

No evidence that stickleback spines directly increase risk of predation by an invertebrate predator

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ABSTRACT

Background: Some populations of stickleback have a reduced number and/or relative size of spines.

Hypothesis: Macroinvertebrate predators such as dragonfly larvae cause selective pressure against spines by capturing more stickleback with pelvic spines than stickleback that are spineless.

Organisms: Ninespine stickleback (*Pungitius pungitius*) and dragonfly larvae (*Aeshna grandis*).

Methods: We used 10 stickleback, five with pelvic spines and five with their pelvic spines removed. We put them in containers with two dragonfly larvae. Every day for 4 days we monitored how many stickleback were captured by the larvae. We repeated this experiment ten times at two different densities of fish and predators. We also developed a model to determine whether selection for spinelessness can be distinguished from drift.

Results: Dragonfly larvae caught as many stickleback with spines as without. The absence of spines was not associated with a decrease in predation risk. We substituted Bayesian estimates of the selection coefficient into quantitative genetic models of allele frequency change, and the results of the models suggest that the selective advantage of spine loss is so small that its effects cannot be distinguished from drift.

Keywords: *Aeshna*, dragonfly larvae, *Pungitius*, morphological defence, predation, prey capture, stickleback.

INTRODUCTION

Identifying the ecological sources of natural selection and its effect on trait evolution has captivated researchers since the time of Darwin (Darwin, 1859; Endler, 1986; MacColl, 2011; Hendry *et al.*, 2013). In contrast to selection and elaboration for novel traits, the underlying causes of

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convergent trait loss is less well understood (Lahti *et al.*, 2009). Reduction or loss of traits may arise either under direct selection if loss of the trait confers a fitness advantage, or via relaxed selection, particularly if the trait is costly to produce (Lahti *et al.*, 2009). Alternatively, loss of a trait may be governed by random processes such as genetic drift in the absence of selection. The influence of random processes can be amplified in isolated populations that have prolonged periods of low population size or bottlenecks, such as in founder populations. Therefore, an important consideration when deciphering the causes of systematic reduction or loss of trait function is to weigh the relative contributions of selection versus neutral processes to the evolution of a particular phenotype.

Stickleback serve as a model system for evolutionary ecology research because they have undergone repeated radiations of phenotypically distinct ecotypes (McKinnon and Rundle, 2002). Perhaps the most characteristic feature of sticklebacks is their spines, from which this group of fishes draws their name. However, some isolated freshwater populations of threespine (*Gasterosteus aculeatus*), ninespine (*Pungitius pungitius*), and brook (*Culaea inconstans*) sticklebacks show a reduction in dorsal spines as well as the loss or a reduction of pelvic spines and the pelvic girdle (e.g. Nelson, 1971, 1977; Bell, 1974; Reimchen, 1980; Blouw and Boyd, 1991; Bell *et al.*, 1993; Ziuganov and Zotin, 1995; Klepaker and Østbye, 2008; Mobley *et al.*, 2011; Baker *et al.*, 2013; Lescak *et al.*, 2013; Reimchen *et al.*, 2013; for a review, see Klepaker *et al.*, 2013). Fossil findings of pelvic reduction (Bell *et al.*, 1993) and the phylogeography of extant populations (Mäkinen and Merilä, 2008; Aldenhoven *et al.*, 2010; Shikano *et al.*, 2010; Mobley *et al.*, 2011; Teacher *et al.*, 2011) indicate that pelvic reduction occurs repeatedly and often independently in these different populations and species.

The function of spines in stickleback is well understood: they primarily serve as a defence against piscivorous fish and birds (Hoogland *et al.*, 1957; Wootton, 1984; Reimchen, 1994). In threespine stickleback, pelvic spine and girdle development is well characterized and is regulated by the pituitary homeobox transcription factor 1 (*Pitx1*) gene (Shapiro *et al.*, 2004). Multiple independently derived populations of threespine stickleback that show reduction of the pelvic spines demonstrate various mutational deletions in the regulatory enhancer (Chan *et al.*, 2010). This regulatory pathway is also likely responsible for pelvic reduction in some, but perhaps not all, ninespine stickleback populations (Shapiro *et al.*, 2006, 2009).

It remains unclear what causes the reduction and loss of spines in some populations of stickleback and several hypotheses have been proposed to explain this phenomenon. Nelson (1969) suggested that stickleback lacking pelvic development have reduced weight and are more streamlined, which may facilitate escape from predation attacks in dense vegetation. Reimchen (1980) hypothesized that a reduction in size or number of spines decreases mortality due to macroinvertebrate predators that, unlike gape-limited piscivorous fish and birds, may use spines to grasp or hold the fish (Reimchen, 1980; Reist, 1981; Shapiro *et al.*, 2004). Alternatively, Giles (1983) hypothesized that low calcium and phosphate concentrations in freshwater environments may result in individuals with reduced bony armour and incomplete spine development (see also Marchinko, 2009). Other explanations include the high developmental cost of producing spines so that reduction of spines would be favoured in populations without vertebrate predators (Bell *et al.*, 1993).

While several experiments have shown that predation by gape-limited fish results in selection for longer spines and increased armour in stickleback (Moodie, 1972; Reimchen, 1992, 1994, 2000; Vamosi and Schluter, 2004), direct evidence that predatory aquatic insects select for spine and armour reduction is generally lacking. Among threespine stickleback, no differences in escape probabilities among spined and non-spined phenotypes have been reported (Reimchen, 1980; Giles and Huntingford, 1984). However, Lescak *et al.* (2012) found a trend towards long-spined

morphs of threespine stickleback escaping capture by dragonfly naiads compared with their short-spined counterparts, but these results were statistically non-significant. In a similar experiment concerning selection on armour in threespine stickleback, Zeller *et al.* (2012) found no evidence for selection against lateral plates by invertebrate predation. In a surprising twist, Reist (1980) found that larvae of water beetles (*Dytiscus* spp.) predated more on brook stickleback possessing five, in contrast to six, dorsal spines. Finally, our best evidence that invertebrate predation does play a significant role in the evolution of defensive structures comes from experiments by Marchinko (2009), who found that adding invertebrate predators (*Notonecta* sp. backswimmers and *Aeshna* sp. dragonfly naiads) to artificial enclosures led to increased mortality of threespine stickleback with complete pelvic girdles among F2 offspring of experimental crosses. However, predation by invertebrates was not directly observed in these experiments, calling into question the precise mechanism by which this increase in mortality arose.

In ninespine stickleback, loss or reduction of spines is usually observed in isolated pond and lake populations in the absence of piscivorous fish (Gross, 1979; Blouw and Boyd, 1991; Ziuganov and Zotin, 1995; Mobley *et al.*, 2011), but reduction of spines may also be observed in isolated populations with natural or introduced predatory fish (Mobley *et al.*, 2011). Ninespine stickleback are intimately associated with aquatic and riparian vegetation where they take shelter as juveniles and nest as adults (Wootton, 1984), and where predation by benthic aquatic insects is likely high. Spines are thought to function as grappling points for invertebrate predators (Reimchen, 1980) and therefore the reduction or absence of spines should provide ninespine stickleback with an escape advantage against aquatic insects.

Here we present an experiment that tested whether the presence of pelvic spines directly increases the risk of ninespine stickleback being captured by dragonfly larvae (*Aeshna grandis*) by manipulating the length of pelvic spines. Other invertebrate predators on small stickleback are known (e.g. *Notonecta* backswimmers), but *Aeshna* larvae readily capture and consume adult stickleback (Foster *et al.*, 1988; Reimchen, 1994) and can live up to 4 years prior to emergence, making them a persistent threat and the primary macroinvertebrate source of mortality in stickleback (Reimchen, 1994). We then test the statistical power of our experiment to detect selection by invertebrate predation on spine length. Finally, we construct a quantitative genetic model to investigate the relative strength of neutral processes versus selection for pelvic spine loss to help explain patterns of convergent spine loss in isolated ninespine stickleback populations.

METHODS

Field collections of stickleback and dragonfly larvae were made during June and July 2011 in the county of Västerbotten, Sweden. Ninespine stickleback were collected near Obbola, Sweden in the Bothnian Bay (63°39.372'N, 20°17.456'E) using a 10 m beach seine with a 0.1 × 0.1 mm mesh catch bag. The collection site is a shallow bay open to the Baltic and is characterized by low salinity (~4 psu) and has a natural complement of fish and invertebrate predators (personal observations). Ninespine stickleback were fully spined with complete pelvic girdles and were morphologically and genetically similar to Baltic coast populations sampled previously (Mobley *et al.*, 2011). Fish were kept at 18°C in circular 75-litre black plastic tanks supplied with a foam filter and airstone, and fed frozen chironomid larvae daily. Dragonfly larvae (*Aeshna grandis*) were collected using dip nets from different locations near Umeå, Sweden including the Obbola collection site, Nydala lake (63°49.585'N,

20°20.129'E), Sofiehemsdammen pond (63°48.040'N, 20°18.287'E), and artificial ponds maintained by the University of Umeå (63°48.572'N, 20°14.55'E). Threespine stickleback, ninespine stickleback or both were present at all collection locations. Dragonfly larvae were placed in individual containers and transported to the laboratory at the University of Umeå where they were fed chironomid larvae daily.

We experimentally modified spine length by removing both pelvic spines near the pectoral girdle from a random sample of fish. Fish were first anaesthetized using MS-222 (tricaine methanesulphonate: 50–75 mg·L⁻¹) for 1 min prior to spine clipping with sterilized scissors. To control for possible effects of handling, the control fish with spines intact were also anaesthetized with MS-222. Fish were allowed to recover for 10 min in fresh, oxygenated water before placing them back in holding tanks. No fish died as a result of spine clipping or anaesthesia.

To determine whether spines affected the capture rate of ninespine stickleback, we placed 10 stickleback, five with spines and five with spines removed, together with two dragonfly larvae in an aquarium. Each tank contained gravel, large rocks, and plastic plants to provide shelter.

Because prey density has the potential to affect capture rate by predators, we performed this experiment in two different containers: (1) at high density in 35-litre glass aquaria ($n = 10$ replicates) and (2) at low density in 75-litre black plastic tanks ($n = 10$ replicates). We also performed control experiments ($n = 4$) in which the same numbers of stickleback and dragonfly larvae were kept in 35-litre aquaria, except that each dragonfly larva was placed inside a perforated petri dish so that visual and chemical cues of the larvae could reach the stickleback, but the larvae could not capture the fish.

Each replicate was run for 4 days. Prior to the experiment, the dragonfly larvae were deprived of food for 24 h. Dead and injured fish were considered 'captured' and removed daily. Captured fish were replaced immediately with similar fish to keep the number of fish with and without spines constant. Both predators and prey were used only once. Fish were fed frozen chironomids once a day during the experiment.

To test for differences in capture rate between spined and non-spined stickleback in the two density treatments, we performed an analysis of variance (ANOVA) using density (high vs. low) as a fixed factor and differential capture (capture of spined stickleback vs. capture of non-spined stickleback) as the response variable. Because density had no effect on differential capture (totals captured in high-density treatment = 16 spined and 17 non-spined; totals captured in low-density treatment = 16 spined and 16 non-spined; $F_{1,18} = 0.015$, $P = 0.903$), we pooled samples across density treatments and performed a two-tailed paired-sample t -test on the number of captured spined versus non-spined stickleback. We also tested whether body size had an effect on capture using an ANOVA on captured versus uncaptured stickleback. All data were normally distributed and means are reported throughout the text \pm one standard error of the mean. All statistical analyses were conducted using PASW18.0 (SPSS Inc., Chicago, IL).

RESULTS

During the experiment, dragonfly larvae were observed to actively pursue and capture stickleback. On average, spined and non-spined fish were equally likely to be captured (totals captured pooled across density treatments: $n = 33$ spined, $n = 32$ non-spined; mean captured per replicate \pm one standard error: spined = 1.60 ± 0.33 , non-spined = 1.65 ± 0.24 ;

$t_{1,19} = 0.127$, $P = 0.900$). No fish died in any of the control treatments, demonstrating that fish in the other experiments all likely died due to capture by dragonfly larvae. Because dragonfly larvae often consumed all the flesh and disarticulated the skeleton of the stickleback, we only reconstructed body length of captured fish for the first four replicates due to imprecision in its measurement. Captured fish (30.7 ± 1.8 mm) were on average slightly smaller than fish not captured (33.4 ± 1.7 mm), but this difference was not significant (ANOVA: $F_{1,37} = 1.319$, $P = 0.258$; first four replicates only).

Since our experimental results suggest that the presence of spines has no effect on the risk of predation by dragonfly larvae, we investigated whether this result may be due to a type II error (β) due to low statistical power. Statistical power is defined as the probability that the test will reject the null hypothesis when the null hypothesis is false or $1 - \beta$ (Sokal and Rohlf, 1995). Let π be the relative predation risk of an individual with pelvic spines, so that if $\pi = 0.5$ spines have no influence on predation risk, while if $\pi = 1$ dragonfly larvae only capture individuals with spines. Each of our replicates can then be regarded as a binomial ($y | n, \pi$) sample, where n is the observed number of captured individuals, and y is the number of captured individuals with spines (so that $n - y$ is the number of captured individuals without spines). Based on data collected from this experiment, the total number of captured fish was 65, so we simulated our experiment as 20 independent binomial trials where the total number of captured individuals was 65. These simulated results were subjected to one-way ANOVA as above. We repeated this simulation 100 times for π ranging from 0.5 (no effect of spines) to 0.75 (strong selection against spines) to calculate the statistical power of our experiment. As can be seen in Fig. 1, if selection against spines is moderate, the chance that our experiment would have detected it is only about 50%. Some further simulations (not shown) reveal that many hundreds of replicate experiments are required to achieve high statistical power to rule out weak selection against spines.

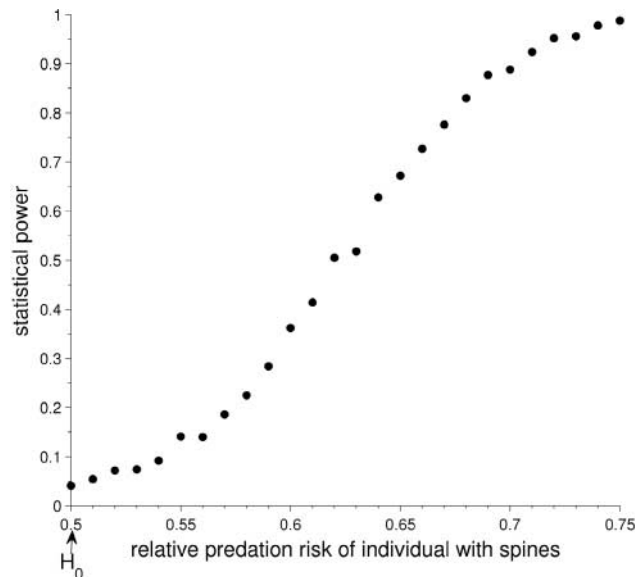


Fig. 1. Statistical power ($1 - \beta$) of the present experiment to reject the null hypothesis (H_0) that spines do not affect predation rate as a function of the relative predation risk of an individual with spines. When the relative predation risk is 0.5, the null hypothesis is true, and the error rate is 0.05.

Because of the above concerns about statistical power, we also employed a Bayesian parameter estimation approach in addition to hypothesis testing. Again, we assumed that the number of captured individuals that had spines y follows a binomial ($y | n, \pi$) distribution. As very little information is available, we assume a uniform prior on $[0,1]$ for the relative predation risk π . It can then be shown that the posterior distribution of π is the $\beta(y + 1, n - y - 1)$ distribution. From the properties of the β -distribution, it then follows that $0.395 < \pi < 0.634$ with 95% confidence.

Subsequently, we performed a simulation to investigate whether selection on reduced pelvic spines from standing genetic variation can be distinguished from genetic drift. Assuming *Pitx1* or a similar one-locus gene is involved in pelvic formation among Baltic ninespine stickleback, we can model selection as a rare recessive allele causing spine reduction. If predation risk π is the only cause of the difference in relative fitness between individuals with and without spines, then the selection coefficient $s = 1 - (1 - \pi)/\pi$. If the initial frequency of this allele is q , then the frequency in the next generation is $(q - sq + sq^2)/(1 - s(1 - q^2))$. Evaluations using different values of q and π reveal that with selection coefficients well within the 95% confidence region of our estimates ($0.395 < \pi < 0.634$), virtual fixation of the spineless allele ($q > 0.999$) can be reached in a few thousand generations, which is far shorter than the age of ninespine stickleback populations originating in the Baltic [$\sim 10,000$ calibrated years before present (Mobley *et al.*, 2011)]. It should be noted, however, that in the absence of selection, drift would lead to fixation of the allele in an expected $4N_e$ generations. In isolated populations that have undergone a population bottleneck and/or a founder effect, if we assume effective population sizes (N_e) between 100 and 1000, we obtain expected times to fixation in the same range as expected under weak selection.

DISCUSSION

In ponds that lack piscivorous fish, dragonfly larvae are the main aquatic predators of small stickleback (Reimchen, 1994). Based on our manipulation experiment, we find little convincing evidence that ninespine stickleback without pelvic spines evade capture by dragonfly larvae any better than those with spines. Furthermore, the results of our simulations show that if selection by invertebrate predation on spinelessness does exist in isolated populations, it is so small as to be indistinguishable from drift.

Larvae of *Aeshna* dragonflies are active visual predators, and detect most of their prey by tactile stimulation or vision (Pritchard, 1965). Reimchen (1980) describes a typical attack: when a fish is within approximately 10 mm of the dragonfly larva, a modified labium is extended in only 15–20 ms. The fish is seized with sharp labial palps, after which the labium is retracted with the prey. The larva then puts its forelegs around the body of the fish, ostensibly to minimize the rapid body movements of the fish and to avoid it escaping. Based on these observations, it is plausible that pelvic spines help the larva grab and hold its prey. We clipped only pelvic spines, because dorsal spines were so small and thin that clipping them required prolonged anaesthesia, which too often led to death of the fish. We do not believe the results of our experiment would have been different if we had also clipped the dorsal spines because *Aeshna* larvae seize stickleback from the ventral body surface (Reimchen, 1980; personal observations).

Previous experimental investigations for selection against spines by invertebrate predators were not manipulative, and therefore it is difficult to distinguish between direct effects of

predation and indirect effects such as survivorship in enclosures (Marchinko, 2009; Bjærke *et al.*, 2010) or behavioural differences correlated with the extent of defensive armour (Reimchen, 1980). For example, Reist (1980) reported that the differential capture success of brook stickleback by water beetles was at least partly due to the closer approach made to the predator by stickleback that naturally had spines. Moreover, Andraso and Barron (1995) demonstrated a trade-off between defensive armour and mechanically induced response performance in brook stickleback but did not test escape response from invertebrate predators directly. Thus, the present experiment provides a direct test of Reimchen's (1980) hypothesis that spines are selected against because they aid capture by invertebrate predators. The experimental set-up used in this study also capitalizes on a manipulative approach to a population that was not naïve to invertebrate predation. Therefore, we were able to disentangle the presence of spines from other potentially covarying morphological and behavioural anti-predator traits (e.g. Mikolajewski and Rolff, 2004; Mikolajewski *et al.*, 2006).

In freshwater populations of ninespine stickleback originating from the Baltic Sea Basin that have been putatively isolated since the Last Glacial Maximum, reduction in pelvic spines relative to body size is found in lakes and ponds lacking piscivorous fish or in populations where predatory fish exist either naturally or as a result of human introductions (Mobley *et al.*, 2011). However, only populations without piscivorous fish have a systematic reduction of relative spine length (as compared to body size) that shows a strong signal of stabilizing selection compared with neutral genetic markers (Mobley *et al.*, 2011). This result implies that a systematic reduction or loss of spines in populations may be advantageous in environments without fish predators but with naturally occurring macroinvertebrate predators. Nevertheless, when trying to answer whether spine loss or reduction in isolated populations is driven by invertebrate predation, the effect of predation risk is most meaningfully regarded in comparison with the effect of genetic drift. The results of our simulation demonstrate that our experiment has little power to exclude the possibility of very weak selection favouring spineless individuals, yet such low levels of selection would be sufficient to explain why some isolated populations of ninespine stickleback lack pelvic spines after 10,000 years. However, drift alone could explain this observation equally well, particularly in populations with low initial effective population sizes. Therefore, our results do provide evidence against a major role for predation by dragonfly larvae but we cannot exclude the possibility that invertebrate predation may play a diminished role in driving the loss of spines in isolated stickleback populations.

Before making generalities about invertebrate predation to all stickleback species, a few caveats to the experiment should be addressed. First, ninespine stickleback are generally smaller and have smaller and less robust spines (both dorsal and pelvic) than their three-spine counterparts (Wootton, 1984), for which the original hypothesis was postulated. For example, comparing pelvic spine length of Baltic ninespine stickleback to previously published allopatric populations of threespine stickleback that occur in freshwater lakes with predators, ninespine stickleback pelvic spines are 40% smaller per unit of body length (Vamosi and Schluter, 2004; Mobley *et al.*, 2011). Therefore, ninespine stickleback may be predisposed to better escape invertebrate predation than threespine stickleback, while threespine stickleback may be better equipped to defend against fish predators (Hoogland *et al.*, 1957). Second, although predation on adult ninespine stickleback was observed in our experiment and body size represented a natural sample of adults captured from the wild during the summer months, selection on spines by dragonfly larvae may be more apparent on juvenile stages or at different times of the year. Additional tests on a broader range of body sizes and at

different times of year would be necessary to resolve this issue. Although *Aeshna* larvae are thought to be the major invertebrate predator of stickleback (Reimchen, 1994), other invertebrate predators such as diving beetles and backswimmer beetles may predate on European ninespine stickleback but their effect on spine selection was not tested in this experiment. Finally, phenotypic plasticity in spine development may affect the strength of selection in stickleback, whereas the expression of such phenotypes may be regulated by environmental factors. However, a recent study investigating phenotypic plasticity in spine length among Baltic populations of ninespine stickleback do not show a phenotypic response to either predation or feeding regimes, lending support to the hypothesis that spine length is genetically determined and not environmentally induced (Välimäki *et al.*, 2012).

Understanding patterns of trait loss is an overlooked part of evolutionary biology but is critical to our understanding of how natural selection operates in the wild. Previous studies suggest salient selection by invertebrate predators on stickleback as a cause for spine reduction and loss, although direct evidence of such selection is scant. The results of our study point to a diminished role of invertebrate predation in ninespine stickleback and that reduction of spines is a less deterministic process, ostensibly affected by genetic drift and, perhaps, relaxed or transient selection on spines or correlated traits by invertebrate predators. We cannot rule out selection by additional ecologically relevant sources of selection such as ion deficiency, thus future studies should also investigate other sources of selection for spinelessness in ninespine and other species of stickleback.

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