

Evolutionary Ecology Research, 2016, **17**: 243–262

Selective agents in the adaptive radiation of Hebridean sticklebacks

Tom Klepaker¹, Kjartan Østbye^{2,3}, Rowena Spence⁴, Mark Warren⁵,
Mirosław Przybylski⁶ and Carl Smith⁵

¹*Department of Biology, Aquatic Behavioural Ecology Research Group, University of Bergen, Bergen, Norway,* ²*Department of Biology, Centre for Ecological and Evolutionary Synthesis, University of Oslo, Oslo, Norway,* ³*Hedmark University College, Faculty of Applied Ecology and Agricultural Science, Evenstad, Norway,* ⁴*School of Psychology and Neuroscience, University of St. Andrews, St. Andrews, UK,* ⁵*School of Biology, University of St. Andrews, St. Andrews, UK and* ⁶*Department of Ecology and Vertebrate Zoology, University of Łódź, Łódź, Poland*

ABSTRACT

Question: What selective agents underpin the adaptive radiation of threespine sticklebacks (*Gasterosteus aculeatus*) on the Outer Hebridean island of North Uist?

Hypothesis: The chief agents of selection for lateral plate number and pelvic score are predatory trout, the availability of dissolved ions, or an interaction of both.

Methods: Adult threespine sticklebacks were collected from 26 lochs on North Uist. Fish were killed, stained, and scored for lateral plate number and pelvic score. We also measured the pH and concentration of dissolved calcium and phosphorous of each loch. We assessed the abundance of predatory trout in a subset of lochs by ‘fly fishing’ using a lure that mimicked the appearance of sticklebacks.

Results: Dissolved calcium and phosphorous predicted stickleback lateral plate number and pelvic score, while trout abundance failed to predict either. Attack rates by trout on stickleback lures were higher in lochs with higher numbers of trout, with high water clarity, and at higher water temperatures. Our findings implicate a role for the bioavailability of dissolved ions in selection for reduced lateral plate number and pelvic score evolution, with indirect evidence for an effect of trout predation on the adaptive radiation of stickleback populations on North Uist.

Keywords: adaptation, ion concentration, natural selection, phenotypic adaptation, Scottish Galapagos, selective landscape, stickleback, trout predation.

INTRODUCTION

Fitness is an outcome of the interaction between phenotype and environment. While the environment is unquestionably the major driver of phenotypic diversity, the ecological agents responsible for generating selection for particular phenotypes are, surprisingly, often

Correspondence: C. Smith, School of Biology, University of St. Andrews, St. Andrews, Fife KY16 8LB, UK.
email: cs101@st-andrews.ac.uk

Consult the copyright statement on the inside front cover for non-commercial copying policies.

incompletely understood or ignored in field studies (Wade and Kalisz, 1990; Schluter, 2001; Sæther and Engen, 2015). The threespine stickleback (*Gasterosteus aculeatus* L.) offers an unusually tractable vertebrate model for identifying the ecological agents of selection for phenotypic adaptations in response to local environmental conditions (Wootton, 1984, 2009; Bell *et al.*, 1993; Schluter *et al.*, 2010; Barber, 2013). This species occupies a wide range of environmental conditions, expresses a range of phenotypes, and is amenable to field and laboratory research (Wootton, 2009; Hendry *et al.*, 2013).

The evolutionary history of the threespine stickleback is one of repeated invasions of freshwater habitats by marine populations, characterized by a rapid change in morph frequency from domination by *complete* to almost entirely monomorphic populations of the *low* plate morph (Bell, 1976; Wootton, 1976; Bell and Foster, 1994), with a reversal in morph frequency in as few as 10 generations (Klepaker, 1993; Bell *et al.*, 2004; Bell, 2009). Adaptation to fresh water corresponds with rapid changes in genotype frequency at the *Eda* locus, which codes for ectodysplasin, a gene product that plays a role in controlling the development of teeth, hair, and bones in mammals (Kangas *et al.*, 2004) and scales in fish (Kondo *et al.*, 2001). Subsequent studies of changes in genotype frequency at the *Eda* locus during experimental introductions of sticklebacks to fresh water were demonstrated to be partially due to selection on lateral plates, but also to selection on additional, unmeasured traits controlled by *Eda* or a tightly linked gene (Peichel *et al.*, 2001; Colosimo *et al.*, 2005; Barrett *et al.*, 2008; Hohenlohe *et al.*, 2010; Rennison *et al.*, 2015). Thus selection on lateral plates only partially explains changes in *Eda* genotype frequencies, implying that the rapid evolution of the *low* morph in fresh water may be the result of both selection on lateral plates and a correlated response to selection on other, hitherto unidentified traits affected by *Eda* (Rennison *et al.*, 2015). Similarly in the case of the pelvic girdle, pelvic reduction is associated with expression of the *Pitx1* gene, with selection for pelvic reduction associated with adaptation to freshwater environments (Shapiro *et al.*, 2004; Bell *et al.*, 2007; Chan *et al.*, 2010). Thus, while the genetic basis to plate and spine reduction in fresh water are at least partially understood, a less tractable problem has been to understand the selective agents that favour the evolution of different plate and spine morphs in contrasting environments. The two most enduring hypotheses are the nature and intensity of predation (Hagen and Gilbertson, 1973; Reimchen, 1980; Bell *et al.*, 1993), and the bioavailability of dissolved ions, particularly calcium (Giles, 1983; Spence *et al.*, 2012).

Unlike most teleost fishes, sticklebacks completely lack scales and instead possess calcified bony lateral plates, dorsal spines, and a spined pelvic girdle. There is considerable variation in the extent of these bony elements among populations. Lateral plates, in combination with pelvic and dorsal spines, function in defence against predators (Hoogland *et al.*, 1957; Reimchen, 1983, 1991, 1994). Pelvic and dorsal spines increase the effective diameter of an individual, making it more difficult to swallow by gape-limited predators, while the bony pelvic girdle and lateral plates protect the body from crushing and puncture (Reimchen, 1983; Bell, 1988). As such, it has been proposed that variation in plate and spine morphs has been driven primarily by predation (Hagen and Gilbertson, 1973). There is evidence to support this narrative. Thus, predation rates and extent of 'armour' are broadly correlated (Hagen and Gilbertson, 1973; Reimchen, 1983, 2000; Bell, 1988; Bañbura *et al.*, 1989; Reimchen *et al.*, 2013). In some cases, changes in the risk of predation are matched by corresponding changes in plate number (Kitano *et al.*, 2008). A caveat is that in the case of invertebrate predators, long spines and robust plates may be disadvantageous if they facilitate the predators grasping their prey or otherwise limit escape responses (Reimchen, 1980, 1992, 2000). Evidence for this hypothesis is mixed (Zeller *et al.*, 2012; Mobley *et al.*, 2013). An experimental study showed that invertebrates can select against dorsal spine

length, but not pelvic spine length or plate number (Marchinko, 2009). Thus, while predation by invertebrates is predicted to limit plate number and spine length, wholly convincing empirical evidence is limited.

There is a striking correlation between plate and spine morphs and the ionic concentrations of the water in which sticklebacks live (Heuts, 1945; Wootton, 1976, 1984, 2009). Thus, the *complete* morph, with a continuous row of calcified plates from immediately behind the head to the caudal peduncle and well-developed dorsal and pelvic spines, is associated with marine and estuarine environments, although some populations migrate into fresh water to spawn in spring, with surviving adults and young returning to the sea in the autumn. In contrast, *low* morphs, with only an anterior row of lateral plates and the remainder of the body naked, usually reside in fresh water throughout their life (Wootton, 1976). Plate thickness in freshwater populations also appears to be thinner than those from marine populations (Wiig, 2014). Other elements of the bony external skeleton of sticklebacks also show variation corresponding with ion concentration, including dorsal, anal, and pelvic spines. This pattern is not universal and resident freshwater populations of the *complete* morph have been recorded from Eastern Europe, eastern North America, north-eastern Asia and scattered populations across their range (Wootton, 1976; Hagen and Moodie, 1982; Bañura, 1994; Klepaker, 1995). Among populations, lateral plate reduction is the most frequently encountered variable armour trait, followed by reduction in spine length. The striking reduction or complete loss of lateral plates and pelvic girdle in some stickleback populations, what Spence *et al.* (2013) termed *minimal* morphs, is rare (Klepaker and Østbye, 2008; Klepaker *et al.*, 2013).

The aim of this study was to investigate the role of brown trout (*Salmo trutta*) predation pressure and dissolved ion availability on stickleback adaptive evolution. On the island of North Uist in the Scottish Outer Hebrides, major morphological variation of threespine sticklebacks is observed among lochs, with the occurrence of extreme plate and spine-reduced sticklebacks (Giles, 1983; Campbell, 1985; Spence *et al.*, 2013). The striking stickleback radiation of North Uist offers an exceptional opportunity to correlate spatial variation of phenotype with putative agents of selection in the environment. North Uist supports numerous populations of both *low*-plated with spines and plate and pelvic girdle reduced or deficient sticklebacks in freshwater lochs that vary in both trout predation pressure and dissolved ion concentration. The west coast of North Uist is characterized by a band of calcium-rich shell-sand grassland, called the *machair*, which supports rich vegetation and alkaline, biologically productive lochs. In the central and eastern regions, the *machair* gives way to blanket peat bogs with acidic and oligotrophic lochs (Beveridge, 2001; Friend, 2012). The pH of freshwater lochs on North Uist ranges from 4.5 to 7.4, with a gradient of increasing pH from west to east. The chief fish predator of sticklebacks on North Uist is the brown trout, which occurs naturally in the majority of lochs. The only other freshwater fishes found are eels (*Anguilla anguilla*), which occur infrequently (two elvers caught in 6 years of sampling), ninespine sticklebacks (*Pungitius pungitius*), and small, localized populations of arctic charr (*Salvelinus alpinus*). In sea lochs and freshwater lochs with open marine connections, migratory salmon (*Salar salar*) and sea trout (*S. trutta*) also occur, although these sites were excluded from the present study because these open systems could not be treated as independent in our analysis. Fishing rights on North Uist are controlled by the North Uist Estate, which maintains detailed fish catch records spanning several decades.

We tested the prediction that in the case that trout predation was the major selective agent for stickleback morphology, stickleback lateral plate number and pelvic score would be greatest at high brown trout abundances. Alternatively, if ion availability was the major

agent of selection for the evolution of stickleback plate and pelvic apparatus, we predicted morphology to vary as a function of dissolved ion concentrations, with sticklebacks displaying the most limited plate number and pelvic score at sites where dissolved ion concentrations were lowest.

METHODS

Stickleback morphology

We collected threespine sticklebacks and water samples from 26 lochs on North Uist in 2013 (Table 1, Fig. 1). A total of 860 sticklebacks were collected using long-handled dip nets,

Table 1. Lochs on North Uist sampled in 2013 with estimates of dissolved calcium and phosphorus concentration, pH, and rank trout abundance (1 = low abundance, 4 = high abundance)

Site number	Site name	OS grid reference	Rank trout abundance	Dissolved calcium ($\text{mg} \cdot \text{L}^{-1}$)	Dissolved phosphorus ($\mu\text{g} \cdot \text{L}^{-1}$)	pH	Sample size
1	Loch Fada	875 705	4	1.7	5	5.9	36
2	Loch á Bharpa*	837 664	1	1.6	1	5.9	18
3	Loch nan Eun*	845 675	4	1.0	2	4.5	19
4	Loch Huna*	813 665	4	2.1	1	6.0	25
5	North Sgadabhagh	868 685	3	0.7	1	5.7	48
6	Loch na Maighdein*	893 683	2	3.4	1	6.4	37
7	Loch na Moracha*	846 665	4	2.6	3	6.1	34
8	Loch á Bhuird*	883 675	4	1.9	7	5.2	50
9	Loch nan Geadh*	888 706	2	2.2	5	6.0	33
10	Loch Tormasad*	820 650	3	4.0	1	6.3	7
11	Loch Bhreagbhat*	882 723	2	1.4	1	5.7	37
12	Loch an Daimh*	889 678	1	2.7	1	5.7	40
13	Loch nan Geireann*	845 725	2	1.9	3	6.0	39
14	Loch Croghearraidh*	716 712	2	19.6	5	7.1	40
15	Loch Sgarraigh*	717 705	2	48.2	2	7.4	50
16	Loch Sanndaraigh*	735 685	2	16.8	7	7.0	30
17	Loch Steineabhat*	875 743	3	4.0	0	6.5	14
18	Loch na Morgha*	870 743	3	0.9	1	6.0	21
19	Loch Hosta*	727 727	4	17.8	3	7.4	50
20	Loch Dubhasairidh*	772 673	2	1.2	1	6.1	42
21	Loch nan Athan*	778 668	3	0.9	1	5.7	55
22	Loch á Charra	779 689	2	1.2	1	6.1	28
23	Loch nan Strùban*	808 647	4	1.5	1	5.7	25
24	Loch an Toim	794 658	4	1.2	1	5.6	23
25	Loch na Creige	883 737	2	3.6	—	5.7	35
26	Bogach Maari	860 719	1	2.4	20	6.1	24
27	Loch Eubhal*	725 713	3	—	—	—	—
28	Loch nan Cethir Eilean*	858 664	2	—	—	—	—
29	Loch Hungabhat*	873 724	3	—	—	—	—
30	Wee Fada*	793 667	1	—	—	—	—

*Populations used in the trout predation assay in 2014.

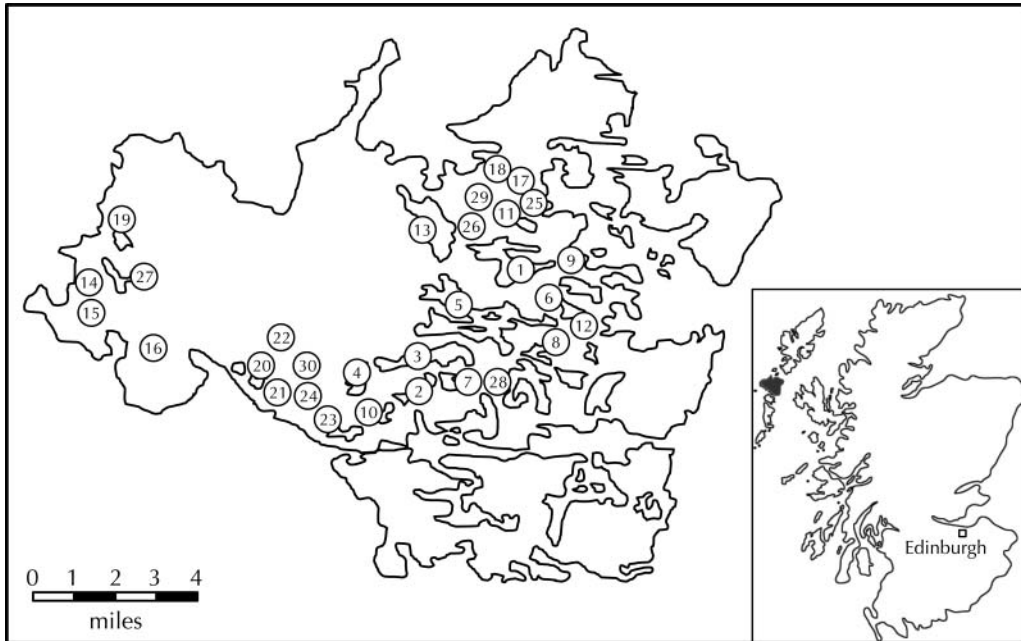


Fig. 1. Map of sampling sites on North Uist, Scotland. Numbered sites correspond with site numbers in Table 1. Inset shows mainland Scotland and Western Isles, with North Uist shaded black.

killed with anaesthetic (benzocaine), and fixed in 4% buffered formalin. Samples were restricted to adult fish >26 mm standard length (SL, measured from the tip of the snout to the origin of the tail) to ensure morphological characteristics were fully developed (Bell, 1981). After fixing, fish were transferred to 70% ethanol for 24 hours, stained with 0.08% alizarin red in 1% KOH for 24 hours, rinsed in water for 24 hours, and stored in 70% ethanol (Brubaker and Angus, 1984).

For each fish a record was made of dorsal spine number, the presence or absence of an anal spine, and the total number of lateral plates. A pelvic score was also assigned to each fish, which comprised a count of the elements of the pelvic structure, with a score of zero indicating complete absence of the pelvic structure and 8 the presence of all the pelvic elements (Klepaker *et al.*, 2013). No lochs showed bimodality in plate or pelvic morph.

Water chemistry

A single water sample was collected from each loch in one-litre sample bottles from below the water surface at a depth of at least 1 m. Dissolved calcium concentration was measured by atomic absorption spectrometric methods (ISO 7980:1986), phosphorus concentration by ammonium molybdate spectrometric methods (EN 1189:1996), and pH was measured electrochemically (ISO 10523:2008). An accredited commercial laboratory performed all water chemistry measurements.

Predator abundance

Brown trout abundance was estimated as a proxy for predation by piscivorous fish using records of brown trout catches by anglers. Lochs were scored on an ordinal scale of 1–4 based on historical catch records over the previous 30-year period, with 1 indicating lochs with the lowest historical brown trout catches and 4 the highest. Lochs were scored independently by the North Uist Estate manager, who is responsible for collating trout catch records. This measure of trout abundance provided a robust index of predation risk for sticklebacks by brown trout among populations, being based on comprehensive long-term records for simple ecological systems, with brown trout the only abundant piscivorous fish in freshwater lochs (Spence *et al.*, 2013). Anecdotal evidence and catch return data indicate little change in brown trout abundances among lochs over several decades (George Macdonald, personal communication). Catch records were based on trout caught by fly fishing, since this is the sole fishing technique permitted on North Uist. ‘Fly’ fishing is a misnomer, since the lures used by fly fishermen more typically imitate small fish than invertebrates, and the favoured fly patterns used on North Uist are fish mimics. Thus, trout catches effectively provided an index of attack rates on small fish, of which sticklebacks are the primary representatives.

To validate the relationship between this ordinal scale of trout abundance and predation risk to sticklebacks from trout, we conducted an additional study. Stickleback lures were prepared by a professional fly-tier, with either red or white hackles to mimic sticklebacks with and without red coloration (www.evolutionary-ecology.com/data/2999Appendix.pdf, Fig. S1). A sample of 24 lochs were subsequently fished by an experienced fly fisherman during May 2014 using a single stickleback lure with a 2.9 m carbon fly rod, floating fly line, and 3 m leader of 0.18 mm monofilament. The perimeter of each loch was fished for one hour and either a red or white stickleback lure was randomly selected for use at each loch. Each lure was used only once and was not reused. The frequency that brown trout were hooked and subsequently lost or hooked and landed was recorded. The fork length (tip of the snout to the end of the middle caudal fin ray) of every landed trout was measured to the nearest 1 mm. When a fish was hooked and either lost or landed, fishing was resumed at least 20 m away from the original site. A record of water temperature was taken at each site and lochs exhibiting the entire range of trout abundances were sampled. Loch variables are presented in Table 1. All trout were released after capture.

Data analysis

Before applying statistical models, a data exploration was undertaken following the protocol described in Ieno and Zuur (2015). The data were examined for outliers in the response and explanatory variables, homogeneity and zero inflation in the response variable, collinearity between explanatory variables, and the nature of relationships between the response and explanatory variables. Data analyses were performed using R (R Development Core Team, 2014).

Water pH and dissolved calcium concentration were collinear, and pH was subsequently dropped from the analysis. Morphological measurements were obtained for only seven fish in one loch (Loch Tormasad), so this site was also omitted from the final analysis. Trout abundance among lochs was unbalanced and was subsequently re-categorized as low (ordinal score 1 and 2) and high abundance (3 and 4).

Stickleback plate number was modelled using a generalized linear mixed model (GLMM) with dissolved calcium (*Calcium*), phosphorous (*Phosphorous*), and trout abundance (*Trout*) as main effects. A random intercept *Loch* was included in the model to introduce a correlation structure between observations from the same loch. Assuming estimates of plate number (*Plate*) for fish *i* in loch *j* were Poisson distributed with mean μ_{ij} , the model was specified as:

$$\begin{aligned} Plate_{ij} &\sim \text{Poisson}(\mu_{ij}) \\ E(Pelvic_{ij}) &\sim \mu_{ij} \\ \log(\mu_{ij}) &= \eta_{ij} \\ \eta_{ij} &= \beta_1 + \beta_2 \times \text{Calcium}_{ij} + \beta_3 \times \text{Phosphorus}_{ij} + \beta_4 \times \text{Trout}_{ij} + a_i \\ a_i &= N(0, \sigma_{Loch}^2) \end{aligned}$$

For pelvic score (*Pelvic*), a residuals plot demonstrated a non-linear pattern in the data. Consequently, these data were modelled using a generalized additive mixed model (GAMM), with loch as a random intercept and a smoother fitted to dissolved calcium concentration. The model took the form:

$$\begin{aligned} Pelvic_{ij} &\sim \text{Poisson}(\mu_{ij}) \\ E(Pelvic_{ij}) &\sim \mu_{ij} \\ \log(\mu_{ij}) &= \eta_{ij} \\ \eta_{ij} &= \beta_1 + f_1(\text{Calcium}_{ij}) + \beta_2 \times \text{Phosphorus}_{ij} + \beta_3 \times \text{Trout}_{ij} + a_i \\ a_i &= N(0, \sigma_{Loch}^2) \end{aligned}$$

$f_1(\text{Calcium}_{ij})$ was included as a smooth function to model non-linear changes in pelvic score in response to calcium concentration. The smoother was parameterized using a thin-plate regression spline (Zuur *et al.*, 2014).

As no plate or spine loss was observed in lochs with dissolved calcium concentrations greater than $10 \text{ mg} \cdot \text{L}^{-1}$, analyses were repeated only for those lochs with dissolved calcium concentrations below this threshold. This additional analysis enabled us to examine the impact of environmental variables on plate number and pelvic score only where calcium was limiting. In the case of pelvic score, data showed no non-linearity and the model was fitted with a GLMM rather than a GAMM.

In the analysis of trout abundance, we applied a Poisson GLM and performed a backward stepwise model selection procedure. A main model was selected *a priori*, which predicted that attack rate by trout on stickleback lures was dependent on the variables: trout abundance (high or low), water temperature, time of day (am or pm), water clarity (clear or peat-stained), and lure colour (red or white). Stickleback morph (*low* and *minimal*) and water clarity (clear and peat-stained) were collinear, and morph was subsequently dropped from the analysis. Working from the main model, a set of 10 biologically plausible candidate models were built and compared using the Akaike Information Criterion (AIC) in the *MuMIn* package and ranked according to their ΔAIC (difference between AIC of the best model and the compared model). A subset of the best models ($\Delta\text{AIC} < 6$) were entered in a model averaging procedure and used to generate model-averaged coefficients.

RESULTS

Stickleback plate number was predicted by dissolved calcium concentration, but not by dissolved phosphorous concentration or trout abundance (Table 2). The relationship with dissolved calcium was positive, with a higher calcium concentration predicting higher plate number (Fig. 2A). For the analysis only of lochs with dissolved calcium concentrations $< 10 \text{ mg} \cdot \text{L}^{-1}$, plate number was not predicted by either dissolved calcium or trout abundance (Table 2), although there was a negative effect of dissolved phosphorous concentration (Fig. 2B).

For pelvic score the pattern was similar. Dissolved calcium and phosphorous predicted pelvic score, while trout abundance did not (Table 2). Dissolved calcium was positively associated with pelvic score (Fig. 3A), while phosphorous showed a negative association (Fig. 3B). For lochs with dissolved calcium concentrations $< 10 \text{ mg} \cdot \text{L}^{-1}$, there was similarly a negative association of dissolved phosphorous concentration with pelvic score (Fig. 3C), while neither dissolved calcium nor trout abundance were significant predictors (Table 2).

The estimate of model-averaged coefficients for trout abundance indicated water temperature had the highest relative importance in the composite model, along with water clarity and trout abundance (Table 3). Trout attack rates were higher in lochs with higher numbers of trout, with high water clarity, and at higher water temperatures (Fig. 4).

Table 2. Summary of GLMM* and GAMM[#] for Poisson distributed data to examine effects of trout abundance, dissolved phosphorus, and dissolved calcium on lateral plate number and pelvic score of threespine sticklebacks (*Gasterosteus aculeatus*) in North Uist lochs

Dependent	Parameter	β	SE	d.f. _{est}	<i>P</i>
Lateral plate number* (full data set) $R^2 = 0.56$	Intercept	1.73	0.31	—	<0.001
	<i>Trout</i> _(high)	-0.57	0.44	—	0.197
	<i>Phosphorus</i>	-0.23	0.22	—	0.291
	<i>Calcium</i>	0.60	0.26	—	0.021
Pelvic score [#] (full data set) $R^2 = 0.39$	Intercept	1.39	0.35	—	<0.001
	<i>Trout</i> _(high)	-0.59	0.45	—	0.348
	<i>Phosphorus</i>	-0.50	0.24	—	<0.001
	<i>s</i> (<i>Calcium</i>)	—	—	1.69	0.006
Lateral plate number* (sites $\text{Ca}^{2+} < 10 \text{ mg} \cdot \text{L}^{-1}$) $R^2 = 0.51$	Intercept	1.39	0.35	—	<0.001
	<i>Trout</i> _(high)	-0.59	0.45	—	0.197
	<i>Phosphorus</i>	-0.50	0.24	—	0.040
	<i>Calcium</i>	-0.06	0.21	—	0.765
Pelvic score* (sites $\text{Ca}^{2+} < 10 \text{ mg} \cdot \text{L}^{-1}$) $R^2 = 0.42$	Intercept	2.09	0.23	—	<0.001
	<i>Trout</i> _(high)	-0.21	0.31	—	0.494
	<i>Phosphorus</i>	-0.67	0.16	—	<0.001
	<i>Calcium</i>	-0.09	0.14	—	0.515

Note: Analyses include the full data set and lochs with dissolved calcium concentrations $< 10 \text{ mg} \cdot \text{L}^{-1}$. Parameter coefficient (β), standard error (SE), estimated degrees of freedom (d.f._{est}) for the GAMM spline function (*s*), and statistical significance (*P*).

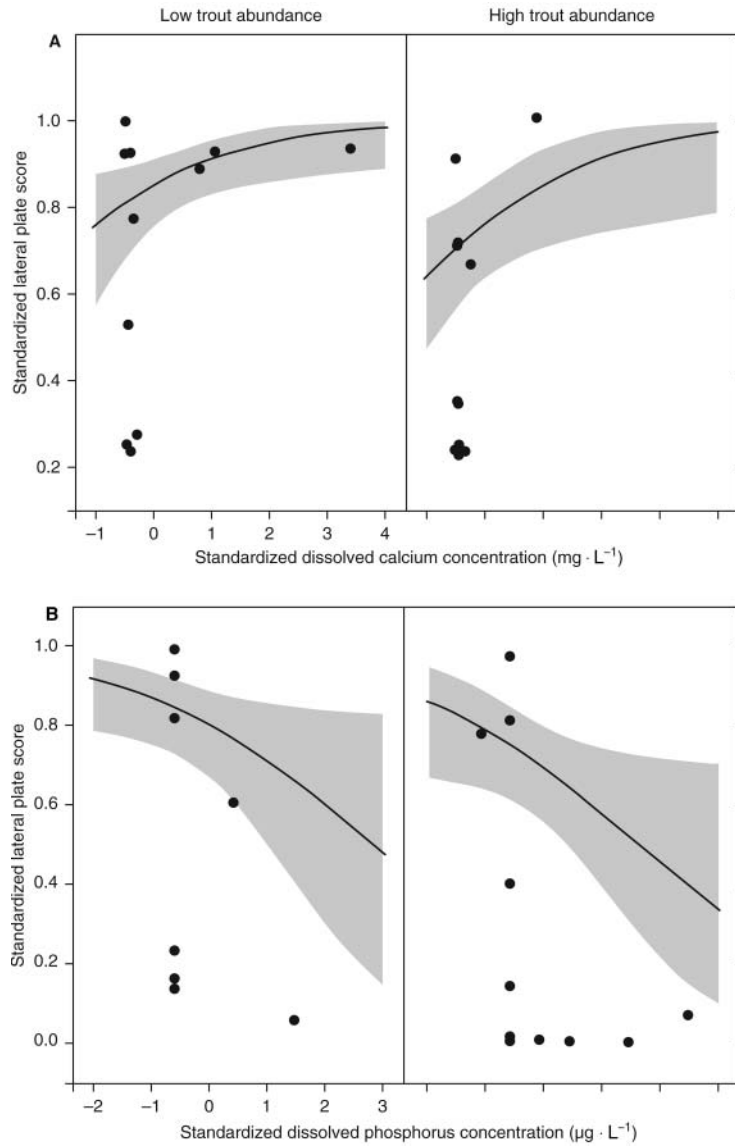


Fig. 2. Fitted values for standardized lateral plate number for threespine sticklebacks from lochs with low and high brown trout abundance modelled using a Poisson GLMM against (A) standardized dissolved calcium concentration ($\text{mg} \cdot \text{L}^{-1}$) for all lochs, and (B) standardized dissolved phosphorus concentration ($\mu\text{g} \cdot \text{L}^{-1}$) for lochs with dissolved calcium concentrations $< 10 \text{ mg} \cdot \text{L}^{-1}$. Grey bands indicate 95% confidence intervals around the fitted line.

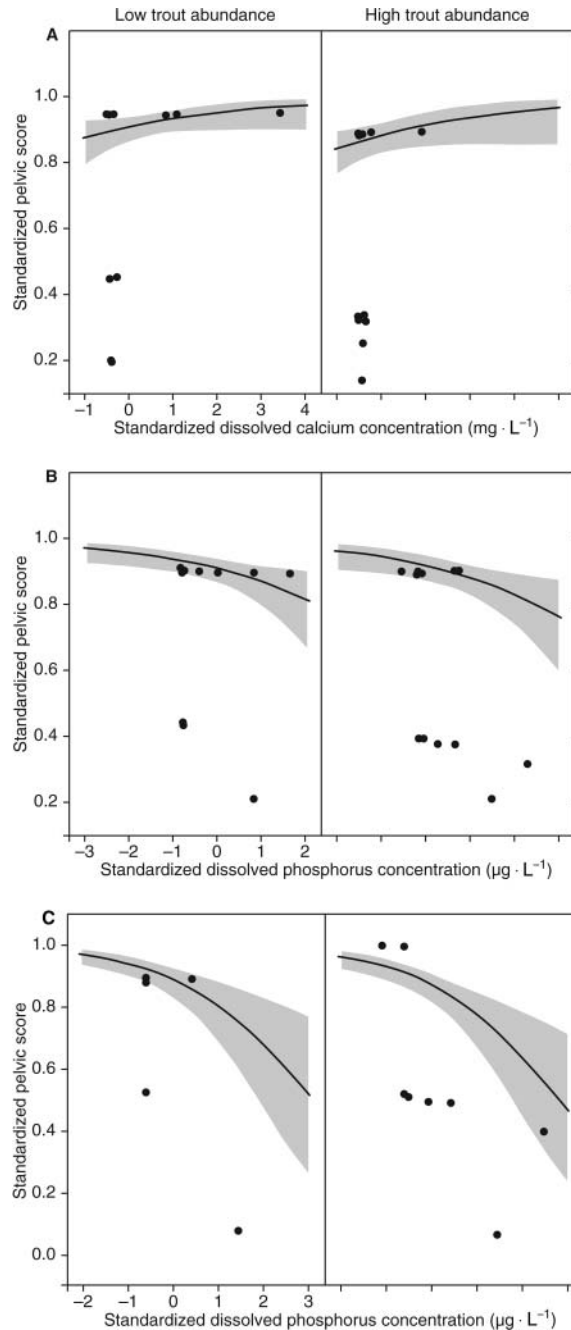


Fig. 3. Fitted values for standardized pelvic score for threespine sticklebacks from lochs with low and high brown trout abundance against (A) standardized dissolved calcium concentration ($\text{mg} \cdot \text{L}^{-1}$) for all lochs modelled using a Poisson GAMM, (B) standardized dissolved phosphorous concentration ($\mu\text{g} \cdot \text{L}^{-1}$) for all lochs modelled using a Poisson GAMM, and (C) standardized dissolved phosphorous concentration ($\mu\text{g} \cdot \text{L}^{-1}$) for lochs with dissolved calcium concentrations $< 10 \text{ mg} \cdot \text{L}^{-1}$ modelled using a Poisson GLMM. Grey bands indicate 95% confidence intervals around the fitted line.

Table 3. Summary of GLM for Poisson distributed data to examine effects of brown trout (*Salmo trutta*) abundance, water clarity, and water temperature on the attack rate of brown trout (*Salmo trutta*) on threespine stickleback (*Gasterosteus aculeatus*) lures

Model parameter	Relative importance	β	SE	<i>P</i>
Intercept	—	-1.97	1.76	0.282
<i>Trout</i> _(low)	0.73	-0.53	0.25	0.046
<i>Water clarity</i> _(peat)	0.89	-0.62	0.25	0.021
<i>Temperature</i>	0.93	0.30	0.11	0.012

Note: Parameter coefficient (β), standard error (SE), and statistical significance (*P*) are shown for a composite model based on models <6 units of AIC compared with the best model (*N* = 10 models). Coefficient of determination (R^2) = 0.55.

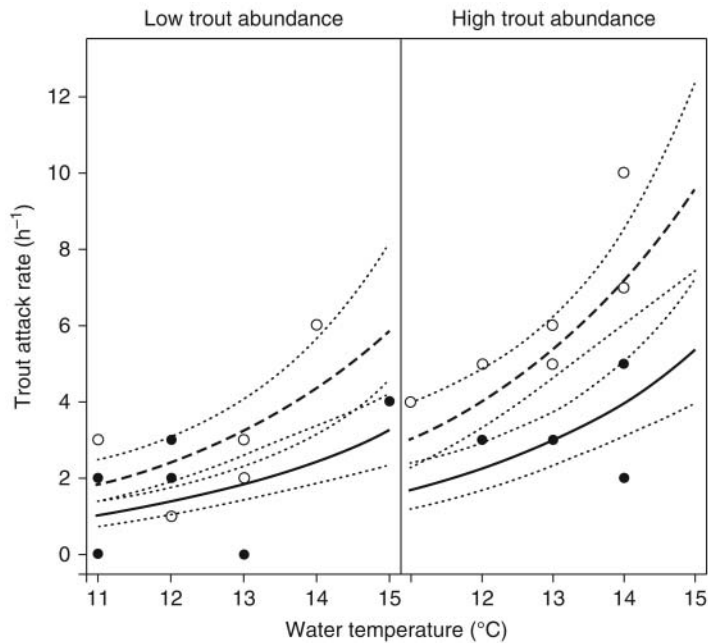


Fig. 4. (A) Fitted values for brown trout attack rate (h^{-1}) on threespine stickleback lures against temperature ($^{\circ}C$) for lochs with low and high trout abundance with peat-stained (solid line, solid circles) and clear water (dashed line, open circles) modelled using a Poisson GLM. Dotted lines indicate 95% confidence intervals around fitted lines.

DISCUSSION

Lateral plate and pelvic evolution in the threespine stickleback provides an exceptional vertebrate model system for investigating the relationship between genotype, phenotype, and fitness, and thereby a full account of the mechanism of adaptive evolution (Wootton, 2009; Schluter *et al.*, 2010). In the present study, dissolved calcium concentration reliably predicted stickleback lateral plate and pelvic score among populations on North Uist, where threespine sticklebacks have undergone a spectacular morphological radiation. Dissolved phosphorous also varied with pelvic score, particularly for those sites in which calcium was limiting. Trout abundance failed to reliably predict either plate or pelvic morphology. The attack rate on lures that mimicked sticklebacks was greater at sites with high trout abundance, implying that selection pressure from predators operated in these populations, but failed to explain plate and pelvic morph. Water temperature and clarity were also positively related to attack rate by trout on stickleback lures.

The results of the present study, and previous work on North Uist lochs (Giles, 1983; Spence *et al.*, 2013) and elsewhere (Bell *et al.*, 1993; Bourgeois *et al.*, 1994), implicate a role for dissolved calcium on plate number and pelvic score in sticklebacks, while evidence for a role for selection driven primarily by trout predation was less pronounced. The correlation between dissolved calcium and stickleback plate and pelvic morph on North Uist is overwhelming (Smith *et al.*, 2014). At all sites in North Uist, threespine sticklebacks with reduced plate and pelvic scores were *only* found in lochs with low dissolved calcium concentrations, irrespective of trout abundance. If trout predation were the chief variable driving morph evolution, highly reduced plate and pelvic morphs – what Spence *et al.* (2013) termed *minimal* morphs – would be predicted in some of the calcium-rich *machair* lochs with few trout; but they were never found in those sites. This pattern of morphology reflects the more widespread step-change between the distribution of plate morphs in marine and freshwater populations, with *complete* and *partial* populations found almost exclusively in the marine environment, while *low* morph fish are exclusively found in fresh water.

Evidence that dissolved calcium can impose selection on stickleback skeletal growth is not wholly correlational. An experiment by Spence *et al.* (2012) demonstrated independent effects of dissolved calcium and salinity on the growth rate of sticklebacks and detected a significant interaction of both with plate morph. Stickleback morphs with the highest plate number and pelvic score showed significantly lower growth rates when exposed to low versus high dissolved calcium concentrations, while sticklebacks with lower plate numbers and pelvic score experienced no impact of calcium concentration on growth. Barrett *et al.* (2008) simulated an invasion of sticklebacks of marine origin that were heterozygous at the *Eda* locus in experimental freshwater ponds. They showed that the *low* morph allele increased in frequency in association with faster growth, better overall survival, and earlier breeding in those fish that carried the allele, though without identifying what generated selection. This study also suggested that the *Eda* gene may have additional, pleiotropic effects on other key traits, or is at least linked to another locus that impinges on fitness (Schluter *et al.*, 2010).

Imposed on this general and almost universal pattern of plate and spine evolution, are some exceptions that challenge our understanding of the evolution of stickleback plate and pelvic morphs. Thus in Central and Eastern Europe, eastern North America, and north-eastern Asia, there are resident freshwater populations of the *complete* morph (Wootton, 1976; Hagen and Moodie, 1982; Bañbura, 1994; Paepke, 1996). There is also evidence that the mechanisms

for calcium and osmotic regulation in these populations may diverge from those of populations displaying the more typical distribution of plate morphs (Spence *et al.*, 2013), and our understanding of why *complete* morph populations of sticklebacks are sometimes resident in fresh water remains incomplete (Smith *et al.*, 2014), though low winter temperatures (Wootton, 1976) and lake volume (Reimchen *et al.*, 2013) may play a role.

In lochs in which calcium was potentially limiting, here defined as sites with dissolved calcium concentrations $< 10 \text{ mg}\cdot\text{L}^{-1}$, dissolved calcium concentration as well as trout abundance failed to predict plate number and pelvic score; dissolved phosphorous did predict morphology, although only weakly in the case of plate number. Bell *et al.* (1993) identified a dissolved calcium concentration of $10\text{--}20 \text{ mg}\cdot\text{L}^{-1}$ as a threshold for pelvic reduction among threespine sticklebacks in Alaska. Notably, in a comparable study to that of Spence *et al.* (2012), the effects of calcium restriction on sticklebacks with contrasting degrees of pelvic expression failed to detect an effect on somatic growth (Rollins *et al.*, 2014). This finding implies that a trade-off between somatic growth and pelvic phenotype does not necessarily occur when calcium is limiting.

At low dissolved calcium concentrations, variation in calcium levels failed to predict plate or pelvic phenotype among North Uist stickleback populations. This outcome is not unexpected, given the limited variation in dissolved calcium concentrations among this subset of lochs. However, two features of our findings are inconsistent with the explanation that dissolved calcium alone explains plate and spine variability among North Uist populations. The first is that some lochs with low dissolved calcium concentrations supported populations of *low* rather than *minimal* morph fish. Second, the degree of loss of lateral plate, spines, and pelvic elements encountered in North Uist populations of three-spine sticklebacks is extremely rare across the entire distribution of the stickleback. Nonetheless, while dissolved calcium concentrations in many North Uist lochs are low, they fall within the range for freshwater lakes elsewhere in Europe and North America that support *low* morph stickleback populations (Reimchen *et al.*, 2013). Thus, while the reduced availability of dissolved calcium is strongly associated with the evolution of *minimal* plate and spine morphs on North Uist, it appears not to be the only variable driving selection.

Detailed morphological data on lateral plate height, width, thickness, and degree of overlap were not recorded. Similarly, expression of the pelvic apparatus and spine length and thickness were scored on a relatively crude scale. A more fine-grained analysis of these traits might have revealed a closer relationship with ionic concentration or predation risk. A study by Wiig (2014) using micro-computed tomography scanning demonstrated marked differences among populations of sticklebacks from different environments in total skeletal bone volume, including plate size and thickness. These findings raise the possibility that sticklebacks can express alternative evolutionary pathways in armour reduction in response to different selective pressures.

Several factors can influence the flux of calcium or other essential elements for skeletal growth in fishes. Notably, fish are able to utilize calcium in their diet. Studies have shown that fish can express normal growth in calcium-free water, provided that they receive adequate calcium from their food (Robinson *et al.*, 1986). Other studies have additionally shown that freshwater fish increase their intestinal absorption of calcium when it is scarce (Ichii and Mugiyu, 1983; Allen *et al.*, 2011). A weakness of the present study was that dissolved calcium alone was included in our model. However, the availability of calcium in the diet, which potentially varied among populations, was not considered. Different modes of feeding may also provide different levels of calcium (i.e. a benthic vs. a limnetic diet). Furthermore, the

bioavailability of calcium in different dietary items can vary, with calcium potentially less readily utilized from some food items (Hossain and Yoshimatsu, 2014).

Another possible explanation for the evolution of *minimal* morphs is reduced efficiency to utilize calcium. Variation in the concentration of the hormone prolactin has been shown to significantly influence the uptake of calcium in fish (Flik *et al.*, 1986; Wongdee and Charoenphandhu, 2013). It has been shown that prolactin secretion is higher in freshwater sticklebacks than in marine populations (Wendelaar Bonga, 1978). Variation among stickleback populations in the production of prolactin will have implications for calcium uptake and, subsequently, how selection acts on skeletal growth when calcium is limiting.

A key finding of the present study was the significant effect of phosphorus on pelvic score, independently of calcium, including in low dissolved calcium lochs. Like calcium, phosphorus plays a key role in the development and maintenance of the skeletal system in fish (Lall and Lewis-McCrea, 2007). Phosphorus concentrations in fresh waters are low and while calcium can be absorbed across the gills, diet is the primary source of phosphorus for fish (Witten and Huyseune, 2009). Phosphorus deficiency results in bone resorption, causing decreased somatic growth, reduced bone mineralization, and skeletal deformities (Lall, 2002). Therefore, bone development and growth are highly dependent on the concentration and availability of dietary phosphorus (Roy and Lall, 2003). Thus like calcium, phosphorus availability has the potential to select for reduced skeletal development. In the present study, however, high pelvic scores were associated with low dissolved phosphorus concentrations, and in low calcium concentration sites, with plate number. Why these seemingly related skeletal structures would show a strong positive association with calcium but a negative one with phosphorus is unclear. However, since dissolved phosphorus may be an unreliable proxy for organic phosphorus, we cannot conclude that variation in phosphorus available to sticklebacks in their diet is a selective factor in plate and pelvic spine evolution. Despite this, it is known that the Ca:P ratio influences growth in fish (Robinson *et al.*, 1987; Hossain and Yoshimatsu, 2014) and the potential role of phosphorus in stickleback morphological evolution is one that warrants further study.

A further complicating issue in this context is in the flux of ions between fish and their environment. The presence of the elements cadmium and zinc has been shown to inhibit calcium uptake through the gills (Sauer and Watabe, 1988; Wong and Wong, 2000), while the presence of hydrogen sulphide may promote calcium uptake (Kwong and Perry, 2015) and fluxes of other ions, such as sodium and chlorine, are influenced by calcium (Evans, 1993). Thus a more detailed analysis of water chemistry of the North Uist lochs is needed to explore the combined effects of calcium and phosphorus with other elements and ions that potentially affect their availability and uptake and their subsequent impact on stickleback plate and spine evolution.

In the present study, just a single water sample was collected at each site, on the assumption that it was representative of the entire loch. Analysis of water samples from multiple sites within lochs may show variation in water chemistry, raising the possibility of intra-loch variation in selection on different plate and spine morphs. However, while stickleback morphs varied markedly among populations, intra-loch variation in morph was limited, with populations effectively monomorphic, suggesting that local variation in water chemistry had limited impact on selection.

Presenting fishing lures that mimicked the appearance of sticklebacks predicted trout abundance (Table 3). This finding supports our assumption that greater trout abundance translated into a higher risk of trout predation. This result also demonstrates the potential

utility of this method as a means of assaying stickleback predation risk from trout. The attack rate on lures by trout was also greatest at higher water temperatures and in lochs with clear water rather than heavily peat-stained water (Fig. 4). The significant positive effect of water temperature on trout attack rates on stickleback lures reflected the strong relationship between temperature and feeding rate in trout (Elliott, 1976; Wootton, 1990). Similarly, the positive effect of water clarity on attack rate was presumably a function of the greater distance at which lures could be detected by foraging trout, which hunt primarily by sight (Bowmaker, 1995). The relationship between reaction distance and the optical characteristics of their habitat is well characterized for salmonids (e.g. Vogel and Beauchamp, 1999).

Predation rates by birds and mammals (principally the Eurasian otter, *Lutra lutra*) were not quantified in the study. However, we had no *a priori* reason to expect that selection pressure imposed by these mobile predators would vary systematically between lochs, and brown trout were predicted to represent the most significant predatory threat to sticklebacks (Giles, 1981; Spence *et al.*, 2013). Otters rarely include sticklebacks in their diet (Watt, 1995), and avian predators, such as grey herons (*Ardea cinerea*), are limited in numbers throughout the Outer Hebrides (Marquiss, 1989). Black-throated (*Gavia arctica*) and red-throated divers (*G. stellata*) are summer visitors to freshwater lochs on North Uist, though at low densities (four pairs seen in 6 years of research) and there is seldom more than a single pair on a loch where they do occur (Gibbons *et al.*, 1997). Common (*Sterna hirundo*) and Arctic terns (*S. paradisaea*) are also summer visitors to the Outer Hebrides and are known to feed on sticklebacks (Wootton, 1984). However, these birds are not as abundant in the Western Isles as further south, and feed on marine rather than freshwater fish species (Buxton, 1985; Clode and Macdonald, 2002). Predatory odonate larvae were encountered during sampling in North Uist. These invertebrate predators were not systematically surveyed in our study, but were encountered more frequently in the calcium-rich *machair* lochs than in low-pH sites. This observation corresponds with the finding that odonate larval survival rates correlate positively with pH (Bell, 1971). Thus these predators were most frequently associated with the *low* rather than *minimal* stickleback morph, which was the opposite outcome to that predicted if these predators were acting as selective agents on plate and spine morphs, since they are predicted to select for reduced plate and spine morphs.

Stickleback morph and water clarity were collinear in our data, with *minimal* morphs associated with peat-stained water. Consequently, we were unable to separate these two covariates in our analysis. Thus, high attack rates on stickleback lures were potentially also associated with higher plate number and pelvic scores. Spence *et al.* (2013) detected a significant difference between stickleback morphs from North Uist in their anti-predator behaviour. Latency to emerge from a refuge among *minimal* morph populations varied significantly with trout abundance, while among better-armoured *low* morph fish it did not. This finding suggests that populations with reduced protection are especially sensitive to predation, possibly because they are more susceptible to trout. An additional finding was that *low* morph fish were overall more cautious than *minimal* morphs, which was explained as a response to differences in water clarity between the typical habitats of the respective morphs, with *low* populations found in clear, alkaline water where fish are more conspicuous, while *minimal* morph populations typically inhabit peat-stained water. These findings provide circumstantial evidence that sticklebacks in clear water lochs, which typically exhibit the *low* morph, experience higher attack rates by trout than those in low-pH, peat-stained lochs, which exhibit *minimal* morphs. A predicted outcome would be an increase in lateral plate number and pelvic score where predation risk is greater and

dissolved calcium and phosphorous are not limiting, but a loss of plates and pelvic structures typical of *minimal* morphs where predators are less efficient (rather than less abundant) and these ions are limiting.

CONCLUSIONS

Based on the data presented here, we cannot identify a simple relationship between environmental factors and the extreme reduction of lateral plates and spine apparatus found among stickleback populations on North Uist. This outcome implies that several factors may interact to drive selection for stickleback plate number and pelvic score. Our findings indicate that the availability of calcium and phosphorous are influential factors, but also provide circumstantial evidence that trout predation, interacting with water clarity, may play a role. An experimental study of the association between selection gradients and environment is needed to understand the interaction of phenotype with fitness and identify which environmental variables are the primary agents of natural selection in the extraordinary adaptive radiation of sticklebacks on North Uist.

ACKNOWLEDGEMENTS

Research was designed by T.K., K.Ø., M.P., C.S., and R.S. Fieldwork was performed by T.K., K.Ø., M.P., C.S., and R.S., morphological and water quality analysis was conducted by T.K., and statistical analyses were designed and performed by C.S. and M.W. C.S. and R.S. wrote the manuscript. We are grateful to George MacDonald of North Uist Estate for granting us permission to sample the Estate's lochs and for his assistance in quantifying brown trout abundances, and to Phil Harding of North Uist Angling Club for permission to sample the club's waters. We are also grateful for helpful comments by Iain Barber, Andrew Hendry, Martin Reichard, Tom Reimchen, Paul Sear, and one anonymous reviewer. Experimental procedures conformed to all legal requirements.

REFERENCES

- Allen, P.J., Weihrauch, D., Grandmaison, V., Dasiewicz, P., Peake, S.J. and Anderson, W.G. 2011. The influence of calcium concentrations on calcium flux, compensatory drinking and epithelial calcium channel expression in a freshwater cartilaginous fish. *J. Exp. Biol.*, **214**: 996–1006.
- Bañbura, J. 1994. Lateral plate morph differentiation of freshwater and marine populations of the three-spined stickleback, *Gasterosteus aculeatus*, in Poland. *J. Fish Biol.*, **44**: 773–783.
- Bañbura, J., Przybylski, M. and Frankiewicz, P. 1989. Selective predation of the pike *Esox lucius*: comparison of lateral plates and some metric features of the three-spined stickle back *Gasterosteus aculeatus*. *Zool. Scripta*, **18**: 303–309.
- Barber, I. 2013. Sticklebacks as model hosts in ecological and evolutionary parasitology. *Trends Parasitol.*, **29**: 556–566.
- Barrett, R.D., Rogers, S.M. and Schluter, D. 2008. Natural selection on a major armor gene in threespine stickleback. *Science*, **322**: 255–257.
- Bell, H.L. 1971. Effect of low pH on the survival and emergence of aquatic insects. *Water Res.*, **5**: 313–319.
- Bell, M.A. 1976. Evolution of phenotypic diversity in *Gasterosteus aculeatus* superspecies on the Pacific coast of North America. *Syst. Zool.*, **25**: 211–227.
- Bell, M.A. 1981. Lateral plate polymorphism and ontogeny of the complete morph of threespine sticklebacks (*Gasterosteus aculeatus*). *Evolution*, **35**: 67–74.

- Bell, M.A. 1988. Stickleback fishes: bridging the gap between population biology and paleobiology. *Trends Ecol. Evol.*, **3**: 320–324.
- Bell, M.A. 2009. Implications of a fossil stickleback assemblage for Darwinian gradualism. *J. Fish Biol.*, **75**: 1977–1999.
- Bell, M.A. and Foster, S.A., eds. 1994. *The Evolutionary Biology of the Threespine Stickleback*. Oxford: Oxford University Press.
- Bell, M.A., Ortí, G., Walker, J.A. and Koenings, J.P. 1993. Evolution of pelvic reduction in three-spine stickleback fish: a test of competing hypotheses. *Evolution*, **47**: 906–914.
- Bell, M.A., Aguirre, W.E. and Buck, N.J. 2004. Twelve years of contemporary armor evolution in a threespine stickleback population. *Evolution*, **58**: 814–824.
- Bell, M.A., Khalef, V. and Travis, M.P. 2007. Directional asymmetry of pelvic vestiges in threespine stickleback. *J. Exp. Zool. B: Mol. Develop. Evol.*, **308B**: 189–199.
- Beveridge, E. 2001. *North Uist*. Edinburgh: Birlinn.
- Bourgeois, J.F., Blouw, D.M., Koenings, J.P. and Bell, M.A. 1994. Multivariate analysis of geographic covariance between phenotypes and environments in the threespine stickleback, *Gasterosteus aculeatus*, from the Cook Inlet area, Alaska. *Can. J. Zool.*, **72**: 1497–1509.
- Bowmaker, J.K. 1995. Visual pigments of fishes. *Prog. Retin. Eye Res.*, **15**: 1–31.
- Brubaker, J.M. and Angus, A. 1984. A procedure for staining fish with alizarin without causing exfoliation of scales. *Copeia*, **1984**: 989–990.
- Buxton, N.E. 1985. The current status and distribution of terns in the Outer Hebrides. *Scott. Birds*, **13**: 172–178.
- Campbell, N.R. 1985. Morphological variation in the three-spined stickleback (*Gasterosteus aculeatus*) in Scotland. *Behaviour*, **93**: 161–168.
- Chan, Y.F., Marks, M.E., Jones, F.C., Villarreal G., Jr., Shapiro, M.D., Brady, S.D. *et al.* 2010. Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a *Pitx1* enhancer. *Science*, **327**: 302–305.
- Clode, D. and Macdonald, D.W. 2002. Invasive predators and the conservation of island birds: the case of American mink *Mustela vison* and terns *Sterna* spp. in the Western Isles, Scotland. *Bird Study*, **49**: 118–123.
- Colosimo, P.F., Hosemann, K.E., Balabhardra, S., Villarreal G., Jr., Dickson, M., Grimwood, J. *et al.* 2005. Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. *Science*, **307**: 1928–1933.
- Elliott, J.M. 1976. The energetics of feeding, metabolism and growth of brown trout (*Salmo trutta* L.) in relation to body weight, water temperature and ration size. *J. Anim. Ecol.*, **45**: 923–948.
- Flik, G., Fenwick, J.C., Kolar, Z., Mayer-Gostan, N. and Wendelaar Bonga, S.E. 1986. Effects on ovine prolactin on calcium uptake and distribution in *Oreochromis mossambicus*. *Am. J. Physiol.: Regul. Integr. Comp. Physiol.*, **250**: R161–R166.
- Evans, D.H. 1993. Osmotic and ionic regulation. In *The Physiology of Fishes* (D.H. Evans, ed.), pp. 315–341. Boca Raton, FL: CRC Press.
- Friend, P. 2012. *Scotland*. London: HarperCollins.
- Gibbons, D.W., Bainbridge, I.P., Mudge, G.P., Tharme, A.P. and Ellis, P.M. 1997. The status and distribution of the red-throated diver *Gavia stellata* in Britain in 1994. *Bird Study*, **44**: 194–205.
- Giles, N. 1981. Predation effects upon the behaviour and ecology of Scottish *Gasterosteus aculeatus* L. populations. PhD thesis, University of Glasgow.
- Giles, N. 1983. The possible role of environmental calcium levels during the evolution of phenotypic diversity in Outer Hebridean populations of the three-spined stickleback, *Gasterosteus aculeatus*. *J. Zool.*, **199**: 535–544.
- Hagen, D.W. and Gilbertson, L.G. 1973. The genetics of the plate morphs in freshwater three-spine sticklebacks. *Heredity*, **31**: 75–84.

- Hagen, D.W. and Moodie, G.E.E. 1982. Polymorphism for plate morphs in *Gasterosteus aculeatus* on the east coast of Canada and an hypothesis for their global distribution. *Can. J. Zool.*, **60**: 1032–1042.
- Hendry, A.P., Peichel, C.L., Matthews, B., Boughman, J.W. and Nosil, P. 2013. Stickleback research: the now and the next. *Evol. Ecol. Res.*, **15**: 111–141.
- Heuts, M.J. 1945. La regulation minerale en fonction de la temperature chez *Gasterosteus aculeatus* L. *Ann. Soc. R. Zool. Belg.*, **76**: 88–99.
- Hohenlohe, P.A., Bassham, S., Etter, P.D., Stiffler, N., Johnson, E.A. and Cresko, W.A. 2010. Population genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. *PLoS Genet.*, **6**: e1000862.
- Hoogland, R.D., Morris, D. and Tinbergen, N. 1957. The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as a means of defence against predators (*Perca* and *Esox*). *Behaviour*, **10**: 205–236.
- Hossain, M.A. and Yoshimatsu, T. 2014. Review: Dietary calcium requirements in fishes. *Aquac. Nutr.*, **20**: 1–11.
- Ichii, T. and Mugiya, Y. 1983. Effects of a dietary deficiency in calcium on growth and calcium uptake from the aquatic environment in the goldfish, *Carassius auratus*. *Comp. Biochem. Physiol.: Mol. Integr. Physiol.*, **74A**: 259–262.
- Ieno, E.N. and Zuur, A.F. 2015. *Data Exploration and Visualisation with R*. Newburgh: Highland Statistics Ltd.
- Kangas, A.T., Evans, A.R., Thesleff, I. and Jernvall, J. 2004. Nonindependence of mammalian denture characters. *Nature*, **432**: 211–214.
- Kitano, J., Bolnick, D.I., Beauchamp, D.A., Mazur, M.M., Mori, S., Nakano, T. *et al.* 2008. Reverse evolution of armor plates in the threespine stickleback. *Curr. Biol.*, **18**: 769–774.
- Klepaker, T. 1993. Morphological changes in a marine population of the threespine stickleback *Gasterosteus aculeatus*, recently isolated in fresh water. *Can. J. Zool.*, **71**: 1251–1258
- Klepaker, T. 1995. Postglacial evolution in lateral plate morphs in Norwegian freshwater populations of the threespine stickleback (*Gasterosteus aculeatus*). *Can. J. Zool.*, **73**: 898–906
- Klepaker, T. and Østbye, K. 2008. Pelvic anti-predator armour reduction in Norwegian populations of the threespine stickleback: a rare phenomenon with adaptive implications? *J. Zool.*, **276**: 81–88.
- Klepaker, T., Østbye, K. and Bell, M.A. 2013. Regressive evolution of the pelvic complex in stickleback fishes: a study of convergent evolution. *Evol. Ecol. Res.*, **15**: 413–435.
- Kondo, S., Kuwahara, Y., Kondo, M., Naruse, K., Mitani, H., Wakamatsu, Y. *et al.* 2001. The medaka rs-3 locus required for scale development encodes ectodysplasin-A receptor. *Curr. Biol.*, **11**: 1202–1206.
- Kwong, R.W.M. and Perry, S.F. 2015. Hydrogen sulfide promotes calcium uptake in larval zebrafish. *Am. J. Physiol.: Cell Physiol.*, **309**: C60–C69.
- Lall, S.P. 2002. The minerals. In *Fish Nutrition* (J.E. Halver and R.W. Hardy, eds.), pp. 259–308. San Diego, CA: Academic Press.
- Lall, S.P. and Lewis-McCrea, L.M. 2007. Role of nutrients in skeletal metabolism and pathology in fish – an overview. *Aquaculture*, **267**: 3–19.
- Marchinko, K.B. 2009. Predation's role in repeated phenotypic and genetic divergence of armor in threespine stickleback. *Evolution*, **63**: 127–138.
- Marquiss, M. 1989. Grey Herons *Ardea cinerea* breeding in Scotland: numbers, distribution, and census techniques. *Bird Study*, **36**: 181–191.
- Mobley, K.B., Ruiz, R.C., Johansson, F., Englund, G. and Bokma, F. 2013. No evidence that stickleback spines directly increase risk of predation by an invertebrate predator. *Evol. Ecol. Res.*, **15**: 189–198.
- Paepke, H.-J. 1996. *Die Stichlinge*. Hohenwarsleben: Westarp Wissenschaften.
- Peichel, C.M., Nereng, K.S., Ohgl, K.A., Cole, B.E.L., Colosimo, P.F., Buerkle, C.A. *et al.* 2001. The genetic architecture of divergence between threespine stickleback species. *Nature*, **414**: 901–905.

- R Development Core Team. 2014. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Reimchen, T.E. 1980. Spine deficiency and polymorphism in a population of *Gasterosteus aculeatus*: an adaptation to predators? *Can. J. Zool.*, **58**: 1232–1244.
- Reimchen, T.E. 1983. Structural relationships between spines and lateral plates in threespine stickleback (*Gasterosteus aculeatus*). *Evolution*, **37**: 931–946.
- Reimchen, T.E. 1991. Trout foraging failures and the evolution of body size in stickleback. *Copeia*, **1991**: 1098–1194.
- Reimchen, T.E. 1992. Injuries on stickleback from attacks by a toothed predator (*Oncorhynchus*) and implications for the evolution of lateral plates. *Evolution*, **46**: 1224–1230.
- Reimchen, T.E. 1994. Predators and morphological evolution in threespine stickleback. In *The Evolutionary Biology of the Threespine Stickleback* (M.A. Bell and S.A. Foster, eds.), pp. 240–277. Oxford: Oxford University Press.
- Reimchen, T.E. 2000. Predator handling failures of lateral plate morphs in *Gasterosteus aculeatus*: implications for stasis and distribution of the ancestral plate condition. *Behaviour*, **137**: 1081–1096.
- Reimchen, T.E., Bergstrom, C. and Nosal, P. 2013. Natural selection and the adaptive radiation of Haida Gwaii stickleback. *Evol. Ecol. Res.*, **15**: 241–269.
- Rennison, D.J., Heilbron, K., Barrett, R.D. and Schluter, D. 2015. Discriminating selection on lateral plate phenotype and its underlying gene, *Ectodysplasin*, in threespine stickleback. *Am. Nat.*, **185**: 150–156.
- Robinson, E.H., Rawles, S.D., Yette, H.E. and Greene, L.W. 1986. Dietary calcium requirement of channel catfish, *Ictalurus punctatus*, reared in calcium-free water. *Aquaculture*, **53**: 263–270.
- Robinson, E.H., LaBomascus, D., Brown, P.B. and Linton, T.L. 1987. Dietary calcium and phosphorus requirements of *Oreochromis aureus* reared in calcium-free water. *Aquaculture*, **64**: 267–276.
- Rollins, J.L., Lohman, B.K. and Bell, M.A. 2014. Does ion limitation select for pelvic reduction in threespine stickleback (*Gasterosteus aculeatus*)? *Evol. Ecol. Res.*, **16**: 101–120.
- Roy, P.K. and Lall, S.P. 2003. Dietary phosphorus requirement of juvenile haddock (*Melanogrammus aeglefinus* L.). *Aquaculture*, **221**: 451–468.
- Sæther, B.E. and Engen, S. 2015. The concept of fitness in fluctuating environments. *Trends Ecol. Evol.*, **30**: 273–281.
- Sauer, G.R. and Watabe, N. 1988. The effects of heavy metals and metabolic inhibitors on calcium uptake by gills and scales of *Fundulus heteroclitus* in vitro. *Comp. Biochem. Physiol.: Toxicol. Pharmacol.*, **91C**: 473–478.
- Schluter, D. 2001. Ecology and origin of species. *Trends Ecol. Evol.*, **16**: 372–380.
- Schluter, D., Marchinko, K.B., Barrett, R.D.H. and Rogers, S.M. 2010. Natural selection and the genetics of adaptation in threespine stickleback. *Phil. Trans. R. Soc. Lond. B: Biol. Sci.*, **365**: 2479–2486.
- Shapiro, M.D., Marks, M.E., Peichel, C.L., Blackman, B.K., Nereng, K.S., Jónsson, B. et al. 2004. Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature*, **428**: 717–723.
- Smith, C., Spence, R., Barber, I., Przybylski, M. and Wootton, R.J. 2014. The role of calcium and predation on plate morph evolution in the three-spined stickleback (*Gasterosteus aculeatus*). *Ecol. Evol.*, **18**: 3550–3554.
- Spence, R., Wootton, R.J., Przybylski, M., Macdonald, K. and Smith, C. 2012. Calcium and salinity as selective factors in plate morph evolution of the three-spined stickleback (*Gasterosteus aculeatus*). *J. Evol. Biol.*, **25**: 1965–1974.
- Spence, R., Wootton, R.J., Barber, I., Przybylski, M. and Smith, C. 2013. Ecological causes of morphological evolution in the three-spined stickleback. *Ecol. Evol.*, **3**: 1717–1726.
- Vogel, J.L. and Beauchamp, D.A. 1999. Effects of light, prey size, and turbidity on reaction

- distances of lake trout (*Salvelinus namaycush*) to salmonid prey. *Can. J. Fish. Aquat. Sci.*, **56**: 1293–1297.
- Wade, M.J. and Kalisz, S. 1990. The causes of natural selection. *Evolution*, **44**: 1947–1955.
- Watt, J. 1995. Seasonal and area-related variations in the diet of otters *Lutra lutra* on Mull. *J. Zool.*, **237**: 179–194.
- Wendelaar Bonga, S.E. 1978. The effects of changes in external sodium, calcium and magnesium concentrations on prolactin cells, skin, and plasma electrolytes of *Gasterosteus aculeatus*. *Gen. Comp. Endocrinol.*, **34**: 265–275.
- Wiig, E. 2014. Variation in armour of three-spine stickleback. MSc thesis, University of Oslo.
- Witten, P.E. and Huysseune, A.A. 2009. A comparative view on mechanisms and functions of skeletal remodeling in teleost fish, with special emphasis on osteoclasts and their function. *Biol. Rev.*, **84**: 315–346.
- Wong, C.K. and Wong, M.H. 2000. Morphological and biochemical changes in the gills of tilapia (*Oreochromis mossambicus*) to ambient cadmium exposure. *Aquat. Toxicol.*, **48**: 517–527.
- Wongdee, K. and Charoenphandhu, N. 2013. Regulation of epithelial calcium transport by prolactin: from fish to mammals. *Gen. Comp. Endocrinol.*, **181**: 235–240.
- Wootton, R.J. 1976. *The Biology of Sticklebacks*. London: Academic Press.
- Wootton, R.J. 1984. *A Functional Biology of Sticklebacks*. London: Croom Helm.
- Wootton, R.J. 1990. *Ecology of Teleost Fishes*. London: Chapman & Hall.
- Wootton, R.J. 2009. The Darwinian stickleback *Gasterosteus aculeatus*: a history of evolutionary studies. *J. Fish Biol.*, **75**: 1919–1942.
- Zeller, M., Lucek, K., Haesler, M., Seehausen, O. and Sivasundar, A. 2012. Little evidence for a selective advantage of armour-reduced threespined stickleback individuals in an invertebrate predation experiment. *Evol. Ecol.*, **26**: 1293–1309.
- Zuur, A.F., Saveliev, A.A. and Ieno, E.N. 2014. *A Beginner's Guide to Generalized Additive Mixed Models with R*. Newburgh : Highland Statistics Ltd.