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The evolutionary ecology of dwarfism in three-spined sticklebacks

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Summary

1. Body size is a defining phenotypic trait, but the ecological causes of its evolution are poorly understood. Most studies have considered only a single putative causal agent and have failed to recognise that different environmental agents are often correlated.

2. Darwin suggested that although trait variation across populations is often associated with abiotic variation, evolution is more likely to be driven by biotic factors correlated with the abiotic variation. This hypothesis has received little explicit attention.

3. We use structural equation modelling to quantify the relative importance of abiotic (pH, metal concentrations) and biotic (competition, predation) factors in the evolution of body size in three-spined sticklebacks *Gasterosteus aculeatus* on the island of North Uist, Scotland. We combine phenotypic data from multiple isolated populations, detailed characterisation of their environment and a common garden experiment that establishes the genetic basis of size differences.

4. Three-spined sticklebacks on North Uist show almost unprecedented intraspecific evolution of body size that has taken place rapidly ($<16\ 000$ years). The smallest fish mature at only 7% of the mass of ancestral, anadromous fish. Dwarfism is associated with reduced abundance of a smaller competitor species, the nine-spined stickleback *Pungitius pungitius*, and with low pH indicative of poor resource conditions. Dwarfism also tends to occur where an important predator, the brown trout *Salmo trutta*, is also small. The abundance of *P. pungitius* and the size of *S. trutta* are themselves related to underlying abiotic environmental variation.

5. Despite the close association between abiotic and biotic factors across populations, our results support Darwin's hypothesis that biotic factors, associated with variation in the abiotic environment, are more important in explaining evolution than is abiotic variation *per se*. This study demonstrates the importance of considering the relationships between environmental variables before conclusions can be drawn about the causes of (body size) evolution on islands.

Key-words: calcium, island rule, nanism, O matrix, selective agent

Introduction

Body size is a defining phenotypic trait of organisms, and evolutionary patterns within and between taxa provide some of the best-known examples of 'rules' in evolution: Bergmann's rule (Bergmann 1847), island rule (Foster 1964; Lomolino 1985) and Cope's rule (Stanley 1973). Cope's rule suggests that body size tends to increase over evolutionary time (Stanley 1973), but in the past decade, there has been a resurgence of interest in the evolution of dwarfism (nanism), with studies of a variety of vertebrate taxa (Brown *et al.* 2004; Kottelat *et al.* 2006; Kraus 2011; Glaw *et al.* 2012; Rittmeyer *et al.* 2012). Studies of fish are conspicuous by their paucity (although see Landry & Bernatchez 2010; Moles *et al.* 2010; Macqueen *et al.* 2011), despite the fact that the world's smallest vertebrate is a fish (Kottelat *et al.* 2006). In addition to changes in body size through time, it is well documented that body size shows particular evolutionary lability on islands (Lomolino 1985;

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Lomolino *et al.* 2012; although see Meiri, Raia & Phillimore 2011). A common pattern among vertebrate taxa is that small species get bigger, while big species get smaller (the island rule, Lomolino 1985). This pattern is not seen in all terrestrial vertebrates and has been hardly examined in fish (although see Herczeg, Gonda & Merila 2009). Despite the interest in documenting patterns of body size variation, we still have a poor understanding of what causes dwarfism, beyond the fact that it often occurs on islands (Lister 1989; Brown *et al.* 2004; Glaw *et al.* 2012; although see Lomolino *et al.* 2012).

Previous work suggests that patterns of body size evolution, especially on islands, are driven by the interplay between resources, competitors and predators (Case 1978; Raia, Barbera & Conte 2003; Raia & Meiri 2006; McNab 2010; Lomolino *et al.* 2012), but the detail remains generally unresolved. Body size therefore provides a good example of how piecemeal is our understanding of the ecological and environmental conditions that drive evolution (MacColl 2011). This is surprising, given that it is a question that vexed Darwin (1859). Darwin clearly thought that the struggle for existence, and hence evolution, was more likely to be driven by biotic factors like predation and competition than by abiotic factors like climate or other aspects of the physical environment (Chapter 3, Darwin 1859). However, in his entangled bank metaphor and elsewhere, Darwin also recognised the 'infinitely complex relations to other organic beings and to external nature' that drive the struggle for existence.

A life-history perspective provides a useful theoretical framework for thinking about how different factors may drive evolution (Palkovacs 2003). In general, reductions in predation (which are thought to be usual on islands) increase survival rates, which is expected to lead to longer life and larger body size. At the same time, decreased competition on islands can increase resource availability, which is expected to lead to increased growth rates and larger body size (Stearns & Koella 1986; Palkovacs 2003). Despite Darwin's observations, what has seldom been taken into account in empirical studies of body size evolution is that different environmental factors may interact, both in their effects on each other, and on phenotype (Stearns & Koella 1986; Grether *et al.* 2001). For example, resource availability may be greater where predators are more common, either because predators reduce competitor density or because productive environments support more predators (Holt 1977). Such relationships between environmental variables, across populations, can be described by a (co)variance matrix that MacColl (2011) has called the 'O' matrix. Relationships between environmental variables may conceal or exacerbate the apparent role of different factors in driving evolution and make it very difficult to interpret the real significance of the many correlative studies that have examined only single putatively causative factors. Because of this, definitive tests of the factors that drive evolution should ultimately rely on experimental manipulation of environmental conditions (putative selective agents) and quantification of subsequent changes in traits. However, the factorial experiments that are necessary should ideally be multigenerational and are therefore difficult and time-consuming to carry out for anything other than the shortest-lived species. Observational evidence obtained from thorough ecological studies can provide insights into the relationships between variation in body size across taxonomic units and systematically quantified environmental variation (Michaux *et al.* 2002; White & Searle 2007; Li *et al.* 2011) and should help the design of future experiments. Observational studies should measure all of the different factors that are hypothesised to affect body size. They require large sample sizes of independent populations so that they can control for the collinearity of environmental factors across populations (Graham 2003) and their potentially confounded effects on evolution (MacColl 2011).

Standard univariate statistical models are not especially good at disentangling multiple interacting processes (Grace 2006), especially when putative mechanisms operate at different levels, for example when underlying environmental variation determines the occurrence of predators and competitors, which in turn determine phenotypic evolution. As hypotheses about the causes of (body size) evolution are inherently complex and multivariate, here we use structural equation modelling to understand how different environmental factors interact (Grace 2006). Specifically, we use a simple structural equation model (SEM) in which abiotic, environmental variables can contribute to variation in body size either directly or indirectly through their influence on biotic variables (competition and predation), which in turn have a direct effect on body size evolution (Fig. 1, see Methods).

Here, we examine the ecological causes of variation in body size in three-spined sticklebacks *Gasterosteus aculeatus* by quantifying variation in body size, competitors, predators and the abiotic resource environment within an adaptive radiation in an archipelago of lochs (lakes) on the Scottish island of North Uist. Among these lochs, there is a strong axis of variation in pH, associated with variation in the concentration of alkaline metals: sodium, potassium, magnesium and especially calcium (see Methods, Waterston *et al.* 1979). This, in turn, is associated with variation in productivity (Waterston *et al.* 1979) and has previously been linked to the evolution of sticklebacks in these lochs (Giles 1983). Because of this previous work, we concentrate on variation in pH and alkaline metals as key aspects of the abiotic resource environment. Alkaline metals are important in major physiological processes, including osmoregulation and the formation of bone, while pH is widely acknowledged as a defining property of freshwaters.

Materials and methods

STUDY SITE

On the island of North Uist (57°35″N; 7°18″W) in the Scottish Western Isles are many lochs that are isolated to a greater or lesser

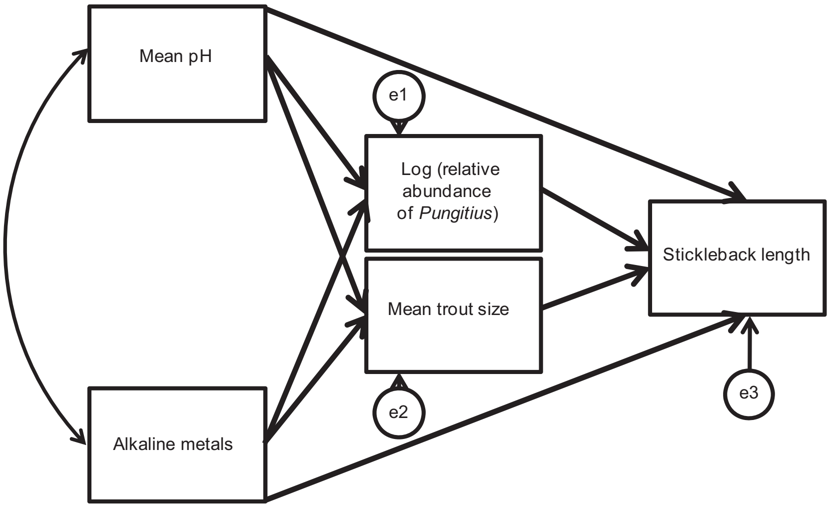

Fig. 1. The *a priori* structural equation model of the direct (solid arrows) and indirect (dashed arrows) relationships between abiotic and biotic variables and stickleback body size. Terms in boxes represent variables, and single-headed arrows imply causal links between them. Circles represent error terms for the endogenous variables. The double-headed arrow indicates correlation between the exogenous variables 'mean pH' and 'alkaline metals', which are assumed to be measured without error.

extent from each other in small, simple catchments (Waterston *et al.* 1979; Giles 1983). The lochs vary substantially in size and are generally shallow (average depth of 11 of the largest (and probably deepest) lochs studied here was 2·8 m a century ago (Murray & Pullar 1910): they are only likely to have become shallower) and well mixed. On the eastern side of the island, oligotrophic lochs lie over peat on predominantly Lewisian gneiss. In contrast, the coastal plain of the western side of the island is strongly influenced by aeolian deposition of marine shell sand, resulting in a 'machair' landscape (Whittington & Edwards 1997), where lochs are hard oligotrophic, mesotrophic or naturally eutrophic, depending on their position and distance from the coast (Waterston *et al.* 1979). The unusual surface geology gives rise to a strong axis of variation in water chemistry across the island. This is characterised by high pH and alkaline metal availability at one end and low pH and alkaline metal availability at the other and is associated with substantial variation in body size of three-spined sticklebacks (Giles 1983). As well as the variation within freshwater lochs, some water bodies are brackish as a result of tidal ingress, adding an additional axis of variation.

The Western Isles were completely covered in ice at the time of the last glacial maximum, approximately 25 000 years ago (Ballantyne & Hallam 2001), but North Uist was probably free of the ice sheet by 15 000 years ago (Ballantyne 2010). In common with freshwater stickleback populations elsewhere in northern Europe (Makinen, Cano & Merila 2006), it is likely that populations of three-spined and nine-spined sticklebacks (*P. pungitius*) in the lochs on North Uist were formed by invasion of anadromous fish from the sea since the ice retreated. Relative sea level rise would have permitted invasion of lochs in the Western Isles soon after the retreat of the ice sheet (Jordan *et al.* 2010), allowing a maximum of about 16 000 years of evolution in freshwater.

Three-spined sticklebacks are common in the lochs of North Uist, where they are a principal component of an otherwise depauperate fish community. Large, anadromous three-spined sticklebacks migrate into coastal lagoons and some freshwater lochs to breed in the spring, but are absent for most of the rest of the year (ADCM, personal observations). In common with previous work on three-spined sticklebacks, we consider that these fish are a good representation of the ancestors of all of the island's three-spined sticklebacks (Bell & Foster 1994). Freshwater lochs are inhabited by phenotypically variable three-spined sticklebacks (Giles 1983). The most obvious difference between freshwater and anadromous fish, apart from size, is that the former generally lack the lateral bony plates that are ubiquitous on anadromous fish. In addition, brackish coastal lagoons contain year-round 'resident' three-spined sticklebacks. Phenotypically similar to freshwater sticklebacks, these breed alongside anadromous fish but are reproductively isolated from them.

In freshwater, nine-spined sticklebacks (hereafter '*Pungitius'*), which are similar (slightly smaller) in body size and trophic position, are the most obvious competitors of three-spined sticklebacks (hereafter 'sticklebacks') (Hart 2003). Brown trout (*Salmo trutta fario*) and European eels (*Anguilla anguilla*) are the only other widespread fish species, although other salmonids also occur (Campbell & Williamson 1979). Arctic charr (*Salvelinus alpinus*) are found in some of the larger, deeper lochs, while migratory Atlantic salmon (*Salmo salar*) and sea trout (*Salmo trutta trutta*) enter those lochs that are accessible to them. Numerous marine fish species are found in tidal lochs (Campbell & Williamson 1979). The lochs on North Uist generally have few invertebrate predators. Water beetle (*Dytiscus semisulcatus*) and dragonfly (*Aeshna juncea* and *Sympetrum* spp.) larvae are present at low density where there is emergent vegetation, which is rare in the North Uist lochs (ADCM, personal observations). The density of avian piscivores is also low (mainly black- and red-throated divers, *Gavia arctica* and *G. stellata*, grey heron *Ardea cinerea* and red-breasted mergansers *Mergus serrator*). Brown trout are therefore likely to be the principal predator of sticklebacks, whose remains are commonly found in their stomachs in at least some lochs (ADCM unpublished data, J.A. MacLeod personal communication). The local North Uist Angling Club has kept a record of all brown trout landed during club fly-fishing competitions from 1956 onwards. We used their data from 1956 to 2006 to estimate the mean mass of captured brown trout ('mean trout size') in lochs where fishing competitions took place.

We are confident that these data provide a good index of the relative sizes of brown trout in these lochs, given the standardised method of capture (only fly-fishing), large sample size of trout, large differences in mean size between lochs (see 'Results') and the length of time over which the data were collected. However, we do not consider that these data provide an unbiased estimate of true mean trout size, because fishing methods are generally targetted at the larger fish in a population. From the perspective of understanding the impact of trout variation on sticklebacks, it is likely to be this fraction of the population that is most important.

STICKLEBACK SAMPLING

We captured sticklebacks in the lochs of North Uist (Table 1) during the breeding season (late April to late May) in 2007, 2008, 2010 and 2011. All of the lochs in this study fall within a total area of 180 km^2, and the greatest distance between any two lochs is 19 km. The area of each loch was estimated from Google Earth using Web-based planimeter software (http://www.freemaptools.com/area-calculator.htm).

Typically, 20 to 30 unbaited minnow traps (Gee traps, Dynamic Aqua, Vancouver, Canada) were set in water approximately 0·3–3 m deep, along a 100–400 m stretch of shoreline. This usually comprised a substantial proportion of the perimeter of a loch (5–25%). Traps were left for one to three (normally two) nights. When lifted, the contents of all traps were emptied into 10-litre buckets and all sticklebacks were counted. A haphazard sample of three-spined sticklebacks (100 or more if these many were available) then had their standard length measured to the nearest 0·5 mm. We call the mean of this measurement for a population 'stickleback length', and we use it as our estimate of body size. We also recorded whether any fish were obviously gravid (females) or showing any nuptial colouration (males). In 2010 and 2011, we recorded the proportion of *Pungitius* in the sample of measured sticklebacks ('Relative abundance of *Pungitius*'). A sample of three-spined sticklebacks was euthanised by overdose of anaesthetic (MS222, tricaine methane sulphonate) and stored in 70% ethanol.

To obtain estimates of size at age, otoliths were extracted from alcohol-preserved sticklebacks for a subset of eight populations

Table 1. Freshwater populations of three-spined sticklebacks on North Uist, with loch name and population 'code', location and environmental characteristics. 'Mean length' gives the overall mean standard length of three-spined sticklebacks in all years in which that population was trapped. Relative abundance of *Pungitius* gives the percentage of trapped sticklebacks that were nine-spined, *P. pungitius*. Mean trout size is the mean mass of brown trout landed during angling contests between 1956 and 2006. '-' in this column indicates that trout are believed to be absent from a loch. We list conductivity in this table because it is very closely correlated (r=0·95, P<0·001) with PC1$_{\text{alkaline}}$, but is a more widely measured and interpretable property of freshwater than principal component scores.

Loch	Code	Mean length	S.E.	N	Grid reference	pH	Conductivity (μS)	Relative abundance of *Pungitius* (%)	Mean trout size (g)	Area (ha)
a'Charra	ACha	36·7	0·58	99	57°36″N; 7°24″W	6·5	163	5	186	8·6
Aonghais	Aong	28·9	0·27	173	57°39″N; 7°16″W	7·0	218	0	428	9·6
Mhic a'Roin	ARoi	40·4	0·88	81	57°35″N; 7°25″W	6·5	173	41	341	6·5
a'Bharpa	Bhar	30·8	0·27	342	57°34″N; 7°17″W	6·0	140	0	304	53·9
Bheireagvat	Bhei	29·2	0·37	101	57°38″N; 7°14″W	6·3	118	0		21·7
a'Bhuird	Bhui	29·2	0·28	70	57°35″N; 7°13″W	6·1	137	5		32·3
na Buaile	Buai	34·0	0·73	70	57°38″N; 7°11″W	6·7	210	0		1·7
Chadha Ruaidh	Chru	29·5	0·35	252	57°36″N; 7°12″W	6·6	149	0	-	2·1
na Creige	Crei	30·8	0·45	70	57°39″N; 7°14″W	6·9	195	0	-	1·6
an Daimh	Daim	35·0	0·26	211	57°35″N; 7°12″W	6·5	162	17		3·6
Dubhasaraidh	Dubh	35·5	0·35	225	57°35″N; 7°24″W	6·8	174	41	548	22·6
Eisiadar	Eisi	30·5	0·30	172	57°38″N; 7°21″W	6·8	130	0	259	11·4
Eubhal	Eubh	37·9	0·99	39	57°37″N; 7°29″W	8·5	395	24	255	34·0
nan Eun	Eun	30·6	0·38	79	57°35″N; 7°17″W	6·1	125	44	135	142·2
nam Feithean	Feit	40·4	1·33	31	57°36″N; 7°30″W	8·3	490	53		15·7
Fhaing Buidhe	Fhai	30·3	0·55	97	57°34″N; 7°23″W	6·6	142	0	322	8·0
nan Geireann	Geir	31·5	0·54	54	57°38″N; 7°17″W	6·7	131	0	299	188·2
Mhic Gille-bhride	Gill	40·4	0·49	226	57°36″N; 7°24″W	6·8	159	14	316	14·0
Grogary	Grog	32·0	0·39	200	57°37″N; 7°30″W	8·2	342	6	328	14·8
Hosta	Host	40·6	0·30	734	57°37″N; 7°29″W	8·3	340	25	449	25·8
na h-Iolaire	Iola	30·1	0·44	87	57°34″N; 7°21″W	6·6	155	0	174	13·2
nam Magarlan	Maga	40·2	0·61	114	57°36″N; 7°29″W	7·8	262	66	384	6·5
Maighdein	Maig	32·3	0·26	185	57°35″N; 7°12″W	7·1	164	0	205	9·6
na Moracha	Mora	33·4	0·34	130	57°34″N; 7°16″W	6·3	174	0	120	37·8
ne Gearrachun	naGe	30·2	1·08	32	57°39″N; 7°25″W	7·0	277	0	-	7·4
na Reival	Reiv	38·1	0·47	616	57°37″N; 7°31″W	9·0	433	5	-	6·1
Sandary	Sann	35·7	0·64	186	57°35″N; 7°28″W	8·3	375	7	517	15·5
Scadavay	Scad	31·3	0·28	366	57°35″N; 7°14″W	6·1	144	3	193	551·6
Scarie	Scar	32·8	0·53	150	57°36″N; 7°30″W	8·5	445	11		10·1
nan Strùban	Stru	30·4	0·29	100	57°34″N; 7°21″W	7·1	165	0	264	19·2
an Toim	Toim	32·5	1·42	25	57°34″N; 7°22″W	6·7	139	38	258	18·4
Tormasad	Torm	30·4	0·17	489	57°33″N; 7°19″W	6·8	181	0	342	21·1
Trosavat	Trof	39·0	1·18	26	57°35″N; 7°25″W	6·7	173	19	663	6·5

spanning the range of environmental and life-history variation. The sagittae were mounted in DPX medium on glass slides and examined at ×400 under a microscope. Sticklebacks were aged following Jones & Hynes (1950).

COMMON GARDEN EXPERIMENT

To determine genetically based variation in body size, we carried out a common garden experiment. In 2008, live three-spined sticklebacks were collected from four lochs (Chru, Torm, Reiv, Host: see Table 1) spanning the range of freshwater variation (de Roij 2010). For each population, F1 offspring were obtained by making eight or nine unrelated full-sib crosses from wild-caught parents using in vitro methods. Briefly, eggs were stripped from a gravid female and were fertilised in a 1‰ salt solution using the minced testes of a euthanised male. Eggs were transported on ice in a 1‰ salt solution to aquaria in a temperature-controlled room (13·5 ± 1 °C) at the University of Nottingham, where they were placed in a plastic cup with a mesh screen on the bottom, suspended in a well-aerated tank containing dechlorinated water and left to hatch. Following hatching, each family was thinned to 15 fry. Fish were maintained under a 16L:8D photoperiod. Fry were fed daily with infusoria (*Colpidium* spp.) for the first 5 days, then twice daily with brine shrimp (*Artemia salina*) naupliae until day 64 post-hatching. Thereafter, fish received chironomid larvae ('bloodworm'; defrosted from frozen) daily. Food was provided to excess. Five fish from each family were haphazardly selected for length and weight measurement at 106 days post-hatching.

WATER CHEMISTRY

The pH and conductivity of lochs was measured using a calibrated pH meter (Multi 340i, WTW, Weilheim, Germany). Most lochs were measured annually over several years. In analyses of pH, we use averages of one to six annual readings per loch (mean = 3·2) taken between April 2006 and May 2011. Two filtered water samples (one acidified with nitric acid, the other not) were collected from each of 33 freshwater lochs in May 2011. These samples were frozen and returned to the University of Nottingham for analysis of metallic cation concentrations by inductively coupled plasma mass spectrometry (ICP-MS), anions using a Dionex DX500 ion chromatograph with an IonPac AS14A (4 × 250 mm) and dissolved organic carbon (DOC) using a Shimadzu TOC-Vcph with an ASI-V autosampler. The geochemical speciation model WHAM(VI) (Windermere Humic Aqueous Model, version 6) was used to predict metal speciation in the water samples and hence calculate the available fraction of alkaline metals (mol L^{-1}). WHAM(VI) includes consideration of binding to colloidal fulvic acid and inorganic ligands in solution (Tipping *et al.* 2003). Fulvic acid (FA) was estimated by assuming that 65% of the DOC was 'active' FA and that FA is 50% carbon (Cheng *et al.* 2005; Buekers *et al.* 2008).

STATISTICAL METHODS

Generalised linear models with normal errors and identity link functions were used to analyse variation in body length between populations. Variation in alkaline metal concentrations was summarised using principal components analysis. We used '$PC1_{alkaline}$' and mean pH as our estimators of variation in the abiotic environment, and as indices of resource availability (Waterston *et al.* 1979). Relative abundance of *Pungitius* and mean trout size were used as indicators of variation in the biotic environment. Pearson correlation was used to examine the relationship between stickleback length, loch area and measures of biotic and abiotic environmental variation.

Structural equation modelling in AMOS 19 (Byrne 2010) was used to quantify relationships between the two abiotic environmental variables ($PC1_{alkaline}$ and mean pH), two biotic variables (mean trout size and log-transformed relative abundance of *Pungitius*) and body size of three-spined sticklebacks (stickleback length). The full *a priori* SEM is shown in Fig. 1. Arrows from abiotic and biotic variables going to stickleback length are described as 'direct' effects on body size, while arrows from abiotic to biotic variables (implying a causal effect) are described as 'indirect' effects on body size. Non-significant paths were removed sequentially from the model. Model fit was assessed with chi square, Akaike's information criterion (AIC) and 'evidence ratios' calculated from the difference in AIC between models (Burnham & Anderson 2002).

Finally, because we were unable explicitly to account for phylogenetic relationships between populations, we examined how differences in stickleback length among the freshwater lochs were related to the abiotic and biotic variables, when their spatial proximity was controlled. Analyses were carried out using generalised least-squares (GLS) models in the package SAM (Rangel, Diniz & Bini 2010). Spatial autocorrelation was modelled with a Matérn model, with parameters estimated from the semi-variogram.

Results

VARIATION IN BODY SIZE BETWEEN POPULATIONS

We measured 6948 sticklebacks from 33 freshwater (Table 1), seven anadromous and seven resident populations (Table 2). Freshwater and resident three-spined sticklebacks in isolated, 'island' populations were much shorter in length than fish in 'continental', anadromous populations, which probably resemble the ancestral condition (Fig. 2, Wald $F_{2,6945} = 4948$, $P < 0{\cdot}0001$, GLM with normal errors, post hoc contrast, mean difference = −30·70, SE = 0·35, $t = -87{\cdot}75$, $P < 0{\cdot}0001$). Residents (sticklebacks with a 'freshwater' phenotype living year-round in saltwater) were also longer on average than freshwater fish (mean difference = 2·10, SE = 0·3471, $t = 6{\cdot}04$, $P < 0{\cdot}0001$). For all 33 freshwater populations, length differed significantly among populations (Wald $F_{32,5417} = 66{\cdot}50$, $P < 0{\cdot}001$). Length also differed significantly among seven anadromous (Wald $F_{6,\ 602} = 28{\cdot}17$, $P < 0{\cdot}001$) and seven resident populations (Wald $F_{6,500} = 5{\cdot}52$, $P < 0{\cdot}001$). For ten freshwater populations with length measurements in all years (2007, 2008, 2010, 2011), lengths fluctuated across years (Fig. 3, Population x Year interaction: Wald $F_{27,\ 2591} = 3{\cdot}41$, $P < 0{\cdot}001$, in a GLM with normal errors). However, differences between populations (~10 mm) were an order of magnitude greater than differences between years (~1 mm, Population: Wald $F_{9,2618} = 161{\cdot}19$, $P < 0{\cdot}001$, Year: Wald $F_{3,2618} = 8{\cdot}11$, $P < 0{\cdot}001$).

Table 2. Populations of three-spined sticklebacks on North Uist that live predominantly in saltwater, with loch name, population 'code' and location. Anadromous populations are identified by the location where they breed, which may be in freshwater. 'Resident' populations are morphologically similar to freshwater populations, but live year-round in saltwater lagoons.

Name	Code	Mean length	S.E.	N	Grid reference
Anadromous					
an Dùin	Duim	68·8	1·37	13	57°39″N; 7°13″W
Fairy Knoll	Faim	68·6	0·35	190	57°38″N; 7°13″W
Grogary	Grom	63·6	0·81	36	57°37″N; 7°30″W
Leodasay	Leom	62·7	0·45	111	57°33″N; 7°20″W
Mòr	Morm	60·8	1·05	14	57°32″N; 7°22″W
Ob nan Stearnain	Obsm	66·5	0·32	223	57°36″N; 7°10″W
Trosavat	Trom	60·7	0·73	22	57°35″N; 7°25″W
Resident					
an Dùin	Duin	34·6	0·71	37	57°39″N; 7°13″W
Fairy Knoll	Faif	37·9	0·34	254	57°38″N; 7°13″W
Leodasay	Leod	36·2	1·68	14	57°33″N; 7°20″W
Ob nan Stearnain	Obse	34·7	0·58	172	57°36″N; 7°10″W
Oban nan Struthan	ObSt	34·5	1·88	10	57°39″N; 7°14″W
Sponish lagoon	Spon	36·5	1·77	10	57°37″N; 7°11″W
Oban Trumaisgarry	Trum	35·0	1·58	10	57°39″N; 7°15″W

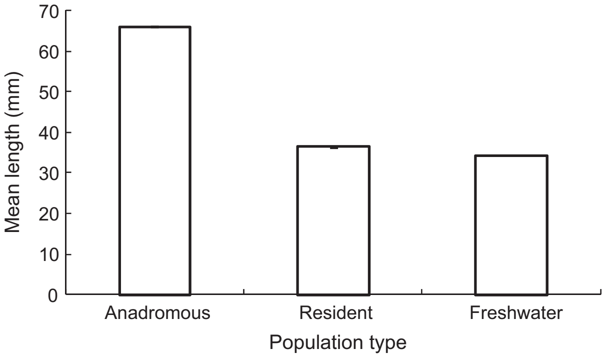

Fig. 2. The mean length (±SE, which are too small to be seen) of three-spined sticklebacks in anadromous (N = 609 fish, seven populations), resident (N = 507 fish, seven populations) and freshwater (N = 5832 fish, 33 populations). Resident fish have a phenotype similar to freshwater fish, but live year-round in brackish lagoons.

Female sticklebacks in oligotrophic lochs on North Uist regularly mature at between 25 and 30 mm in length. Males in some populations show nuptial colouration at even shorter lengths than this, down to 23 mm (data not shown). The average somatic mass (without ovaries) of gravid females from the population with the smallest fish at maturity (Bhei) was 0·29 ± 0·02 g (mean ± SE), N = 6. The somatic mass of mature females from the (anadromous) population with the largest fish (Faim) was 3·47 ± 0·22 g, N = 12.

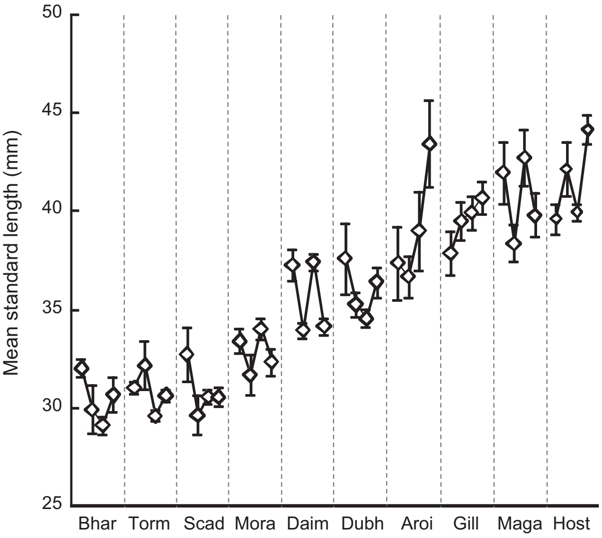

Fig. 3. Mean lengths (±SE) of three-spined sticklebacks in ten freshwater lochs on North Uist in 2007, 2008, 2010 and 2011.

AGE—SIZE RELATIONSHIPS

For 101 sticklebacks from eight freshwater and resident populations spanning the range of water chemistry (Table 1: ARoi, Bhar, Daim, Faif, Gill, Host, Reiv, Scad) that were aged using otoliths, there were significant differences between populations in the length of one-year-old fish (*i.e.* with one otolith ring; for population, Wald $F_{7,93} = 7{\cdot}00$, $P < 0{\cdot}001$). For these populations, there was a significant correlation between length of 1-year-old fish and mean length in the population (0·82, $P = 0{\cdot}012$). For two populations representing the extremes of the freshwater environmental variation (Reiv, alkaline and Scad, acid), there was a significant interaction between population and age in determining length (Fig. 4, Population x Age (otolith): Wald $F_{1,113} = 5{\cdot}59$, $P = 0{\cdot}020$, Population:

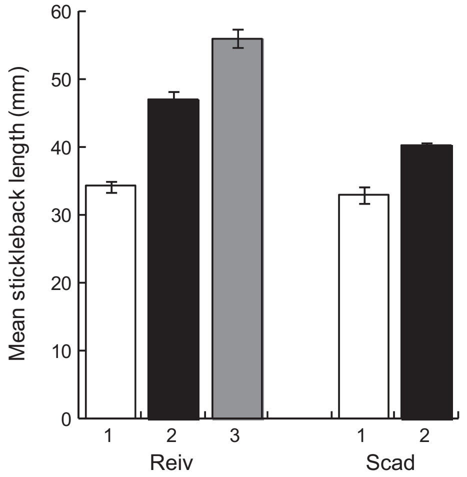

Fig. 4. Mean length at different ages of three-spined sticklebacks from two populations on North Uist, Reiv and Scad, spanning the extremes of environmental conditions. Sticklebacks were aged from otoliths.

Wald $F_{1,114} = 13·38$, $P < 0·001$, Age (otolith): Wald $F_{2,114} = 74·90$, $P < 0·001$).

COMMON GARDEN EXPERIMENT

For three-spined sticklebacks from four freshwater populations from contrasting natural environments on North Uist (Chru, Host, Reiv, Torm), which were reared in common conditions in the laboratory, there were significant differences between populations in both family mean length and weight at 106 days post-hatching (for length, Wald $F_{3,24} = 7·17$, $P = 0·001$, for weight, Wald $F_{3,24} = 8·54$, $P < 0·001$). For these four populations, length in the laboratory at 106 days was highly correlated with mean length of wild-caught fish (correlation=0·99, $P = 0·0065$).

ENVIRONMENTAL VARIATION

Loch area varied by more than two orders of magnitude (Table 1). Data for the mean size of trout caught in angling contests were available for 23 freshwater lochs. Size of brown trout in lochs with available data varied from 120 to 663 g between lochs, based on an average of 185 trout per loch. A further four freshwater lochs probably do not contain trout (J.A. MacLeod personal communication, ADCM personal observations, Table 1).

For 26 lochs in which the relative abundance of *Pungitius* was recorded in both 2010 and 2011, there was a strong positive correlation between years ($r = 0·70$, $P < 0·001$). For these lochs, we used the average of the 2 years as our estimate of *Pungitius* relative abundance, when investigating associations with stickleback body size. For other lochs, we used our estimate from a single year.

The first principal component of an analysis of alkaline metal concentrations ($PC1_{alkaline}$) accounted for 88·5% of the variation in Ca^{2+}, K^{+}, Mg^{2+} and Na^{+} concentrations. All metals contributed approximately equally to this axis: Calcium, 0·50; Potassium, 0·50; Magnesium, 0·52; and Sodium, 0·48.

Variation in alkaline metals was closely associated with variation in mean pH, and in general, our principal environmental variables (above) were strongly interrelated (collinear) with each other (Table 3). However, neither the measured environmental variables nor stickleback length was correlated with loch area. In a principal components analysis for 23 freshwater lochs for which a complete set of the environmental variables (pH, $PC1_{alkaline}$, trout size and relative abundance of *Pungitius*) was available, the first principal component accounted for 60% of the variation, and the second principal component for a further 22%. All variables had positive loadings on the first principal component, but it was most strongly related to variation in water chemistry (pH, $PC1_{alkaline}$, Table 4). The second principal component was more strongly related to variation in biotic factors (trout size and relative abundance of *Pungitius*).

STRUCTURAL EQUATION MODELLING

The best fitting model (Fig. 5) was one in which stickleback size is directly determined by variation in the relative abundance of *Pungitius*, and to a lesser extent by the direct effect of variation in pH. Variation in alkaline metals exerted a significant indirect effect on stickleback body size through its effect on the abundance of *Pungitius*. Alkaline metal variation also contributed significantly to variation in trout size. The effect of trout size on stickleback length was not significant ($P = 0·059$), although a model including it was not significantly less good at explaining the data (evidence ratio = 1·79, AIC 34·79 vs. 33·62). We can infer from this that competition and the direct and indirect effect of abiotic factors were important in determining stickleback body size, while there is some support for trout size having direct effects on stickleback body size. A spatially explicit model (GLS) was consistent with the SEM in as much as the (log) relative abundance of *Pungitius* remained the best predictor of variation in stickleback length (Table 5), although stickleback length was not significantly related to any of the other biotic and abiotic factors.

Discussion

Our results provide support for Darwin's hypothesis (1859) that biotic factors, associated with variation in the

Table 3. Univariate Pearson's correlations (r, lower left, values in bold are significant $P<0·05$. For values in italics $0·1>P>0·05$) and P values (upper right) between mean stickleback length and measured environmental variables across 33 freshwater populations on North Uist

Variable	Variable number						
Mean stickleback length	1	–	0·24	0·04	0·04	<0·001	0·04
Area	2	−0·26	–	0·19	0·14	0·79	0·19
PC1 alkaline	3	**0·44**	−0·28	–	<0·001	0·08	0·06
Mean pH	4	**0·44**	−0·32	**0·93**	–	0·18	0·09
Log (relative abundance of *Pungitius*)	5	**0·72**	−0·06	*0·38*	0·29	–	0·12
Trout size	6	**0·42**	−0·28	*0·39*	*0·36*	0·33	–
	Variable number	1	2	3	4	5	6

Table 4. Weighting on principal component axes 1 and 2, of four environmental variables that characterise environmental variation in 23 freshwater lochs on North Uist. The first and second principal components account for 59·7% and 21·5% of the variation, respectively.

	Weighting of principal components	
	1	2
PC1alkaline	0·60	0·34
Mean pH	0·58	0·44
Log (relative abundance of *Pungitius*)	0·38	−0·68
Mean mass of brown trout	0·41	−0·48

abiotic environment, are more important in explaining evolution than is abiotic variation *per se*. This is consistent with other recent studies that have investigated multiple causes of body size evolution (Meiri, Cooper & Purvis 2008; Raia & Meiri 2011). Specifically, dwarfism in three-spined sticklebacks is associated with reduced abundance of a smaller competitor species, the nine-spined stickleback *P. pungitius*, and with low pH indicative of poor resource conditions. Dwarfism also tends to occur where an important predator, the brown trout *Salmo trutta*, is also small and may act more as a large competitor. The abundance of *P. pungitius* and the size of *S. trutta* are themselves related to underlying abiotic environmental variation.

Sticklebacks in freshwater 'island' populations almost always evolve to smaller body size than their 'continental' marine relatives (this study, Baker 1994; although see Moodie 1972). This effect is especially pronounced in some of the archipelago of freshwater lochs on North Uist, where sticklebacks mature at smaller size than has been recorded anywhere else in their large range (Baker 1994; Baker *et al.* 2008). Females in the smallest (freshwater) population matured at only 7% of the mass of females in the largest anadromous population. In the size range of the species, the smallest freshwater populations can therefore legitimately be described as dwarfed. However, among the lochs on North Uist, there was no correlation between loch ('island') size and the body size of sticklebacks, despite the fact that variation in loch size spanned more than two orders of magnitude. Obviously, a large part of the reduction in size between 'continental' anadromous sticklebacks and freshwater populations is associated with change in salinity. However, it is likely that most of this difference is attributable to some other aspect of the change in environments than the change in salinity *per se*. 'Resident' sticklebacks, which are phenotypically difficult to separate from freshwater sticklebacks but live year-round in coastal saltwater lagoons, are substantially smaller than anadromous ones but are nonetheless generally larger than freshwater sticklebacks.

Among freshwater three-spined sticklebacks dwarf sizes are especially likely to evolve where nine-spined stickleback *P. pungitius* are uncommon or absent, and where pH and the availability of alkaline metals is low. Small size of brown trout may also contribute to the occurrence of dwarfism. This strongly suggests that the evolution of body size among freshwater populations of three-spined sticklebacks is determined mainly by the resource environment: fish are bigger where productivity (as controlled by water chemistry) is higher and smaller where it is lower, but they only evolve to very small sizes in the absence of a smaller competitor species. The relative abundance of competitors (and the size of predators) is itself determined by underlying abiotic variation. This is consistent

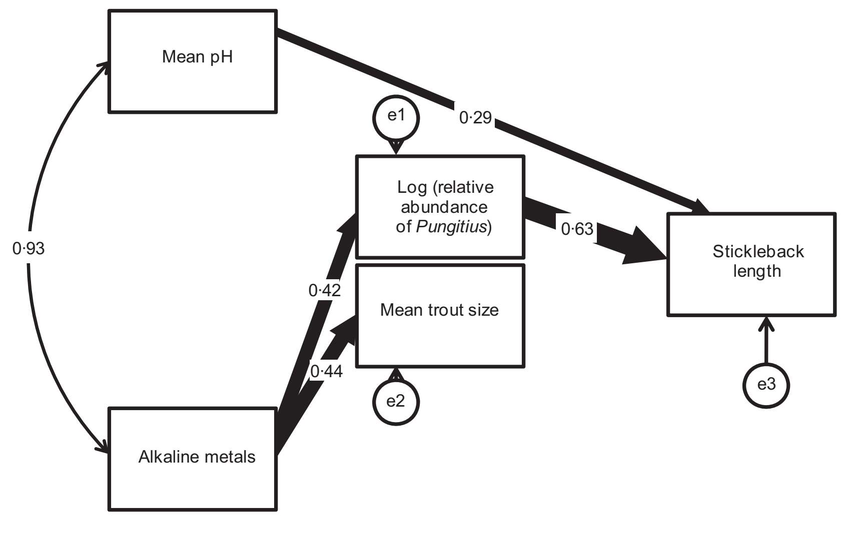

Fig. 5. Final structural equation (path) model, showing the effect of abiotic and biotic variables on each other, and on body size (standard length) of sticklebacks in freshwater populations on North Uist, Scottish Western Isles. Single-headed arrows indicate significant paths, weighted by their standardised path strengths (all are positive). The curved, double-headed arrow shows the correlation between abiotic variables.

Table 5. The results of a generalised least-squares model examining the effect of different putative abiotic and biotic factors on stickleback length after spatial autocorrelation has been controlled. Spatial autocorrelation was accounted for with a Matérn model. R^2 (predictors plus space) = 0·62.

Variable	GLS coefficient	SE	*t*	*P*
PC1 alkaline	0·55	0·68	0·80	0·43
Mean pH	1·70	1·51	1·13	0·27
Log (relative abundance of *Pungitius*)	1·49	0·72	3·93	< 0·001
Trout size	0·002	0·003	0·61	0·55

with emerging ideas that the evolution of body size on islands is more to do with the exact resource and biotic conditions that prevail (especially the presence of competitors and predators) than with being on an island *per se* (Case 1978; Raia, Barbera & Conte 2003; Raia & Meiri 2006; McNab 2010). It is also consistent with the notion that the evolution of body size in fish may be especially responsive to competitor and predator regimes (Robinson & Wilson 1994; Herczeg, Gonda & Merila 2009).

The differences in body size we recorded are probably at least partly genetic, given differences in size between populations reared in common garden conditions in the laboratory, but differences in growth rates and longevity in the wild also contributed to overall differences between populations. The great majority of breeding fish from acid lochs are just a year old (0+ age class), with only occasional 1+ fish (ADCM unpublished data). In contrast, populations from alkaline lochs often showed evidence of a 1+ and sometimes a 2+ (3 year-old) age class. Most populations show inter-annual fluctuations in body size, which are presumably the result of variation in growth conditions (resources and temperature), but these are generally small in comparison with the differences between populations.

Our data are not obviously consistent with a life-history explanation of body size variation (Palkovacs 2003). On North Uist, adult mortality is high in the acid lochs (the fish are annual) where growth rate is low. The most appropriate theory (Stearns & Koella 1986; Stearns 1992) predicts that where adult mortality is inversely related to growth rate, we should expect organisms to mature later, at smaller size. Instead, on North Uist, they mature earlier at (much) smaller size. It is possible that the shape of the size–age reaction norm (L-shaped) that leads to the Stearns and Koella prediction is different in the lochs on North Uist. For example, if juvenile mortality was also inversely related to growth rate, the expected shape of the reaction norm would be 'keel' shaped (Stearns 1992) which would lead to different predictions. However, we consider it to be unlikely that juvenile mortality is higher in the acid lochs, because in these lochs, the sticklebacks generally lay fewer, larger eggs (ADCM, unpublished data), which suggests that juvenile mortality is likely to be lower. Alternatively, size and age at maturity in endothermic organisms can be the result of differences in temperature (Zuo *et al.* 2012). This seems an unlikely explanation for sticklebacks on North Uist where size variation does not correlate with spot readings of temperature in loch shallows during the breeding season or with loch depth which, in well-mixed lakes like those on North Uist, should be a proxy for annual average water temperature (ADCM, unpublished data).

Our data are also not consistent with a simple interpretation of the effect of competition or predation. Release from competition is normally expected to result in body size increasing, to 'take advantage' of an expanded niche. In addition, theory suggests that small predators should favour the evolution of rapid growth and large body size, to allow evolutionary 'escape' from predation, whereas large predators should favour rapid maturity at small size (Reznick & Endler 1982). In contrast, on North Uist, release from competition by *Pungitius* facilitates the evolution of smaller body size. This may be explained if trout are more important as competitors than as predators. Larger trout in the (alkaline) lochs on North Uist certainly eat sticklebacks, but the fact that adult survival of sticklebacks is higher in these lochs than in the acid ones suggests that the effect of trout predation on adult survival is not a primary factor driving body size evolution and that its effect, if anything, may select for larger size (Moodie 1972). We do not yet know whether the small trout in the acid lochs eat sticklebacks, but in other oligotrophic northern temperate and boreal lochs piscivory among (small) trout is rare: they are more likely to be planktivorous or insectivorous (Kahilainen & Lehtonen 2002; Museth *et al.* 2003). This suggests that small trout also compete with sticklebacks and that this competition may limit the upper size of sticklebacks in resource-poor, acid lochs where trout are small. The absence from acid lochs of *Pungitius*, which apparently cannot tolerate the environmental or resource conditions, frees up the small body size niche, which is then occupied by three-spined sticklebacks.

Dwarfism may be common among freshwater fish (Riget *et al.* 2000; Landry & Bernatchez 2010; Moles *et al.* 2010; Macqueen *et al.* 2011), at least in North temperate lakes, but there has been little previous study of its causes (although see Riget *et al.* 2000; Landry & Bernatchez 2010). One previous study of the evolution of gigantism in (nine-spined) sticklebacks (Herczeg, Gonda & Merila 2009) is consistent with ours in that body size variation was principally associated with biotic variation between populations. However, they found that reduced competition and predation led to increased body size. Although this appears to contradict the patterns we observed, in fact it is very consistent given that Herczeg, Gonda & Merila (2009) studied body size evolution in the smaller *P. pungitius*. Thus, it appears that three-spined and nine-spined sticklebacks have reciprocal effects on each other: absence of the larger three-spined allows nine-spined sticklebacks to become 'giants', while absence of the smaller nine-spined allows three-spined to become 'dwarfs'. This is consistent with the idea that competition is an important agent of character displacement in freshwater fishes (Robinson & Wilson 1994).

The relationships between different abiotic and biotic factors in our study highlight the shortcomings of trying to understand the causes of evolution by examination of single factors and emphasise the importance of quantifying the O matrix of relationships between environmental variables as completely as possible (Graham 2003; MacColl 2011). In our study, decreased competition from one species may go hand in hand with increased competition from another species and reduced resource availability, while improved environmental circumstances are associated with larger predators. Previous studies that have linked predation with body size evolution have usually only characterised the presence or absence of predators or the number of predator species (Michaux *et al.* 2002; Herczeg, Gonda & Merila 2009; Li *et al.* 2011). In our study, the large variation in trout size between populations shows that this may be inadequate to fully understand the contributions of variation in predation to evolution of 'prey'. Indeed, it is quite likely that any indirect measure of predation will be confounded with other environmental factors. Even measures of predator size are unlikely to be a reliable index of the extent of predation: predator diets can vary substantially between populations, depending on what prey is locally available (Kahilainen & Lehtonen 2002; Museth *et al.* 2003). In future work, we intend to investigate variation in piscivory among North Uist brown trout populations using stomach sampling.

Overall in our study, body size variation within freshwater three-spined sticklebacks was more strongly affected by biotic (competition) than abiotic (pH) variation, but both clearly play a role. Darwin's hypothesis (1859) that biotic interactions have a more important influence on evolution than do abiotic ones may therefore be simplistic. It seems more likely that an 'entangled bank' model, in which evolution is driven by complex interactions between ecology and the physical environment, is better able to explain variation in body size in three-spined sticklebacks. This study demonstrates the importance of considering the whole O matrix before drawing conclusions about the causes of body size evolution on islands.

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