Evolutionary Ecology Research, 2013, **15**: 413–435

Regressive evolution of the pelvic complex in stickleback fishes: a study of convergent evolution

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ABSTRACT

Background: Regressive evolution of the pelvic complex has been observed in populations of three of five stickleback genera, *Gasterosteus*, *Pungitius*, and *Culaea*, from young freshwater habitats (<18,000 years old). It has been hypothesized to be selected for by ion limitation, lack of predation by fishes, and increased predation by large aquatic insects. Stickleback pelvic reduction is often caused by deletion mutations of the *Pitx1* gene, which prevent its expression during pelvic development. Lack of *Pitx1* expression may be partly compensated for by *Pitx2*, which is preferentially expressed on the left side of the body, causing the left pelvic vestige to be larger than the right. Thus, left-biased directional asymmetry of pelvic vestiges implicates null *Pitx1* alleles.

Aim: Review the geographical patterns of pelvic variation in the three stickleback genera and consider their implications for hypotheses regarding the genetics and evolution of pelvic reduction in the Gasterosteidae.

Data: We review published data on pelvic reduction and add previously unpublished information from populations in Scotland and Norway.

Methods: We assign individual stickleback to one of three pelvic phenotypes – normal, vestigial, and lost pelvis – and explore the distribution of these three broad classes among populations. We study bilateral symmetry of the pelvis by scoring pelvic phenotypes on the left and right sides.

Results: Pelvic reduction is rare in sticklebacks and occurs only in fresh water. Sticklebacks with pelvic reduction tend to occur in the western and northern parts of their ranges in Eurasia and North America, but this pattern is less evident for the ninespine stickleback. Pelvic phenotype frequency distributions within populations differ among stickleback genera. The vestigial pelvic phenotype dominates in many populations of *Gasterosteus aculeatus*, rarely in *Pungitius*, and never in *Culaea inconstans*. In *Culaea*, the lost and normal pelvic phenotypes are often dimorphic, but a vestigial pelvis is rare. This dimorphism does not occur in the other two genera. Monomorphism of the lost pelvic phenotype does not occur in *Gasterosteus*, and is

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rare in the other two genera. Asymmetry of pelvic vestiges is common in all three genera, but left-larger directional asymmetry predominates only in *Gasterosteus*. This bias could be due to the suggested regulatory mutation in the *Pitx1* gene and/or mutations in downstream targets of *Pitx1*, and suggests involvement of other genes for pelvic reduction in the other genera.

Keywords: convergent evolution, directional asymmetry, Gasterosteidae, pelvic reduction, regressive evolution, stickleback.

INTRODUCTION

Evolution has produced many examples of adaptive morphology in new or changing environments. These examples can suggest hypotheses for the environmental correlates of morphological variation and even the identity of selection agents (Endler, 1986). They may lead to hypotheses concerning intrinsic (e.g. Arnold, 1992; Walsh and Blows, 2009) and extrinsic (e.g. Slatkin, 1985) constraints on the evolutionary response to natural selection. They can provide the source of genetic variation for adaptation (e.g. Colosimo *et al.*, 2004; Cresko *et al.*, 2004; Shapiro *et al.*, 2004) and the mechanisms by which that variation has arisen (Colosimo *et al.*, 2005; Chan *et al.*, 2010). These adaptations can usually be studied in related species only after divergence is complete. Therefore, modification of major traits within extant species and even populations offers valuable opportunities to investigate evolutionary processes. Stickleback fish (Gasterosteidae) are well suited to study intraspecific morphological diversification, and their pelvic skeleton is among their most conspicuous variable traits.

Sticklebacks have a northern distribution, and the majority of freshwater stickleback habitats formed after the last glacial retreat began about 18,000 years ago (Peilou, 1991). Despite their relative youth, astonishing morphological differentiation occurs among freshwater populations (e.g. McPhail, 1963; Münzing, 1963; Nelson, 1969; Hagen and Gilbertson, 1972; Bell, 1976; Gross, 1977; Campbell, 1984; Reimchen, 1994; Ravinet *et al.*, 2013). The skeletal armour functions in post-capture defence against gape-limited predators and varies both within and among populations.

The pelvic girdle of fishes primitively supports a pair of fins located ventrally and posteriorly on the trunk. Early studies suggested that they play a limited role in swimming, but more recent studies show that they are important for manoeuvring and balance (e.g. Standen and Lauder, 2007). Nevertheless, they have frequently been modified for walking, digging, suction, flying, climbing, sensing, signalling, reproduction, and predator defence by both camouflage and spines, with or without venom (Yamanoue *et al.*, 2010). About 8% of extant fishes have lost pelvic fins completely, commonly accompanied by a reduction or loss of the internal pelvic girdle (Nelson, 1993).

Four of the stickleback genera exhibit highly divergent pelvic skeletons (Nelson, 1971a; Wootton, 1984; Bowne, 1994). It is a bilateral structure (Fig. 1) with a median suture extending most of its length. A strong, serrated spine on each side articulates to the pelvic girdle by a trochlear joint. A small pelvic fin, supported by the spine and one or a few fin rays, appears when the spine is erect. The pelvic girdle is composed of three parts: a posterior process and an anterior process forming a ventral shield, and a lateral ascending branch that overlaps with lateral plates, when present. The largest and strongest pelvic complex occurs in marine threespine stickleback, while the sizes of the different pelvic structures vary within and among species. In sticklebacks, the pelvic complex contributes to post-capture defence

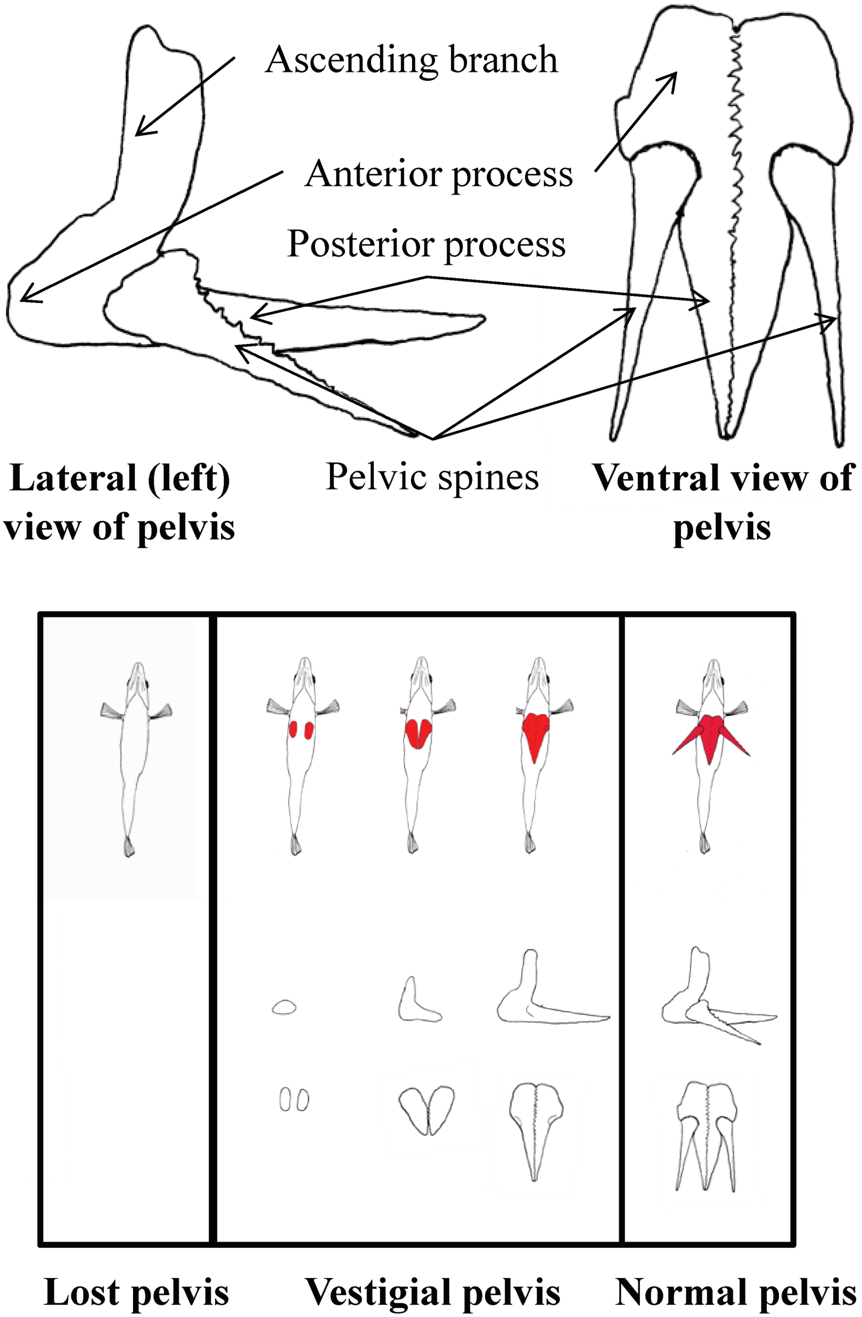

Fig. 1. (Top) Pelvic structures of Gasterosteidae (represented by *G. aculeatus*), lateral (left) and ventral (right) aspects. (Bottom) Structures of the three pelvic phenotypic classes (lost, vestigial, and normal), lateral and ventral aspects.

against gape-limited predators, especially fishes and birds (Reimchen, 1994). Locking pelvic and dorsal spines (Hoogland, 1951) makes the stickleback difficult to ingest and swallow (Hoogland *et al.*, 1957; Reimchen, 1994), and the pelvic girdle and adjacent armour plates protect the abdominal organs from compression during capture. Although pelvic reduction and loss is common in fishes, it rarely varies within and among populations (Nelson, 1993). However, it does vary within and among populations of three of the five stickleback genera, *Pungitius*, *Culaea*, and *Gasterosteus*. Pelvic reduction was first reported in the ninespine stickleback from Ireland by Day (1878), and since 1970 it has been reported in extant populations of all three genera (e.g. Nelson, 1969, 1971b, 1977; Bell, 1974; Reimchen, 1980; Reist, 1981; Giles, 1983; McPhail, 1992; Bell and Ortí, 1994) and in fossil *Gasterosteus* (Bell, 1974, 1987; Bell *et al.*, 2006).

Evolution of vestigial phenotypes or complete loss may be due to relaxation of selection based on removal of a selection agent and resulting changes in the cost–benefit relationship between maintenance and reduction of the trait (Ortí *et al.*, 1994; Lahti *et al.*, 2009). Pelvic reduction in sticklebacks appears to represent some combination of changes in the net fitness effects of the robust pelvic girdle versus reduction, and it probably reflects variation among populations of all three stickleback genera of the importance of several selection agents (e.g. Nelson and Atton, 1971; Reimchen, 1980; Reist, 1980; Bell, 1987, 1988; Bell and Ortí, 1994; Lesack and von Hippel, 2011; Lescak *et al.*, 2013). Thus, pelvic reduction in sticklebacks provides an excellent opportunity to investigate the interactions of selection agents with both positive and negative effects on fitness (Lahti *et al.*, 2009).

Recent results on the molecular genetics of pelvic reduction in the threespine stickleback have greatly increased interest in this phenomenon. Cole *et al.* (2003) first showed that failure of the homeodomain transcription factor, *Pitx1*, to be expressed is associated with pelvic reduction. Cresko *et al.* (2004) and Shapiro *et al.* (2004) mapped pelvic reduction to the *Pitx1* locus and the latter authors inferred that a regulatory mutation at this locus causes pelvic reduction. Chan *et al.* (2010) showed that *Pitx1* alleles for pelvic reduction arose independently in several populations of *Gasterosteus aculeatus* and all have deletions that share a 484 base pair region, within which the *pel* enhancer is located. Its paralogue, *Pitx2*, is expressed preferentially, on the left, which causes directional asymmetry of internal organs and, when there is a null *Pitx1* allele, of the pelvis (Campione *et al.*, 1999; Lin *et al.*, 1999; Lu *et al.*, 1999; Marcil *et al.*, 2003; Shapiro *et al.*, 2006). Thus, a large adaptive morphological difference in natural populations of threespine stickleback can be caused by a single gene. Furthermore, Shapiro *et al.* (2009) demonstrated that pelvic reduction in one ninespine stickleback population mapped to a different region than *Pitx1* and may result from another gene that maps to this region and is known to be involved in fin and limb development. Bell *et al.* (2007) estimated directional asymmetry in several populations with vestigial pelvic phenotypes, and found four that did not exhibit a tendency to be larger on the left. Thus, *Pitx1* is a recurrent cause of pelvic reduction in threespine stickleback, but different genes can be the major factor for convergent pelvic reduction in different stickleback populations.

In this study, we review existing information on stickleback populations with pelvic reduction, including their geographical distribution, phenotype frequency distributions within populations, and correlations with other armour traits. We present data on pelvic reduction in populations from Norway and Scotland that have not been studied before and new data from previously studied populations in Alaska, USA (Bell and Ortí, 1994). We address the following questions: Is it exclusively a freshwater phenomenon? Is pelvic reduction geographically structured? How common is it? Do pelvic phenotype frequencies within populations differ among the three genera? Are pelvic phenotype frequencies within populations stable over time? Is loss of pelvic structures symmetrical? Is pelvic reduction correlated with ecological factors?

MATERIALS AND METHODS

Our data come from two sources. We searched the literature on stickleback armour for populations with pelvic reduction (evolutionary-ecology.com/data/2802Appendix.pdf). Second, we analysed samples from populations with pelvic reduction that had not been reported previously or for which published data were unsuitable for our analysis. The new data describe the frequency distribution of pelvic reduction and numbers of dorsal spines and lateral plates in four Norwegian and 11 Scottish threespine stickleback populations (Table 1).

Reports on populations with pelvic reduction vary from detailed descriptions of pelvic phenotype frequencies with illustrations to mere mention of its occurrence. Pelvic reduction is often described as spine loss without reference to other parts of the pelvis, precluding an analysis of all populations that includes the degree of pelvic reduction. However, data from numerous populations of all three genera can be included by classifying pelvic phenotypes into three categories: (1) *normal pelvis*, all pelvic elements present (i.e. anterior process, ascending branch, posterior process, pelvic spine; Fig. 1) (Nelson, 1971a); (2) *vestigial pelvis*, some but not all pelvic elements present; and (3) *lost pelvis*, all elements missing. Populations with pelvic reduction vary extensively for the frequency of specimens within these three categories and can be divided into four types: Type 1, mostly *normal pelvis*; Type 2, mostly *vestigial pelvis*; Type 3, mostly *lost pelvis*; and Type 4, only *lost pelvis*. Only populations with sample sizes ≥20 were included in this analysis.

Pelvic asymmetry was recorded when sufficient data were available in published sources. In many cases, the presence of asymmetry was noted, but the direction of asymmetry was not. Thus, new data were collected from samples from 16 populations of threespine stickleback (i.e. 5 Alaskan, 6 Scottish, 5 Norwegian) to obtain data on directional asymmetry of pelvic phenotypes. Loss of pelvic elements was recorded bilaterally using a 5-point scale on each side (Bell, 1987; Bell *et al.*, 1993; Bell and Ortí, 1994) (Fig. 1): 4, no pelvic elements missing; 3, pelvic spine missing; 2, pelvic spine and posterior process missing; 1, pelvic spine, posterior process, and ascending branch missing; and 0, all elements missing. Differences between the right and left scores and the direction of asymmetry were recorded. Only specimens with a vestigial pelvis were included in this study, since the normal pelvis and lost pelvis morphs are symmetrical by definition when symmetry is scored this way.

All geographical coordinates were verified on maps, and inaccurate published coordinates were corrected. Coordinates for sampling sites are presented as decimal degrees to thousandths of a degree in the appendix (2802Appendix.pdf Table 1).

RESULTS AND DISCUSSION

Is pelvic reduction exclusively a freshwater phenomenon?

Threespine and ninespine sticklebacks inhabit both marine and freshwater habitats. There are several reports of pelvic reduction in these species from fresh water (see below), but few studies have commented on pelvic reduction in marine populations. A large sample ($N = 3440$) of anadromous threespine stickleback from Cook Inlet, Alaska, USA contained

Table 1. Pelvic phenotypes, dorsal spine numbers, and lateral plate numbers of Norwegian and Scottish threespine stickleback populations

					Pelvic phenotype			Pelvic score		Dorsal spine number		Lateral plate number (left)	
Site	Country	Latitude	Longitude	*N*	Normal	Vestigal	Lost	Mean	s.d.	Mean	s.d.	Mean	s.d.
Geitungsvatn	Norway	60.397N	05.071E	71	12.7	87.3	0.0	5.08	1.82	3.00	0.29	4.16	0.75
Nesavatn	Norway	59.553N	05.441E	428	0.0	15.4	84.6	0.28	0.78	2.28	0.48	0.19	0.61
Liavatn	Norway	59.552N	05.416E	735	26.3	32.9	40.8	3.08	3.39	2.77	0.44	0.20	0.58
Vigdarvatn	Norway	59.520N	05.363E	492	92.3	5.9	1.8	7.63	1.48	2.97	0.16	0.75	1.10
Bogach Maari	Scotland	57.627N	07.263W	41	0.0	9.5	90.5	0.46	1.10	2.95	0.22	0.02	0.16
Loch Fada	Scotland	57.617N	07.237W	16	0.0	0.0	100.0	0.00	0.00	1.13	0.62	0.00	0.00
Loch nan Geadh	Scotland	57.616N	07.215W	44	0.0	6.8	93.2	0.91	0.36	1.20	0.80	0.02	0.15
Loch na Maighdein	Scotland	57.601N	07.202W	47	0.0	100.0	0.0	2.40	1.06	1.57	0.65	0.00	0.00
Loch Scadavey	Scotland	57.599N	07.257W	61	0.0	98.4	1.6	2.05	0.69	1.69	0.56	0.00	0.00
Loch nan Eun	Scotland	57.586N	07.302W	17	0.0	100.0	0.0	2.00	0.00	1.18	0.81	0.00	0.00
Loch nan Ceitir Elean	Scotland	57.578N	07.257W	7	0.0	100.0	0.0	5.86	0.38	3.00	0.00	4.14	0.38
Loch Buird	Scotland	57.578N	07.224W	98	5.1	28.6	66.3	1.37	2.42	2.05	0.33	0.00	0.00
Loch na Moracha	Scotland	57.577N	07.263W	47	0.0	76.6	23.4	1.49	0.88	1.09	0.75	0.02	0.15
Loch Huna	Scotland	57.577N	07.336W	10	0.0	50.0	50.0	1.00	1.05	1.50	0.53	0.00	0.00
Loch Bharpa	Scotland	57.294N	07.294W	115	1.7	97.4	0.9	2.12	0.88	2.14	0.51	0.00	0.00

Note: Populations are listed by descending latitude.

only one individual with pelvic reduction, i.e. left spine missing (Aguirre *et al.*, 2008). In Scotland, Campbell (1984) found threespine stickleback with pelvic reduction in a few estuarine samples, but these differed morphologically from marine stickleback and most likely originate from adjacent freshwater habitats. Campbell also surveyed 69 other saline sampling sites, and no pelvic reduction was found. There are no reports of pelvic reduction in marine ninespine stickleback. Pelvic reduction seems to be confined to fresh water.

Is pelvic reduction geographically structured?

Threespine and ninespine sticklebacks are widely distributed in fresh waters along the eastern, western, and Arctic coasts of Eurasia and North America. In contrast, brook stickleback are endemic to the fresh waters of north-central North America.

By far the greatest number (1–95, numbers in 2802Appendix.pdf Table 1) of threespine stickleback populations with pelvic reduction come from lakes around Cook Inlet, Alaska, USA. Only 33 additional populations with pelvic reduction have been reported from throughout the rest of the world, including the west coast of California, USA (96) and Canada (97–100), Quebec, Canada (101–102), Iceland (103), Scotland (104–120), and Norway (121–128).

Several nominal species of ninespine stickleback (genus *Pungitius*) have been described, and pelvic reduction occurs in three of them. Pelvic reduction occurs in lake populations distributed around Cook Inlet, Alaska, USA (129–136), northern (137–142) and eastern (143) Canada, Ireland (144–147), Sweden (148–149), Finland (150–151), north-west Russia (152–156), Norway (157), Greece (158), and Japan (159–160).

A total of 46 brook stickleback populations (161–206) with pelvic reduction have been reported from Canada and mostly from Alberta (2802Appendix.pdf Table 2) (Fig. 2). In addition, Nelson and Atton (1971) referred to an unconfirmed report of a southern population in Merrick Lake, New Mexico, USA (almost certainly introduced).

Some general patterns are apparent in the geographical distributions of pelvic reduction in sticklebacks. First, there seems to be a tendency for clustering in all three genera. A clustering pattern can be due to sampling effort; for instance, the high numbers of pelvic-reduced populations around Cook Inlet, Alaska, are likely to some extent due to intensive sampling for the phenomenon. A number of these populations, especially those with a low frequency of pelvic reduction, would probably not have been found in a general survey. Most adjacent areas of Alaska are inaccessible and have never been sampled for stickleback; pelvic-reduced populations may be common elsewhere in Alaska.

However, we find it unlikely that sampling effort alone can explain this clustered pattern. A plausible mechanism for geographical clusters is gene flow among adjacent populations. Since several of the populations are within the same drainage systems or have been connected in recent time, the origin of pelvic reduction in one population with subsequent spread to nearby populations may explain the existence of population clusters. However, sequencing of the *pel* enhancer region in *Pitx1* alleles for pelvic reduction revealed that three to five separate deletion mutations produced pelvic reduction just within the Matanuska-Susitna Valley in Alaska (Chan *et al.*, 2010). Further genetic analyses are needed to determine whether pelvic reduction in adjacent populations has a common origin or evolved independently of each other based on independent mutations.

Another apparent geographical pattern is a tendency for restriction of pelvic-reduced populations to northern and western portions of continents. The vast majority of

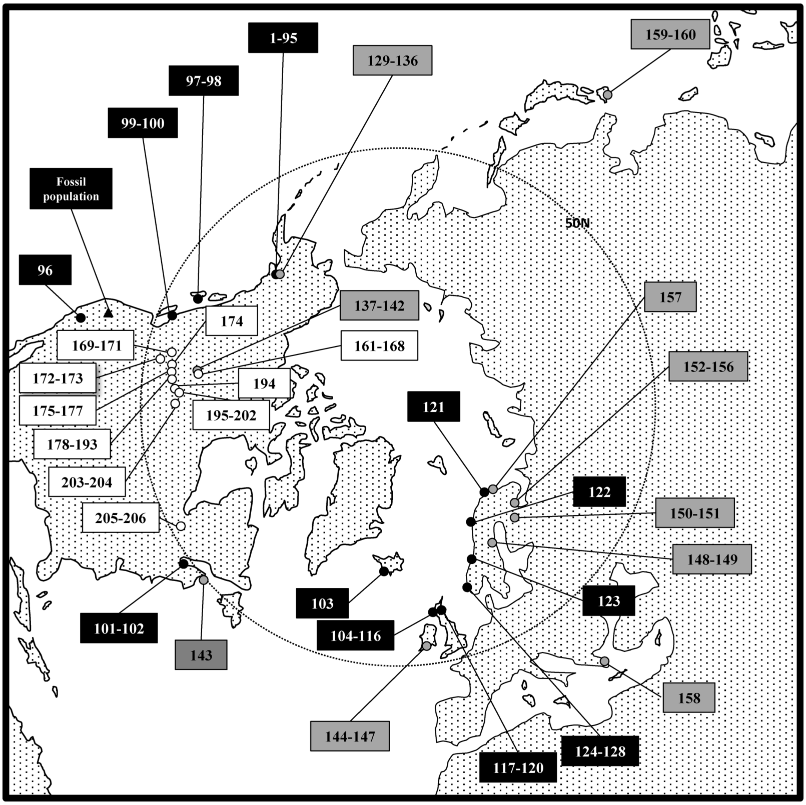

Fig. 2. The distribution of threespine (black), ninespine (grey), and brook stickleback populations (white) with pelvic reduction. Each point may represent more than one population. The black triangle represents the location of the fossil case. The numbers refer to site numbers in the Appendix (2802Appendix.pdf Tables 1 and 2).

populations occur close to or north of 50° north latitude; pelvic reduction has been observed only twice further south (Fig. 2). Furthermore, all but two populations of threespine stickleback have been found on the west coasts of North America and Europe. Almost all known populations of the brook stickleback with pelvic reduction are from Alberta and Saskatchewan, from the northern and western part of the species' range. This pattern may be partially explained by the distribution of resident freshwater populations of both species; they are more common to the north and west.

Another possible factor influencing this pattern may be sampling effort. However, the lack of observations of pelvic reduction in threespine stickleback in Asia and southern and eastern Europe, in spite of morphological studies using populations from these areas (Münzing, 1963; Penczak, 1965; Gross, 1978; Mori, 1987), indicates geographical structure. Extensive sampling in California, near the southern edge of the range of western North American threespine stickleback, produced only one population with pelvic reduction, and it is rare and moderate, when it occurs (Bell, 1987; unpublished data). Similarly, southern and eastern populations of the brook stickleback have almost exclusively normal pelvic morphology (Nelson, 1969; Moodie, 1977). In contrast, ninespine stickleback tend to have a more northern distribution than the other two genera, and pelvic reduction occurs in southern and eastern populations from Greece and Japan. The latter are recognized as separate species (*P. hellenicus*) or subspecies (*P. pungitius tymensis*) of *P. pungitius* (Keivany and Nelson, 2000). The lack of a pelvis is a diagnostic character for *P. hellenicus*. Thus, pelvic reduction in European *Pungitius* has a broad distribution. Overall, pelvic reduction in sticklebacks appears to exhibit a geographical pattern, but more information, especially from the regions where pelvic reduction has not been observed, is needed to adequately characterize this distribution.

A geographical pattern can be caused by phylogeographic history; populations in different regions may possess genetic variation that is lacking in others. Freshwater populations of the three genera have contrasting phylogeographic histories. Nearly all extant threespine stickleback populations with pelvic reduction have evolved repeatedly from marine ancestors since deglaciation, forming a phylogenetic raceme (Bell and Foster, 1994). An mtDNA study revealed an endemic clade in the Sea of Japan, and a second larger clade with Pacific and Atlantic subclades (Ortí *et al.*, 1994). Pelvic reduction has evolved repeatedly in the latter two subclades (Bell, 1987; see above). European threespine stickleback comprise three major clades based on mtDNA analysis (Mäkinen and Merilä, 2008), and pelvic reduction is restricted to one clade. But this clade covers the major parts of the distribution area in Europe, and cannot explain the distribution of pelvic reduction.

Ninespine and brook sticklebacks are less tolerant of high salinity water, and the post-glacial dispersion originated from a few ice-free freshwater refugia through freshwater connections. The ninespine stickleback can be divided into three North American clades (Aldehoven *et al.*, 2010) and two in northern Europe (Teacher *et al.*, 2011), but no relationship can be seen between these clades and pelvic reduction. The presence of pelvic reduction in Greek and Japanese populations, on the southern borders of the Eurasian distribution of ninespine stickleback, may be relicts from ancient isolated refugia, but we have no genetic data to support this interpretation.

McPhail and Lindsey (1970) concluded that the present distribution of brook stickleback reflected dispersal from a single refugium in the upper Mississippi basin, but an mtDNA study by Gach (1996) identified clades from two glacial refugia. Interestingly, these clades have north-western and south-eastern distributions, and pelvic reduction is restricted to the former. The only south-western population that might have pelvic reduction is from Merrick Lake, New Mexico. Gach (1996) concluded that this population was probably introduced and belonged to the south-eastern clade, not supporting the hypothesis that pelvic reduction is restricted to one clade. There is no evidence that phylogeographic history has played a major role in the distribution of pelvic-reduced populations. Rather, genetic variation for pelvic reduction has arisen repeatedly both within and among clades within each genus. Chan *et al.* (2010) provide strong evidence that null mutations in *Pitx1* for pelvic reduction arose repeatedly, even within restricted, recently deglaciated regions. However, we cannot exclude the possibility of a common origin of the alleles producing pelvic reduction in the brook and ninespine sticklebacks; further studies of the genetics of pelvic reduction in these species are needed to decide this issue (Shapiro *et al.*, 2009).

How common is pelvic reduction?

Pelvic reduction has been reported from about 200 populations of the Gasterosteidae worldwide (2802Appendix.pdf Tables 1, 2) (Fig. 2). This is a small number, considering that the absence of a pelvic spine is conspicuous, pelvic reduction has attracted a great deal of attention, and numerous populations have been examined for various purposes. But care must be taken to sample stickleback populations randomly for pelvic reduction to estimate the frequency of its occurrence, and this kind of data is limited.

A random sample of 123 threespine stickleback populations from Norway produced only one with a substantial frequency of individuals with pelvic reduction, and three more with low frequencies (Klepaker and Østbye, 2008) (2802Appendix.pdf Table 1). In the Haida Gwaii (Queen Charlotte) archipelago of British Columbia, Canada, Reimchen *et al.* (2013; see also Reimchen, 1980) reported extensive pelvic reduction in only two of 102 populations of *G. aculeatus*. Of 204 threespine stickleback populations sampled in Cook Inlet, Alaska, 97 had pelvic reduction, but this survey targeted habitats with ecological conditions that favour evolution of pelvic reduction (Bell and Ortí, 1994). However, it may indicate that pelvic reduction is more common in this area than in others because so many cases were found. On the island of North Uist, Scotland, pelvic reduction occurred in 13 of 40 populations sampled (Giles, 1983), making it more common on this island than on the Scottish mainland, where Campbell (1984) reported only four populations with pelvic reduction from 164 freshwater populations sampled.

Ninespine stickleback with pelvic reduction have been observed in a total of 32 widespread populations (2802Appendix.pdf Table 2) (Fig. 2). Mobley *et al.* (2011) examined 18 populations from a restricted area in Sweden and found one population with pelvic reduction. Fifteen of 66 ninespine stickleback populations from lakes around Cook Inlet, Alaska exhibit pelvic reduction (M.A. Bell, unpublished data; see von Hippel, 2008). *Pungitius* is the genus with the lowest number of reported populations with pelvic reduction, indicating that it is rare, but it seems to be more frequent in the other two genera in some areas.

Nelson (1977) reported that 37 of 81 populations of brook stickleback sampled in Alberta, Canada had pelvic reduction, but Moodie (1977) examined pelvic phenotypes in 45 populations in Manitoba, Canada without finding a single case of pelvic reduction. These observations indicate that pelvic reduction is relatively rare but might be locally frequent.

Do pelvic phenotype frequencies within populations differ among the three genera?

Stickleback from populations with pelvic reduction can be placed into the three broad classes of pelvic phenotypes (i.e. normal, vestigial, lost) and frequencies of these phenotypes can be used to place populations into four classes: Type 1, normal dominates; Type 2, vestigial dominates; Type 3, lost dominates; and Type 4, lost fixed (Table 2).

Most (58%) threespine stickleback populations ($n = 116$) belong to Type 1, usually with more than 90% normal pelvic phenotypes (2802Appendix.pdf Table 1). Thirty-four per cent of the populations are Type 2, and they may be subdivided into two groups, vestigial plus normal (51%) and vestigial plus lost. Only 8% of populations belong to Type 3, and they usually contain a small fraction of the vestigial phenotype, but the normal phenotype also may be present. The lost pelvic phenotype occurs in only 5% of the populations but rarely at a high frequency (Type 3) compared with the other two genera, and never fixed (Type 4).

Only 18 ninespine stickleback populations have large enough samples to assign them to population type (Table 2). As in threespine stickleback, Type 1 populations are the most common. Although Type 1 populations are more frequent in ninespine stickleback than in threespine stickleback, they tend to have lower frequencies of the normal and higher frequencies of the vestigial pelvic phenotype (2802Appendix.pdf Table 2). There are three

Table 2. Pelvic phenotype frequencies in populations of threespine, ninespine, and brook sticklebacks

Population type	Populations (*n*)	Pelvic morph Normal mean % (S.D.)	Vestigial mean % (S.D.)	Lost mean % (S.D.)
Threespine stickleback				
1. Normal pelvis dominates	67	91.1 (12.0)	8.5 (11.4)	0.4 (1.3)
2. Vestigial pelvis dominates	39	12.3 (18.8)	81.4 (19.3)	6.3 (10.8)
3. Lost pelvis dominates	9	6.5 (10.3)	23.0 (13.7)	70.5 (18.1)
4. Lost pelvis fixed	1			100
Ninespine stickleback				
1. Normal pelvis dominates	6	67.2 (4.5)	24.4 (7.1)	8.5 (3.9)
2. Vestigial pelvis dominates	2	2.7 (0.9)	83.4 (18.9)	14.0 (19.8)
3. Lost pelvis dominates	5	8.6 (10.2)	7.1 (7.9)	84.3 (12.1)
4. Lost pelvis fixed	5			100
Brook stickleback				
1. Normal pelvis dominates	22	71.6 (12.4)	5.4 (6.1)	23.0 (13.6)
2. Vestigial pelvis dominates	1	7.7 (—)	61.5 (—)	30.8 (—)
3. Lost pelvis dominates	12	9.1 (11.3)	9.5 (13.9)	81.6 (17.3)
4. Lost pelvis fixed	4			100

Type 2 populations, and the vestigial phenotype is fixed in two of them. Five populations are dominated by the lost pelvic form (Type 3), while the lost pelvis is fixed (Type 4) in only four adjacent populations from north-west Russia (Ziuganov and Zotin, 1995). Populations with high frequencies of the lost pelvis (Types 3 and 4) are more frequent in ninespine stickleback than in the other two genera.

Pelvic phenotype frequency distributions in brook stickleback populations ($n = 39$) differ from the other two genera; the vestigial pelvic phenotype is rare and dominates (Type 2) in only one population. Populations are usually dominated either by the normal pelvic phenotype or the lost pelvic phenotype (Table 2). Unlike the other two genera, most brook stickleback populations with pelvic reduction have bimodal frequency distributions for the normal and lost categories (2802Appendix.pdf Table 2). For example, in Astotin Lake, the normal pelvic phenotype constitutes 52% and the lost pelvic phenotype 44% (Nelson and Atton, 1971) (2802Appendix.pdf Table 2). Four Type 4 populations have been recorded, but the sample sizes are too small to exclude the possibility that other pelvic phenotypes occur at low frequencies.

Our crude classifications of pelvic morphology into three phenotypic classes (i.e. normal, vestigial, lost) and of populations into four groups ignore important details of variation among populations with pelvic reduction, but some interesting differences among genera emerge. First, one of the three phenotypes tends to dominate in threespine and ninespine stickleback populations, but ninespine populations tend to have greater pelvic reduction. One possible explanation is that pelvic reduction evolves faster in ninespine than in threespine stickleback. Indeed, Ziuganov and Zotin (1995) emphasized that the lakes where they found complete loss of pelvis are relatively young. Another major difference among stickleback genera is that brook stickleback populations are usually bimodal for the normal and lost phenotypes. Phenotypic bimodality also occurs in threespine stickleback (Bell and Ortí, 1994) and could indicate any of several causes, including strong disruptive selection, the presence of two separate demes, or genetic dominance of one of the modal phenotypes (Cresko *et al.*, 2004; Shapiro *et al.*, 2004, 2009).

Is the distribution of pelvic phenotypes within populations stable over time?

There are few studies of temporal variation of pelvic phenotype frequency change in extant stickleback populations, and they usually span limited time intervals. Pelvic phenotypes of threespine stickleback from Wallace Lake, Alaska were stable between 2001 and 2008, but a sample from 1990 had a significantly higher frequency of the normal pelvis (Lescak *et al.*, 2013). Threespine stickleback from Lake Storvatnet, Norway had stable pelvic phenotype frequencies in samples from three consecutive years (Klepaker *et al.*, 2012), and three large samples made 20 years apart from three other Norwegian populations with low frequencies of pelvic reduction showed no change (Klepaker and Østbye, 2008; T. Klepaker, unpublished data). Nelson and Atton (1971) compared pelvic phenotype frequencies in samples separated by 2–9 years from four populations of brook stickleback. They were stable in two populations and the frequencies of reduced phenotypes increased in the other two.

The lack of extensive time series for pelvic phenotype frequencies in extant populations makes it impossible to infer whether their variability represents transient or balanced polymorphism. However, Bell *et al.* (2006) presented a time-series for pelvic reduction in fossil *Gasterosteus doryssus* over 20,000 years, during which a transition took place for pelvic phenotypes: normal pelvic monomorphism, gradual pelvic reduction with high variability, dominance by a small vestige, and finally appearance of the lost pelvic phenotype. Evolution of greater pelvic reduction occurred over at least 8000 years. The highest rates of evolution occurred during the first 2000–3000 years, and then slowed. Hunt *et al.* (2008) showed that the pattern of change in this time-series is far more likely to have resulted from directional selection than genetic drift. If this case is typical, the rate of pelvic reduction would be too slow to demonstrate phenotypic change in time-series of 50–100 years. Finally, the fossil population never became monomorphic for pelvic loss, even thousands of years after small vestigial phenotypes became numerically dominant, showing that multiple pelvic phenotypes can co-exist in natural populations for extended periods. Thus, type 2 populations may persist for extended periods, and transient polymorphism can appear to represent balanced polymorphism. This observation is consistent with the existence of numerous populations that are polymorphic for pelvic reduction (2802Appendix.pdf Table 1).

Is loss of pelvic structures symmetrical?

Asymmetry of vestigial pelvic structures in sticklebacks has been reported from extant populations of all three genera and from the fossil record of *Gasterosteus*. Left-biased directional asymmetry (i.e. larger left vestige) is the signature for *Pitx1* in vertebrate pelvic reduction (Shapiro *et al.*, 2006), so it is important to determine whether asymmetry is directional. However, a lack of asymmetry does not necessarily rule out the involvement of *Pitx1*.

The majority of threespine stickleback populations that have been examined for pelvic symmetry exhibit left-biased directional asymmetry (Reimchen, 1980; Bell *et al.*, 1985, 2007; Shapiro *et al.*,

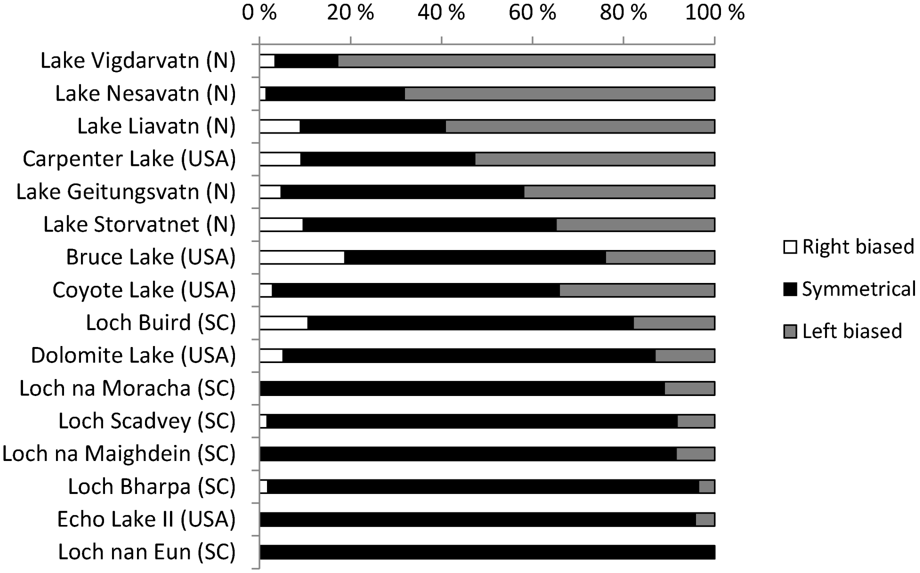

Fig. 3. Frequency distributions of symmetry classes of pelvic vestiges in threespine stickleback populations from Alaska (USA), Scotland (SC), and Norway (N). The populations are ranked by decreasing asymmetry.

2004; Klepaker *et al.*, 2012; Lescak *et al.*, 2013). Pelvic asymmetry is also common in ninespine and brook sticklebacks, but it generally appears to represent fluctuating asymmetry (Nelson, 1971b; Nelson and Atton, 1971; Takata *et al.*, 1985; Blouw and Boyd, 1992). However, right-biased directional asymmetry occurs in three populations of threespine (Bell *et al.*, 2007) and one population of ninespine stickleback from Cook Inlet, Alaska, USA (Shapiro *et al.*, 2009).

Figure 3 shows the directionality of bilateral asymmetry of the pelvic scores in threespine stickleback populations from Alaska, Scotland, and Norway. The proportion of left-biased specimens is consistently higher than that of right-biased ones, but there may be variation among regions. Asymmetry is common in Norwegian populations, and the proportion of left-biased specimens ranges from 40 to 80% of the specimens with a vestigial pelvis. The proportion appears to be lower (<20%) in Scottish populations, and sticklebacks with symmetric scores are much more common. The Alaskan populations tend to have intermediate levels of directionally asymmetric pelvic scores. In contrast, Bell *et al.* (2007) observed a wide range of directionality of pelvic asymmetry within Alaskan populations, but this difference may be due to their use of size differences between sides estimated by inspection, and not a difference in score.

The differences observed between threespine stickleback and the other two genera for pelvic asymmetry suggest that the genetics of pelvic reduction differ among genera. In threespine stickleback, left-biased asymmetry occurs in all populations in which *Pitx1* has been shown to be the major gene for pelvic reduction (Cresko *et al.*, 2004; Shapiro *et al.*, 2004; Bell *et al.*, 2007; Chan *et al.*, 2010). Shapiro *et al.* (2006) found that *Pitx1* is probably the major gene for pelvic reduction in one ninespine stickleback population from the Northwest Territories, Canada, but not in the population with right-biased asymmetry from Cook Inlet in which the major locus for pelvic reduction maps to a different linkage group than *Pitx1* (Shapiro *et al.*, 2009). The independent origin of pelvic reduction in disparate populations of threespine and ninespine stickleback suggests that alleles for pelvic reduction have arisen independently many times and may involve different genes. Genetic analyses have detected different genes for pelvic reduction within a small sample of threespine and ninespine stickleback populations. Furthermore, deletions in *Pitx1* of different lengths but all including the same 484 base pairs show that alleles for this gene have arisen many times independently. Variation of symmetry among populations suggests extensive diversity for the genetics of pelvic reduction in sticklebacks.

Is pelvic reduction associated with reduction of other anti-predator armour traits?

The stickleback pelvis is part of an integrated skeletal defence, formed by the dorsal spines, lateral plates, and pelvis, which protects the fish from gape-limited predators (e.g. Bell, 1987, 1994; Reimchen, 1994). Thus, pelvic reduction should be associated with reduction of other armour structures. This association has been observed in some ninespine (Keivany *et al.*, 1997; Herczeg *et al.*, 2010) and threespine stickleback populations (Bell, 1974; Campbell, 1984; McPhail, 1993; Bell *et al.*, 2006; T. Klepaker, unpublished data). The most extreme examples come from the Scottish threespine stickleback populations on North Uist, where almost all populations with pelvic reduction lack lateral plates, and several have lost dorsal spines (Table 1). Similarly, a fossil stickleback lineage that evolved pelvic reduction lacked lateral plates entirely and evolved a reduction in dorsal spines and pelvic reduction almost simultaneously (Bell, 1974, 1994, 2009). However, this association is not universal. Although the lateral plates and dorsal spines of *Gasterosteus* populations with pelvic reduction in Cook Inlet lakes are small, and they are more likely to have reduced numbers of dorsal spines than populations with normal pelvic structures, plates and spines are never entirely lost (Bell *et al.*, 1985). Norwegian populations with pelvic reduction do not show any reduction in dorsal spine or lateral plate numbers compared with other freshwater populations (Klepaker and Østbye, 2008). The most striking deviation from correlated reduction in anti-predator traits is in Quebec, Canada, where two threespine stickleback populations have a reduced pelvis but the majority are partially plated (Edge and Coad, 1983; Lacasse and Aubin-Horth, 2012).

Is pelvic reduction correlated to ecological factors?

Calcium limitation and predation regime have been proposed to influence the evolution of pelvic reduction in sticklebacks. Bone is mineralized by calcium salts, most of which teleost fishes obtain from ambient water (Simkiss, 1974), and the normal pelvis is massive bone. Low concentrations of calcium and possibly other ions could exert selection against pelvic development. Giles (1983) and Bell *et al.* (1993) found that pelvic reduction was associated with low calcium concentrations in Scotland and Alaska. Bell *et al.* (1993) found that pelvic reduction often occurs at a calcium (or some correlated ion) concentration below 12 $\mathrm{mg \cdot L^{-1}}$, provided that predatory fish are absent from the lake. But pelvic reduction in sticklebacks also occurs in water with much higher calcium concentrations. Wood Buffalo National Park, Canada has limestone bedrock, and calcium concentrations exceed 120 $\mathrm{mg \cdot L^{-1}}$ in lakes where both brook and ninespine sticklebacks have pelvic reduction (Nelson and Paetz, 1972, 1974). Numerous other stickleback populations from waters with low calcium concentration throughout their range have not evolved pelvic reduction. For example, the calcium concentration in most Norwegian lakes is well below 12 $\mathrm{mg \cdot L^{-1}}$, but stickleback pelvic reduction occurs in only a few populations. Furthermore, while pelvic reduction in some populations is accompanied by a reduction in dorsal spine numbers and lateral plates, as expected if low calcium concentration increases the cost of skeletal development, compared with typical freshwater populations, many pelvic-reduced populations do not show significant reduction in other bony armour traits (Edge and Coad, 1983; Klepaker and Østbye, 2008).

The calcium limitation hypothesis predicts that stickleback with a larger pelvic skeleton should grow more slowly in water with a low calcium concentration than those with pelvic reduction. Marchinko and Schluter (2007) compared growth rates experimentally between lateral plate morphs with contrasting amounts of bone. The completely plated morph (more bone) grew slower in low-calcium water than the low-plated morph (less bone), suggesting that allocation of calcium to bony armour elements limited growth. If this is correct, it is reasonable to expect that it should apply to other defensive armour structures, but so far neither experimental nor field studies of growth in relation to pelvic structure have detected an effect of pelvic structure on growth rate in low-calcium water (Lescak *et al.*, 2013; J. Rollins, unpublished data).

The pelvic spines and girdle are parts of the stickleback's structural defence against gape-limited predators (Hoogland *et al.*, 1957; Reist, 1980; Reimchen, 1991). Therefore, it is reasonable to hypothesize that predation regime influences selection on pelvic structure. If gape-limited predation is low or absent, selection for maintenance of pelvis and armour protection should be relaxed (Lahti *et al.*, 2009). Bell *et al.* (1993) found that an absence of native predatory fishes was a second, necessary factor (with low calcium concentration) for the occurrence of pelvic reduction, supporting this hypothesis. Further support comes from the occurrence of pelvic reduction in threespine stickleback from Little Quarry Lake, British Columbia, Canada, where piscivorous fish are absent (Gow *et al.*, 2008). Hoogland *et al.* (1957) observed that stickleback from which spines had been removed were much more vulnerable to fish predation than those with intact spines. Similarly, Lescak and von Hippel (2011) used rainbow trout (*Oncorhynchus mykiss*) as predators and stickleback with pelvic phenotypes ranging from normal to lost from Wallace Lake, Alaska. Specimens without pelvic spines suffered more trout predation than those with spines, but they could not exclude other phenotypic factors (e.g. behavioural differences). However, the hypothesis that an absence of piscivorous fish is the primary cause of pelvic reduction in threespine stickleback is contradicted by sympatry of pelvic-reduced threespine stickleback and predatory fish in Scotland (Giles, 1983), Norway (Klepaker and Østbye, 2008), Quebec, Canada (Edge and Coad, 1983), and Paxton Lake, British Columbia, Canada (McPhail, 1992).

Pelvic reduction also occurs in populations of the other stickleback genera that are sympatric with predatory fishes. Ninespine stickleback populations with pelvic reduction occur in sympatry with northern pike (*Esox lucius*) in the Salt River system in Wood Buffalo National Park, Canada (Nelson, 1971b). Similarly, a large proportion of brook stickleback in Wakomao Lake, Alberta, Canada, where northern pike predation was confirmed by stomach content analyses, have pelvic reduction (Reist, 1981). Although an absence of fish predation is a factor in stickleback pelvic reduction, its occurrence in sympatry with predatory fishes either requires that other factors interact with it to select for pelvic reduction or indicates that the association of absence of predatory fishes and pelvic reduction among some sets of populations is a spurious correlation.

Another consequence of reduced fish predation is increased density of large predatory insects, including backswimmers (*Notonecta* sp.), giant water bugs (Belostomatidae), and dragonfly nymphs (*Aeshna* spp.), which unlike gape-limited predatory vertebrates, grasp and hold stickleback. Spines offer no protection against these predators and may facilitate their grip on stickleback (Reimchen, 1980). Vamosi (2002) did an experiment in Paxton Lake, in which benthic (reduced pelvis) and limnetic (normal pelvis) juvenile sticklebacks were subjected to predation from backswimmers, dragonfly nymphs, and adult stickleback. Juvenile limnetics exposed to backswimmers and adult stickleback had lower survival than benthics, but since these two sympatric populations differ for numerous traits, it is impossible to attribute selective predation to pelvic armour alone. Marchinko (2009) used F2 hybrids from crosses between marine and freshwater threespine stickleback with pelvic reduction in a similar experiment. He found that possession of a short pelvic girdle and short dorsal spines was correlated with survival, but, surprisingly, there was a negative association between absence of a pelvis and survival. Two recent studies on threespine (Zeller *et al.*, 2012) and ninespine stickleback (Mobley *et al.*, 2013) also failed to demonstrate an advantage of reduced armour (fewer plates and shorter spines) against insect predation.

Although fish predation appears to impose strong selection favouring a robust pelvic skeleton (Hoogland *et al.*, 1957; Reimchen, 2000; Lescak and von Hippel, 2011), there is no convincing experimental or comparative evidence for selective differences among individuals with normal and reduced pelvic structures, and there are many exceptions to associations between proposed environmental causes and pelvic reduction. However, there may be a very good reason for this. If the evolutionary transition from normal to reduced pelvis is as slow as observed in the fossil record, where the transition from a normal pelvis to reduced pelvis took more than 2000 years (Bell *et al.*, 2006), the relative fitness difference between normal and reduced pelvic phenotypes may be very small. It is unlikely that very small fitness differences among phenotypes could be detected in a short-term experiment comparing a limited number of specimens and adaptation of natural populations to the predation regime may be very slow. This trait may be different from lateral plate morphs, which can evolve very rapidly (Klepaker, 1993; Bell *et al.*, 2004; Kitano *et al.*, 2008; Le Rouzic *et al.*, 2011), and experience strong short-term directional selection (Barrett *et al.*, 2008, 2009; Barrett, 2010; Bell and Aguirre, 2013).

Evolution of pelvic reduction

Phenotypic traits and their causal alleles will increase in frequency over time if their fitness benefits exceed their fitness costs (Lahti *et al.*, 2009). Their net fitness effect can be depicted as a peak on an adaptive or fitness landscape, and the slope and elevation of the peak represent the intensity of selection keeping the population at the peak (Svensson and Calsbeek, 2012). For example, there is apparently a high, steep-sloped adaptive peak for robust armour defence for marine threespine stickleback. Any phenotype even a short distance from the adaptive peak will experience strong negative selection, and the corresponding phenotypes will be quickly eliminated from the population. Thus, they invariably have robust armour, including the pelvis, and exhibit very limited variation for armour traits within and among populations (Aguirre *et al.*, 2008). In freshwater stickleback, the cost–benefit relation of robust armour is different. Robust armour should have greater fitness costs. Calcium concentration is 50 times lower in fresh water than in the ocean (Sverdrup *et al.*, 1970), and dense armour increases the demands on buoyancy regulation (Myhre and Klepaker, 2009). The benefits of armour may also be lower in fresh water compared with the marine environment. Freshwater stickleback often live close to cover, increasing the value of pre-capture evasion, which is improved by loss of armour (Reimchen, 2000). Thus, the adaptive peak for armour expression is displaced to lower values in freshwater stickleback, and its position is more variable because predation regime varies among freshwater habitats (Reimchen, 1994).

The occurrence of pelvic reduction requires two conditions: (1) genetic variants that produce reduced pelvic phenotypes must be present, and (2) natural selection must favour individuals with reduced pelvic structures or some phenotype that is genetically correlated with it (Hohenlohe *et al.*, 2012). Geographical clustering of populations with pelvic reduction, which occurs in all three genera, could be due to gene flow rather than to recurrent mutation. Populations downstream of other populations with pelvic reduction in Alaska, Canada, Scotland, Norway, and Japan may exhibit low-frequency pelvic reduction, even though environmental conditions do not seem to favour that phenotype (Edge and Coad, 1983; Takata *et al.*, 1985; Bell and Ortí, 1994; R.N. Campbell, unpublished data; T. Klepaker, unpublished data). The occurrence of pelvic reduction in these populations suggests that gene flow adds alleles for pelvic reduction and selection removes them, producing a gene flow–selection equilibrium (Levene, 1953). Interestingly, the benthic-feeding species of *G. aculeatus* in Paxton Lake, Canada has pelvic reduction, but only normal pelvic structures occur in the sympatric limnetic (i.e. planktivorous) species and in two lake populations immediately downstream (McPhail, 1993). Thus, it appears that reproductive isolation can prevent introgression of reduced alleles for pelvic reduction into other populations. Moreover, different alleles of *Pitx1* cause pelvic reduction in threespine stickleback from different adjacent lakes in Cook Inlet, showing that genetic variation for this trait can arise independently in different populations (Chan *et al.*, 2010). Gene flow probably provides the genetic variation for pelvic reduction in some closely connected populations, but reproductive isolation and selection against pelvic reduction in recipient populations can limit the impact of such gene flow, and independent mutations in *Pitx1* can also provide the genetic variation for evolution of pelvic reduction.

Relaxed selection for maintenance of armour is plausible when predation by gape-limited predators is low or absent. But it may also happen when gape-limited predation is important; if predators are sufficiently large, gape width will not limit their ability to swallow sticklebacks and spines may not provide any protection (Reimchen, 1991). If risk of predation is very high, cryptic behaviour may be more important, and reduced encounters with predators may reduce the benefit of armour. Lahti *et al.* (2009) argued that the net vector for selection on a trait can incorporate the effects of multiple selection agents acting on the trait and genetically correlated traits. The same resultant vector can be produced by different magnitudes and directions of the individual vectors. This could explain why it is so difficult to identify individual factors that are common to populations sharing a trait. A further illustration of this is that even if the same agents are likely to affect fitness of pelvic phenotypes in all three genera, it is uncommon to find pelvic reduction in different stickleback genera in sympatry (Nelson, 1971b; M.A. Bell, unpublished data). Subtle differences may maintain selection against pelvic reduction in one genus, while it is relaxed in another.

Relaxed selection by gape-limited predators and positive selection by other environmental factors may be a sufficient explanation for evolution of pelvic reduction in most ninespine and threespine stickleback populations, but the bimodal population structure observed in brook stickleback may require more complicated evolutionary causes. Bimodality could result from the existence of two alternative predator defence strategies, only one of which relies on the defensive function of the pelvic skeleton. Since crosses within each extreme phenotype of brook stickleback (i.e. normal × normal, lost × lost) can produce the whole range of phenotypes (Nelson, 1977), some kind of frequency-dependent selection may be involved. Alternatively, directional selection on phenotypes based on alleles for sharply contrasting phenotypes with dominance would produce phenotypic bimodality during the transition from the ancestral to the derived pelvic phenotype. Bell *et al.* (2004) observed such a bimodal evolutionary transition for lateral plate phenotypes that are determined largely by one gene with dominance (Colosimo *et al.*, 2004; Cresko *et al.*, 2004), and this mechanism may have caused the bimodal phenotypic transition for pelvic reduction in fossil stickleback (Bell, 2009). Finally, the two phenotypic extremes in brook stickleback may indicate the co-existence of two sympatric demes with restricted genetic exchange (i.e. biological species) within the same lake. Such sympatric benthic–limnetic species pairs of threespine stickleback have been observed in British Columbia (McPhail, 1992, 1993, 1994). This possibility was proposed by Nelson (1977) himself but not studied further.

The phenomenon of pelvic reduction in the Gasterosteidae provides an excellent opportunity to study evolution on the most detailed level because there is extensive variation both within and among populations, and it has evolved independently within three genera. However, it also demonstrates that an understanding of evolution at this level can be challenging because it may depend on the interaction of both genetic and selection mechanisms that may vary among populations. More than 40 years of effort since Nelson (1971b) first reported pelvic reduction in sticklebacks has left many questions unanswered.

ACKNOWLEDGEMENTS

This paper is dedicated to Joseph S. Nelson, who passed away one year before it was first written. His efforts provided most of our knowledge on pelvic reduction in the ninespine and brook sticklebacks. We also thank colleagues who have provided information on pelvic-reduced populations: Matt Arngard, Folmer Bokma, Tuomas Leinonen, Gábor Herczeg, Juha Merilä, Nadia Aubin-Horth, and Keisuke Takata. Jennifer Rollins allowed us to cite her unpublished data on growth in relation to pelvic phenotype. Carl Smith and his co-workers generously provided samples from the North Uist populations. Erin Kunz re-scored Alaskan samples for pelvic symmetry. This is contribution #1228 from Ecology and Evolution at Stony Brook University.

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