaining a density of 10 – 12 adult fish per tank). The husbandry facility was maintained at 17.5°C on an 8 hr light : 16 hr dark cycle to prevent fish from entering breeding condition. Once stickleback were large enough to consume them, they were maintained on a diet of frozen blood worms (chironomid larvae) and frozen adult brine shrimp. Fish from 2005-2009 were reared at Clark University, and fish in 2010 were reared at The College of New Jersey, which has a husbandry facility identical to that at Clark University.

Experimental Procedures

Subjects to be tested on a given day were held overnight in a holding tank in the testing room, either collectively by population (all wild-caught subjects), collectively by family (lab-reared subjects 2005, 2011) or individually (2007 – 2009). Fish in 2007 – 2009 were held individually overnight because they were used in another experiment in which their activity in the holding tank was passively monitored two hours before anti-predator trials. These fish were held overnight in a mesh cylinder (7 cm diameter x 25 cm height) within a 37-L aquarium, and then released into the aquarium the following morning where they swam freely for the next two hours with no other disturbance. All holding tanks (individual or collective) were enriched with gravel substrate and a plastic plant. We used general linear models to determine whether being held individually or collectively impacted subsequent anti-predator behaviour. The logistics of obtaining live fish prevented us from testing fish from all populations in all years, but three populations were used in both types of holding conditions, and were thus included in this analysis (Anchor River, Rabbit Slough, Willow Lake; Table 1). Predator, population and acclimation conditions (alone or in a group) were used as fixed factors in the models. Neither log-transformed latency to recovery from an attack ($F_{1, 152} = 2.57$, $P = 0.111$, partial $\eta^2 = 0.010$) nor log-transformed time spent frozen ($F_{1, 152} = 0.022$, $P = 0.883$, partial $\eta^2 < 0.001$) differed between holding conditions. Once a fish was ready for testing, it was transferred individually to either the trout model or tern model arena (see below). Trials would commence between 15 and 60 minutes after the focal fish began freely exploring the arena. Variation in acclimation time resulted if trials in other arenas happened to take extended periods of time. All arenas (two per predator treatment) were visually isolated from one another. Each fish was tested once in only one of the two predator experiments. Approximately 30 fish per population, per rearing history (lab vs. wild) were tested in each condition. See Table 1 for exact sample sizes.

Simulated Trout Attack

Exposure to a simulated trout attack took place in a 150-L aquarium (92 cm x 32 cm x 43 cm tall) filled with 110 L of dechlorinated tap water at 3 ppt salinity. The tank was divided into a front compartment for the focal individual and a back compartment for a model trout, which remained hidden from view until the “attack.” The focal fish arena had a gravel substrate. A moveable, clear plastic partition prevented the focal fish from entering the trout chamber. A 7-cm tall feeding tray was placed in center of focal fish compartment (Fig. 1A). The model trout (33.5 cm long) was made from fiberglass and painted realistically. It was suspended just above the water level of its compartment, out of view of the focal fish. When the trout was activated via a long rope, it would swing down into the water, rapidly passing through the middle of the aquarium (and right past the focal fish), and then up and out of the water at the other end of the aquarium. By positioning the trout's snout just at the water surface, we eliminated any splashing that might otherwise scare the focal fish. Black plastic shields allowed the stickleback to view only the portion of the trajectory in which the trout model was moving nearly horizontally through the center of the aquarium. This was considered a realistic approximation of attacks by trout as *in situ* observations demonstrate that trout attack quickly, and then swim rapidly from sight (S. Foster & Ploch, 1990). In the 2005 wild-caught trials detailed in (Messler et al., 2007), the trout did not leave the view of the focal stickleback, potentially leading to difference in behaviour between those fish and the rest of the fish in this study. The data were thus analysed with and without these subjects (see below).

Each trial began with the addition of several bloodworms to the feeding tray. The observer sat 1.5 metres from the arena and remained stationary. Once the stickleback was positioned at the feeding tray, the partition between the focal fish compartment and trout compartment was gently slid aside, via a long pole attached to plastic rod that was affixed to a rod on the top of the partition. The removal of the partition enabled focal fish to not only see the onrushing trout, but to also detect its motion. If a subject did not feed for 10 minutes, more food

was added near the fish to encourage feeding and exploration. If they did not eat this food, but freely explored the tank, the partition was removed. Following the removal of the clear partition, fish continued to explore the tank. As soon as they were in the proper position, the rope was pulled to trigger the trout attack. Proper position was defined as the focal fish being at least 2 body lengths from the sides or bottom, and oriented towards the back of the aquarium with at least one eye facing the direction of the soon to be on-rushing trout. This positioning ensured that the focal fish could dart in any direction, would see the oncoming trout, and would not be looking at the observer, who would have to move slightly to trigger the trout. When the trigger was pulled, the trout would swing briefly (~ 1.5 seconds) into view towards the focal fish, and then would move up and out of the water on the other end of the aquarium. Because the entry of the trout was hidden by a black plastic shield, from the perspective of the fish the trout was moving nearly horizontally through the arena.

Response variables recorded included time spent frozen in place, the presence/absence of an initial dart(s), and latency to recover (cutoff at 300 seconds), which was defined as resumption of eating, or continuous exploratory swimming. If a focal fish darted behind the opaque trout partition and could not be viewed from an angle, then trial was aborted and data were not used.

Simulated Bird Attack

Exposure to a simulated bird attack took place in a 37 L aquarium with gravel substrate, and a 40 watt work lamp suspended 1.5 metres above the center of the tank. A 7 cm tall feeding tray was placed in the middle of the arena. A wooden bird (41 cm wingspan) cut in the shape of an arctic tern was suspended via two pulleys to a rope above the test aquarium (Fig. 1B). When triggered, the bird slid down the rope, passing just above the aquarium and below the lamp, casting a shadow over the focal fish. The pre-trial acclimation procedure, trial

initiation with food, and response behaviours recorded following the attack, were identical to those of the trout model experiment. Once a fish had eaten the worms and subsequently moved into proper position, the bird was triggered by the observer and responses were recorded.

Statistical Analyses

Latency to recover and time spent frozen following simulated attacks were log-transformed to better meet the assumptions of analysis of variance (ANOVA). Mixed model ANOVA (Quinn & Keough, 2002) was used to determine whether each of these dependent variables differed significantly between predator models, individual origin (laboratory versus wild), among predation environments, among populations within predation environments, and due to all two- and three-way interactions. Predator model, origin and predation environment were modeled as fixed factors, and population (which was nested within predation environment) was modeled as a random factor. A separate ANOVA was performed for each dependent variable (log latency to recover, log time spent frozen). While the two response variables are highly correlated (Pearson $r = 0.766$, $P < 0.001$), many fish with a long latency to recover nevertheless did not remain completely frozen. Considering latency to recover therefore captures the anti-predator responses of all individuals, not just those who exhibited a freeze response. On the other hand, time spent frozen has the benefit of being a more objective measure of behaviour than latency to recover (determining that a fish has "recovered" is a subjective judgment, whereas a fish is either moving or not). Therefore we present results of both analyses. Degrees of freedom were estimated using Satterthwaite's procedure (Satterthwaite, 1946). Both ANOVAs were performed with and without the populations used in Messler *et al.* (2007) to account for the fact that the wild-caught populations experienced a slightly different experimental setup than the one employed here (the flyover trials were identical, but the trout model did not disappear from view following attack as in the present study). Because the analyses did not yield qualitatively different results (compare Table S1 to Table 2), those three populations were included in the

present analysis to improve statistical power, add balance to the experimental design, and to generally improve the robustness of our conclusions.

We also investigated whether the nature of a stickleback's response was related to its rearing origin or predation environment. Log-linear analysis of frequencies was used to determine whether a stickleback's propensity to perform a dart, freeze, both a dart and freeze, or neither response was related to its rearing origin, predation environment, or their interaction. All statistical analyses were performed with Statistica v.11 (Statsoft, Inc.).

Ethical Note

Throughout the study, care was taken to minimize stress and discomfort of study subjects. Plastic plants and gravel substrate was provided in home aquaria for habitat enrichment, and animals were handled as little as possible. Water quality was monitored regularly, and a regular schedule of water changes was followed (at least a 20-25% water change every other week). At the conclusion of the study, all subjects were euthanized with an overdose of buffered MS-222, a fish anesthetic. All protocols were approved by the Clark University and The College of New Jersey IACUC committees (Clark IACUC protocol #007R, TCNJ IACUC protocol #1105-001MW1), and animal protocols conformed to the ASAB/ABS Guidelines for the Use of Animals in Research.

RESULTS

Stickleback treated the predators as distinct stimuli, which is evident by the significant predator x origin, predator x population and predator x predation environment x origin interactions (Table 2). For example, in response to the model bird, laboratory-reared fish had longer latencies to recover and spent more time frozen than wild caught fish. In response to trout, the opposite was true, particularly in terms of latency to recover. Wild-caught fish had longer latencies to recover from a trout attack than laboratory-reared fish (Figure 2). The

significant predator x population interactions indicate that stickleback from different populations responded to the predators in distinct ways.

The main effect of predation environment was associated with the largest effect size of any factor (Table 2). On average, stickleback from freshwater populations that co-occur with piscivorous fish, whether native or recently introduced, took significantly longer to recover and spent significantly more time frozen than did either oceanic stickleback or stickleback from freshwater populations lacking such predators (Figures 3 and 4). This pattern of difference was more clear in response to the trout model than the bird flyover model. In response to the trout model, freshwater fish that co-occur with trout are most fearful, and There was a significant population (nested within predation environment) x origin interaction for both latency to recover and time spent frozen, indicating that rearing environment (lab versus wild) had a differential effect on the behaviour of fish from different populations (Table 2).

Log-linear analysis of escape behaviour frequencies indicated that overall, stickleback disproportionately exhibited both a dart and a freeze following a model trout attack, relative to just a dart, just a freeze, or neither response (Marginal Association $X^2_3 = 513.4$, $P < 0.001$; Fig. 5A). There was also a significant origin x behaviour interaction, with wild-caught fish being even more prone to both darting and freezing than their laboratory-reared counterparts (Marginal Association $X^2_3 = 27.9$, $P < 0.001$; Fig. 5A). Fish from different predation environments did not differ in the nature of their responses to a model trout attack (Marginal Association $X^2_9 = 13.3$, $P = 0.150$).

Similar to their responses to the model trout, stickleback disproportionately exhibited both a dart and a freeze following a model bird flyover, relative to just a dart, just a freeze, or neither response (Marginal Association $X^2_3 = 668.9$, $P < 0.001$; Fig. 5B). A model including a three-way origin x predation environment x behaviour interaction was significantly different from the null model of no difference (Pearson $X^2_9 = 39.29$, $P < 0.001$). A model including two-way interactions was not significantly different than the null model, indicating that the predation

environment x behaviour and origin x behaviour interactions were not significant (Pearson $X^2_{15} = 24.0$, $P = 0.065$).

DISCUSSION

We set out to determine whether the loss of predation from piscivorous fish is associated with reduced anti-predator responses, and if so, whether these responses could re-emerge following the re-imposition of these predators. Following a simulated trout attack, stickleback from lakes lacking piscivorous fish responded weakly relative to stickleback from lakes that contain trout, which would appear to support the hypothesis that relaxed selection leads to a reduction in anti-predator behaviour. However, because ancestral, oceanic stickleback exhibited anti-predator responses similar to trout-free freshwater populations, relaxed selection from these predators does not seem to have altered the anti-predator behaviours we considered. Instead, it appears that the colonization of freshwater environments led to an increase in anti-predator responses where predatory fish are present. Stickleback from reverse-selected populations behaved similarly to those from lakes where trout are native, indicating that enhanced anti-predator responses can evolve quite quickly—trout stocking was initiated in these three lakes only within the past 20 years (Bear Paw Lake initially stocked in 1999, Boot Lake initially stocked in 2001, Kalmbach Lake initially stocked in 1994, <http://www.adfg.alaska.gov>). Concluding that these populations rapidly responded to reverse selection assumes that they exhibited weak responses prior to trout stocking. This is an interpretation we think is reasonable given the weak responses of the relax-selected populations, which reflect the prior state of the reverse-selected populations, and the fact that reduced responses are the likely ancestral condition.

Several explanations might account for relatively weak responses by oceanic stickleback, despite the fact that large, predatory fish are abundant in the ocean. First, they are much more heavily armored than freshwater stickleback (T.E. Reimchen, 1983; T. E. Reimchen, 1992), and more robust morphological defenses may reduce the need for behavioural defenses that might otherwise lead to opportunity costs for foraging or reproduction (trait compensation, Cotton, Rundle, & Smith, 2004; Dewitt, Sih, & Hucko, 1999; Mikolajewski & Johansson, 2004). Alternatively, the marine environment might favor a different suite of behavioural tactics than freshwater lakes. For example, a dart and freeze, the most common behaviour we observed across all conditions, might be disadvantageous when there is no substrate or habitat structure in which to hide. Instead, swimming erratically, or swimming a long distance might be the best strategy to avoid an attacking predator in open water. Schooling behavior might also be an important anti-predator adaptation (Ioannou, Guttal, & Couzin, 2012), and oceanic stickleback show greater schooling tendencies than their freshwater counterparts (Di-Poi, Lacasse, Rogers, & Aubin-Horth, 2014; Wark, Greenwood, Taylor, Yoshida, & Peichel, 2011). The present study represents one of only a few assessments of anti-predator behaviour in oceanic threespine stickleback fish (along with Di-Poi et al., 2014; Messler et al., 2007), and is the first to consider both laboratory-reared and wild-caught individuals to assess potential contributions of learning and genetic divergence. It would be informative to consider behaviour in oceanic stickleback from other regions around the globe, as recent evidence suggests that the marine population is not necessarily as phenotypically uniform in all respects throughout their range as has been observed for morphology (Susan A Foster, 2013; Hohenlohe, Bassham, Currey, & Cresko, 2012; Orti, Bell, Reimchen, & Meyer, 1994; Wund et al., 2012). For example, establishing the ancestral condition for stickleback in the Northeast Atlantic would help establish evolutionary polarity of the behavioural variation observed among high and low predation populations in the United Kingdom (Giles & Huntingford, 1984; F. A. Huntingford et al., 1994).

Stickleback are exposed to many predators in addition to piscivorous fish, including birds such as arctic terns (*Sterna paradisaea*), loons (*Gavia immur* and *Gavia pacifica*) and grebes (*Podiceps grisegena*) as well as invertebrates such as water scorpions (*Ranatra* spp.) and dragonfly naiads (*Aeshna* spp.) (Thomas E. Reimchen, 1994). Because of their dispersal ability, these predators are present even in areas where predatory fish are absent. If common neurophysiological mechanisms underlie responses to all predators, then consistent predation by birds and invertebrates might impose sufficient selection to maintain responses to piscivorous fish under conditions of relaxed selection (the "multipredator hypothesis," Blumstein, 2006; Blumstein, Ferando, & Stankowich, 2009). Our data clearly reject this hypothesis in our system, and might even suggest the opposite -- that the intensity of responses to attacking birds may be in part driven by the presence or absence of piscivorous fish. In response to the model bird flyover, the general patterns of responses to an attacking bird mirrored those for an attacking trout--wild-caught stickleback from lakes free of stickleback exhibited relatively weak responses to a bird flyover, despite the fact that we regularly observe birds capturing stickleback at these three lakes. The possibility that a lack of selection by piscivorous fish is leading to reduced responses to predatory birds is surprising, and warrants further investigation. However, complex interactions among predator model, rearing origin and predation environment led to less of a clear distinction among predation environments in response to the bird model than to the trout model, so we interpret these results with caution.

Our results indicate that variation in anti-predator responses to trout attack likely result from a combination of genetic divergence and learning. The overall pattern of differences among predation environments was maintained in naïve, laboratory-bred fish, indicating a strong genetic component. However, responses of these naïve fish were overall lower than the responses exhibited by their wild-caught counterparts, indicating that experience plays a modifying role in the development of these behaviours. Laboratory-bred fish were also more likely to freeze without an initial dart than were wild-caught fish, suggesting that experience in

the wild influences not just the intensity of the response, but also the type of response produced. In the present study, rearing history impacted behaviour in some populations, but not others, as evidenced by a significant population x rearing origin effect. This indicates possible evolutionary divergence among populations in learning behaviour, which is a fascinating possibility. There is evidence in stickleback for divergence in how learning impacts the development of anti-predator behaviour. In the United Kingdom, stickleback from one high predation population fully develop anti-predator responses only if they interact with fathers at the nest, but this parental effect is absent in a low predation population (F. A. Huntingford et al., 1994; Tulley & Huntingford, 1987). Given that this experiment only considered one population from each predation environment, it is not clear whether divergence in learning resulted from differential selection by predators in the two populations, or from some other unmeasured factor. (1987) In the present study, the impact of experience we observed was not associated with a particular predation environment, but rather was idiosyncratic among populations. While divergence in spatial learning has been identified among stickleback populations (Odling-Smee, Boughman, & Braithwaite, 2008), the possibility that learned predator recognition has diverged requires further investigation.

In a world dominated by human activity, natural populations face unprecedented rates of change in the selective regimes they experience. Many species have responded with some combination of range shifts (Myers, Lundrigan, Hoffman, Haraminac, & Seto, 2009), adaptive phenotypic plasticity (Hendry, Farrugia, & Kinnison, 2008; Marnocha, Pollinger, & Smith, 2011) and adaptive evolutionary responses (J. A. Baker, Heins, King, & Foster, 2011; De León et al., 2011; Phillips, Brown, & Shine, 2004; Sih, Ferrari, & Harris, 2011; Stockwell, Hendry, & Kinnison, 2003; Sunday et al., 2013; Thomas, Quofl, Hartmann, & Eckmann, 2009). Human-assisted dispersal will increasingly alter community composition, and it is important to understand how native populations might respond to the loss and re-imposition of predators. The unplanned introduction of invasive pike (*Esox lucius*) has already resulted in the demise of

some stickleback populations (Haught & von Hippel, 2011; Patankar, von Hippel, & Bell, 2006), and the planned introductions of rainbow trout have led to volatile population dynamics in others, a problem that is most pronounced in populations exhibiting the relatively rare pelvic reduced phenotype (John A. Baker et al., 2010). Even where stickleback populations persist through a combination of learned and evolved behavioural strategies, as our results suggest, we must still consider the consequences of lost or reduced behavioural diversity, as the stickleback radiation becomes ever more homogenized (Susan A. Foster, Baker, & Bell, 2003).

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Table 1. Populations and Sample Sizes

Predation Environment	Population	Coordinates	Years Tested		N Lab Reared		N Wild-Caught	
			Lab-	Wild-	Trout	Tern	Trout	Tern
			Reared	Caught	Model	Model	Model	Model
Oceanic (Ancestral), Native Piscivorous Fish	Anchor River	59.77444, -151.86530	2008, 2011	2007	26	25	30	30

Table 2. Results of mixed model ANOVAs for responses to simulated trout and bird attacks.

Log₁₀ Recovery Time	Effect	df	<i>F</i>	<i>P</i>	Partial η^2
Predator	Fixed	1, 8.0	0.00	0.987	0.000
Predation Environment	Fixed	3, 8.0	4.39	0.042	0.622
Origin (lab vs. wild)	Fixed	1, 8.0	1.40	0.271	0.148
Population w/in PredEnvt	Random	8, 9.5	0.22	0.979	0.155
Predator*PredEnvt	Fixed	3, 8.0	0.28	0.094	0.084
Predator*Origin	Fixed	1, 1311.0	20.32	0.000	0.152
Predator*Population	Random	8, 1311.0	2.16	0.028	0.013
PredEnvt*Origin	Fixed	3, 8.0	0.36	0.784	0.119
Origin*Population	Random	8, 1311.0	10.45	0.000	0.060
Predator*PredEnvt*Origin	Fixed	3, 1311.0	3.00	0.086	0.005
Log₁₀ Time Frozen	Effect	df	<i>F</i>	<i>P</i>	Partial η^2
Predator	Fixed	1, 8.0	0.02	0.905	0.002
Predation Environment	Fixed	3, 8.0	6.39	0.016	0.705
Origin (lab vs. wild)	Fixed	1, 8.0	1.13	0.319	0.124
Population w/in PredEnvt	Random	8, 10.5	0.25	0.971	0.158
Predator*PredEnvt	Fixed	3, 8.0	0.49	0.701	0.154
Predator*Origin	Fixed	1, 1311.0	9.17	0.003	0.007
Predator*Population	Random	8, 1311.0	2.43	0.013	0.015
PredEnvt*Origin	Fixed	3, 8.0	1.10	0.405	0.291
Origin*Population	Random	8, 1311.0	6.31	0.000	0.037
Predator*PredEnvt*Origin	Fixed	3, 1311.0	3.15	0.024	0.007

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FIGURE LEGENDS

Figure 1. Schematic diagram of the (A) trout testing arena (top view) and (B) bird flyover arena (front view). Small fish represent the focal stickleback and the large fish and bird represent the model predators. Dashed lines represent the path of the model predator. Circles represent the feeding tray. The gray line in (A) represents a transparent barrier that was removed prior to the trout attack. Black lines on the interior of the trout model arena were opaque, thus hiding the trout from view until it attacked.

Figure 2. Responses of stickleback to the model bird and trout, illustrating the interaction between predator model and rearing origin. A) Latency to recover, and B) time spent frozen. Bars are means $\pm$ SEM. All populations are pooled. See Table 1 for sample sizes.

Figure 3. Responses of stickleback to the attacking trout model. Both laboratory-reared (black bars) and wild-caught (gray bars) individuals were assessed from each of four predation environments (three populations per type) that differ in predation history. A) Latency to recover, measured as time from the initial attack to the resumption of exploratory and/or feeding behaviour. B) Time spent frozen between the initial attack and recovery. If recovery was not achieved after 300s, the trial was concluded. Bars are means $\pm$ SEM. See Table 1 for sample sizes.

Figure 4. Responses of stickleback to the attacking bird model. Both laboratory-reared (black bars) and wild-caught (gray bars) individuals were assessed from each of four predation environments (three populations per type) that differ in predation history. A) Latency to recover, measured as time from the initial attack to the resumption of exploratory and/or feeding behaviour. B) Time spent frozen between the initial attack and recovery. If recovery was not

625 achieved after 300s, the trial was concluded. Bars are means $\pm$ SEM. See Table 1 for sample
626 sizes.

627
628 Figure 5. Categorical responses of stickleback to an attacking trout model (A) and bird model
629 (B). Black bars represent laboratory-reared stickleback and gray bars represent their wild-
630 caught counterparts. Percentages above the bars reflect the fraction of individuals in a particular
631 rearing group (lab or wild) performing a particular behaviour.

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Figure 1a

Figure 1b

Figure 2a

Figure 2b

Figure 3a

Figure 3b

Figure 4a

Figure 4b

Figure 5a

Figure 5b