GENETIC, MORPHOLOGICAL AND BEHAVIOURAL VARIATION IN SCOTTISH THREE-SPINED STICKLEBACK (*GASTEROSTEUS ACULEATUS* L.): INSIGHTS FROM DIFFERENTLY ARMOURED POPULATIONS

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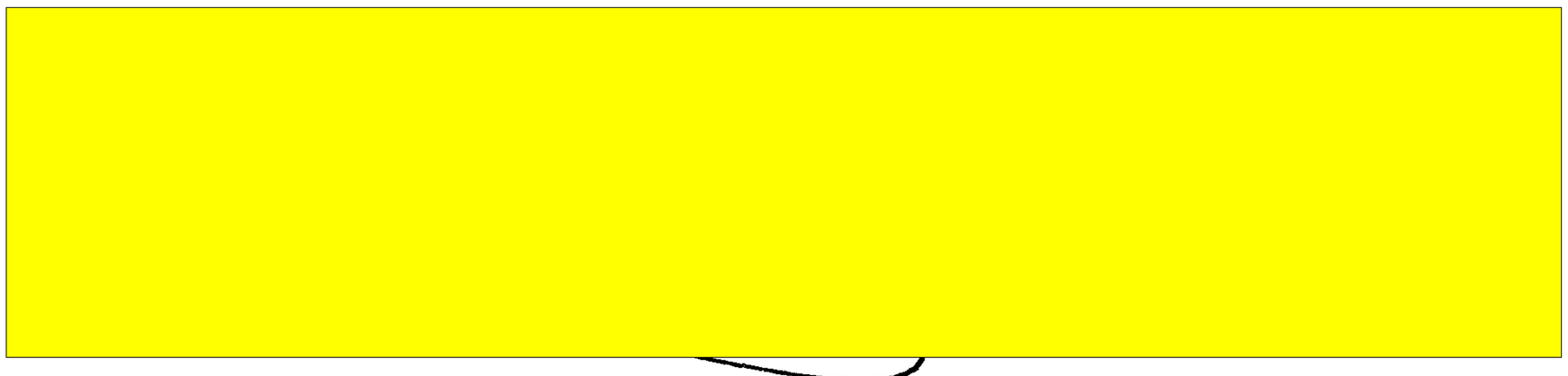
Stumpies in breeding colouration

"In recent times, we are looking in Scotland upon evolution in its course."

James Ritchie

Candidates Declaration

I declare the work in this thesis is entirely my own, and of my own composition. No part of this work has been submitted for any other degree.



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This thesis is dedicated to my grandmother

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Abstract

- This thesis addresses a central theme in evolutionary biology, namely understanding the process of adaptive radiation, using the three-spined stickleback, which has become a model system in this context. In particular, the work concentrated on sticklebacks from several unique populations in the Outer Hebrides that have lost the body armour after which this species was named, lacking dorsal spines, pelvic girdle and spines and lateral plates.

- A first specific aim was to examine an existing hypothesis about the selective force responsible for armour loss in these populations, namely that it is an adaptive response to low calcium levels. This is in contrast to the selective force favouring armour loss in North American populations, where predation by piscivorous fish is thought to favour armour development and predation by invertebrates to favour armour loss. This was studied by relating variability in protective body armour to calcium concentration at 10 sites with calcium concentrations ranging from 1.2mgCa^{2+}/L to 50.5mgCa^{2+}/L and spread over a wide geographical range. The results confirmed previous studies, providing partial support for the low-calcium hypothesis for Hebridean populations. Thus armour reduction is only found in sticklebacks from low-calcium sites, although not all fish from such sites are unarmoured. Piscine, avian and invertebrate predators were present at all sites, ruling out the predation regime hypothesis (Chapter 3).

- A second aim was to relate variability in risk-taking to variable armour expression, both within and between populations of stickleback. 180 wild-caught fish from 7 sites across Scotland (but mainly concentrated in the Hebrides) were screened for risk-taking behaviour using a well-established testing protocol (quantifying rates of exploration of and movement in a novel and potentially dangerous environment) that is broadly predictive of some aspects of the sticklebacks' response to a predator. No significant relationship was found between individual risk-taking score and the extent of body armour, either across populations or among individuals within populations (Chapter 4). The risk-

taking test (chosen because it is simple and easily controlled) only reflects one aspect of anti-predator behaviour and may have been too focused to identify subtle individual differences in risk-taking.

- During this study, it became apparent that the novel environment test, utilised to identify risk-taking behaviour, was an unsuitable test for two reasons. Firstly, temperature was found to have a significant effect on risk-taking, low temperature producing risk-averse fish and secondly, consistency in risk-taking between tests was weak and repeatability absent (Chapter 2). One difference between this and other studies (Huntingford, 1973, 1976; Dingemanse *et al.*, 2002) was that fish were held in isolation. A possibility for the failure to find repeatability may be that interactions within social groups sustain differences in risk-taking. The supposition then that the novel environment test provides results that are always consistent and repeatable should be treated with prudence.

- This finding compromised a major aim of the study, namely to investigate the inheritance of risk-taking behaviour by generating a large F_2 generation for QTL mapping, using the stickleback genome-wide linkage map (Chapter 5). All parental, F_1 and F_2 fish were screened for exploration/activity in a novel environment. Family effects were found in F_1 and F_2 fish, but they were unrelated to parental phenotype. Again, social effects within groups are a possible explanation for such family effects or possibly maternal stress. Mothers of broods experiencing differing degrees of stress, transferring variable amounts of cortisol to their eggs that in turn influenced the behaviour of their offspring. In light of these findings, QTL analysis of risk-taking behaviour was not carried out. Further attempts to identify genes for this behaviour should therefore proceed with caution.

- Another major aim of the work described in this thesis was to reveal the molecular basis of variation in protective body armour in the Hebridean populations of three-spined stickleback. The mechanisms underlying the inheritance of dorsal spines, the pelvic girdle and pelvic spines, lateral plates and the anal spine were investigated. Pelvic girdle and lateral plate inheritance had a relatively simple Mendelian mode of inheritance. However, the inheritance of

dorsal spines and the anal spine, presented a much more complicated picture (Chapter 6). An interesting finding was that the same gene, *Pitx1*, underlying the reduction of the pelvic complex in Scottish populations of three-spined stickleback is the same as that found in pelvic reduced North American and Icelandic populations, even though the selective regime is different. This finding adds to mounting evidence of parallel evolution, in geographically separated populations of three-spined stickleback.

- A final aim was to use microsatellite markers to determine the extent to which the study populations (and in particular, the armour-reduced Hebridean populations) are genetically distinct, using 8 populations and 16 microsatellite markers. These populations not only differed in armour expression but also ranged in geographical distance from one another from 5km to 375km. Discrimination between all populations was achieved, although only half of the microsatellite markers were utilised. Interestingly, one site (Loch Charrasan, on the island of Lewis) was found to have more than one population present. Although populations were found to be clearly distinct, the extent of genetic differentiation did not relate to geographic distance, morphological distinctness or to differences in calcium concentration (Chapter 7). This finding has implications for conservation of these populations. As the populations are genetically distinct they are not interchangeable and so each should be viewed as evolutionary significant units.

- Clear findings of this study are that:
  - The calcium hypothesis holds up when a wider geographical range of sites are used.
  - Differently armoured populations in the Hebrides are genetically distinct.
  - Inheritance of the pelvic complex is under control of one major locus, *Pitx1*, though populations are under different selection pressures. Lateral plates show a simple mode of inheritance but inheritance of dorsal spines and the anal spine do not.
  - A commonly used measure of risk-taking (exploration of a novel environment) may be strongly influenced by external factors that compromise repeatability.

- Risk-taking behaviour is not inherited in any simple sense.

These results are put into a broader context and discussed in detail in chapter 8.

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Chapter 1. General introduction

1.1 Adaptive radiation and evolutionary change

Recognition of the process of adaptive radiation (diversification of an organism from a common ancestor in response to a new environment) was one of the key events in Darwin's elucidation of the theory of evolution by natural selection. Classic and well-studied examples include beak size and shape in Galápagos finches (family Emberizidae) (Grant & Grant, 2006) and the wide range of foraging specialisations in the cichlid 'superflock' (family Cichlidae) in Lake Victoria, East Africa (Verheyen *et al.*, 2003). The origins and maintenance of such variation continues to fascinate evolutionary biologists and an area of active interest in the genomic age is the identity of the number, location and function (or change in function) of genes underlying the evolution of new traits. The understanding of the molecular basis of phenotypic variation usually involves large scale breeding programs. Animals that differ in a trait of interest are crossed and large numbers of first and second-generation animals are reared. There after a candidate gene of interest is identified and a molecular map used to identify the location and function of the gene of interest (Peichel, 2005).

To use molecular tools to elucidate the mechanisms underlying adaptive radiation requires a study system in which phenotypic diversity (whether in morphology, physiology or behaviour) is well documented and its consequences for fitness understood. It also requires large scale breeding programmes to be feasible and the genome of the organism concerned to be well characterised. Amongst the small but growing number for which these criteria are met is the three-spined stickleback (*Gasterosteus aculeatus* L.), which is the subject of the present thesis.

1.2 The biology of the three-spined stickleback

1.2.1 Post-glacial radiation in sticklebacks

The three-spined stickleback is a very common, small teleost fish. It is easy to keep and rear in captivity, tolerates handling well and has a relatively short generation time (Hagen & Gilbertson, 1972). As a model species, the stickleback has for many years been the subject of intense research to address many complex questions concerning evolutionary biology at the individual and population level (Wootton, 1976, 1984; Bell & Foster, 1994; Ostlund-Nilsson *et al.*, 2006). In the 1950's, one of the founding fathers of ethology, Nikolaas Tinbergen, studied the complex reproductive biology of three-spined stickleback (Tinbergen, 1951). Based on this research, he jointly won the Nobel Prize in Physiology and Medicine in 1973 for the study of social animal behaviour. More recently the fish has become the darling of molecular geneticists eager to unlock to secrets of vertebrate diversity.

At the end of the last ice age, less than 20,000 years ago, oceanic heavily armoured three-spined stickleback successfully invaded newly created freshwater habitats. These fish had (and mostly still have today, see Figure 6.1, Chapter 6) three barbed dorsal spines, a robust pelvic girdle and spines (pelvic complex), up to 35 lateral plates running along the lateral sides from the front of the pectoral fin to the tail and a keeled (plated) caudal peduncle (Wootton, 1976). In the relatively short period of time since this invasion of freshwater, the newly established inhabitants have subsequently diverged morphologically, physiologically and behaviourally in response to the local environment (Røed, 1979; Giles, 1987; Klepaker, 1993; Bell, 1995; Kristjánsson *et al.*, 2002; Bell, 2005). The distinct differences both within and between populations are one of the things that make this species of interest to evolutionary biologists and molecular geneticists.

1.2.2 Adaptive radiation in body armour in three-spined stickleback

One of the most striking features of three-spined stickleback is the great degree of variation in development of body armour. In fact, such is the great variation in armour morphology seen in this fish, that for many years it was classified as over 40 different species.

The 'typical' three-spined stickleback has 3 dorsal spines (after which the fish is named, see Chapter 6), two pelvic spines attached to robust pelvic girdle and a row of lateral plates running along the flanks of the fish (Wootton, 1976). All spines can be locked into a rigid position, forming a triangle of protection against gape-limited fish and bird predators. Spines are known to be an effective defence in shielding stickleback from piscivores. The dorsal spines and pelvic girdle are supported by the anterior lateral plates, which run from the back of the head to the second dorsal spine. Plates not only buttress the pelvic and dorsal spines, preventing them from bending or fracturing during predator handling (Reimchen, 1983), but also protect vital organs from puncture by toothed predators (Reimchen, 1992). Upon capture, anterior plates increase the stickleback's chances of escaping a predator, by interfering with the retraction of the pharyngeal jaw and disrupting swallowing in piscivorous fish (Reimchen, 2000). However, there is a trade-off between armour and escape speed. For example, in brook stickleback (*Culaea inconstans*) fish lacking a pelvic girdle and pelvic spines had a faster escape response and bent deeper in the early phase of the response than fish with a fully developed pelvic complex (Andraso, 1997).

In many populations, a significant reduction in protective body armour has occurred in the freshwater descendents of marine colonists and this reduction has occurred in less than 20,000 generations. In such cases, dorsal spines are shorter, reduced in number or absent, pelvic complex is smaller or (occasionally) absent, the keel has disappeared and typically lateral plate number is between 0 and 7 (Schluter & Nagel, 1995). In North American populations, armour reduction and/or loss is most likely a response to low calcium concentration and local predation regimes. Thus, sticklebacks gain protection from gape limited and toothed predators by having longer spines and more plates (Gross, 1977; Reimchen, 1995). In the presence of avian or invertebrate predators, plates and spines are selected against. Lateral plates provide less protection from avian attack where fish are compressed, and spines provide a grasping site for invertebrate predators (Bergstrom, 2002; Reimchen & Nosil, 2002). The total absence of predatory fish has been implicated in the reduction and loss of spines, but all lakes where spine-less morphs are found also have avian predators (Reimchen, 1980). Therefore, the type of predator, rather than simple presence or absence of predation risk, may be a major driving force in reduction and loss of body armour (Cresko *et al.*, 2004).

The situation is different, however, in stickleback in the Outer Hebrides, Scotland, where loss of body armour occurs in several populations that coexist with abundant predatory fish (Giles, 1983). Here the proposal is that calcium concentration is the selective agent in the reduction of protective body armour, since low armoured fish from these islands are only found in lochs with low levels of calcium (<5 mg Ca^{2+}/L). These nutrient-poor, peaty moorland water bodies sit upon impenetrable Lewisean gneiss and are characterised by tea-stained water low in cations, cations such as calcium, being retained on insoluble humic acids (Szalay & Szilágyi, 1968). However, the correlation between reduced armour and low calcium levels is not perfect, since, while Giles (1983) only found fish with reduced armour in calcium-poor lochs, he found normally armoured fish at some sites with low calcium levels. One aim of this thesis is to examine the hypothesis that, in some sites at least, low calcium levels are the selective agent favouring armour loss, by collecting data from a wider geographical range of populations than those studied by Giles.

1.3 Behaviour and behavioural syndromes

1.3.1 The study of risk-taking behaviour: past and present

The study of boldness (also termed risk-taking) from a functional perspective dates back to the mid-1960's when Benzie (1965) compared risk-taking in three- and nine-spined stickleback in a novel environment and in the presence of a predator. She found that boldness was related to how well-armoured a fish was, with the better-protected three-spined stickleback being more risk-prone than the less-well protected nine-spined stickleback. A second aim of this thesis is to examine whether armour expression is related to variable risk-taking.

Following on from Benzie, a study carried out on individual three-spined stickleback from several different sites by Huntingford (1976) described striking repeatable individual differences in risk-taking and showed there was a positive correlation between an individual's boldness toward a predator, territorial aggression toward a conspecific and exploration of a novel environment. At the population level, risk-taking was found to vary in response to predation risk, with fish at high

risk of predation being more risk-averse than those fish at low risk of predation (Giles & Huntingford, 1984; Tulley & Huntingford, 1988). From a developmental perspective, differences in risk-taking were shown to have a heritable component with predator-naïve fry (progeny of fish from high risk sites) showing marked predator avoidance behaviour (Giles, 1983). However, further research revealed a more complicated picture of the inheritance of risk-taking, with paternal care promoting anti-predator responses in fish from high predation sites but not low predation sites (Tulley & Huntingford 1987). The results from the above studies have contributed to risk-taking in three-spined stickleback becoming a case study for behavioural syndromes.

After a gap of several years, the general topic of individual differences in risk-taking was revitalised in a paper by Wilson *et al.* (1994), which addressed the question of why humans and animals differ in their propensity to take risks, by developing the idea of the shy-bold continuum found in natural populations of juvenile pumpkinseed sunfish (*Lepomis gibbosus*) (Wilson *et al.*, 1993). In their study they used the novel object test, first used by child psychologists rather than behavioural biologists to identify shyness and boldness in children, and found that individual levels of risk-taking (tendency to approach the novel object) were not fixed, but rather depended on environmental conditions.

This raises the question of what boldness is and how it can be measured. Boldness in the simplest terms is the propensity to take risks and shyness the propensity to avoid risk (hereafter referred to as risk-taking and risk-averse responses). For example, risk-takers are often more active in a novel environment (Bell & Stamp, 2004), engage in anti-predator inspection more often (Murphy & Pitcher, 1997), are more likely to take novel food items (Wilson *et al.*, 1993) and instigate fights with conspecifics more readily than risk-averse fish, even if a predator is present (Brick & Jakobsson, 2002). The tendency to behave in a risky manner may have serious implications for the reproductive success and even the survival of the individual (Armitage, 1986; Wilson, *et al.*, 1993). Risk-taking can be assessed using a standard test of exploration/activity in a novel environment, as used by Verbeek *et al.* (1994) to measure exploration rate in Great tits (*Parus major*). This test and the response to a novel object test are well-established techniques for quantifying risk-taking in many species of fish (Trinidad killifish [*Rivulus hartii*], Peociliid [*Brachyraphis episcopi*], Brown trout [*Salmo trutta*] and Rainbow trout

[*Oncorhynchus mykiss*]) and in stickleback is broadly predictive of some aspects of the sticklebacks' response to a predator (Huntingford, 1976; Bell & Stamps, 2004; Bell, 2005).

The fact that suites of correlated behaviours are often consistent across different contexts led to the identification of 'behavioural syndromes' (Sih *et al.*, 2004) and the suggestion that these might have important evolutionary consequences (Wilson, 1998; Gosling, 2001; Dingemanse, 2003; Bell, 2005; Sinn & Moltschaniwskyj, 2005). Specifically, if two behavioural traits are inevitably correlated, depending on how natural selection acts on each, this might place constraints on evolutionary change in either single trait. Thus, selection favouring aggressive individuals (as during competition for limited resources) might not be effective if such individuals take excessive risks when faced with a predator. However, Bell (2005) found that in some populations of sticklebacks aggression and boldness were uncoupled, suggesting that this particular behavioural syndrome represents an adaptive response to local selective pressures (possibly predation regime) rather than an evolutionary constraint.

A separate body of literature, but converging on the same issues, comes from a physiological perspective. It has been well documented for a number of species that, when exposed to a standard stressor, individuals of the same species show strikingly different patterns of response, both behavioural and physiological (Reviewed by Koolhaas *et al.*, 1999). These different stress response patterns are usually designated proactive (characterised by bold/aggressive individuals, rigid in their behaviour, that flourish in rich, stable environments and respond to stress through the adrenal-sympathetic system) and reactive (characterised by shy/non-aggressive individuals, flexible in their behaviour, that flourish in poor, variable environments and respond to stress through the hypothalamic-cortisol system) coping strategies.

A number of studies using different species have shown that risk-taking in a novel environment has a heritable component (Great tits: Dingemanse *et al.*, 2002; Drent *et al.*, 2003; Three-spined stickleback: Bell, 2005). Bell (2005) showed that variable risk-taking in sticklebacks (at least as reflected in tendency to approach and inspect a predator) has a significant heritable component. Aggression, with which risk-taking is often correlated, is also an inherited trait in sticklebacks (Bakker, 1986). Development of the genome-wide linkage map for sticklebacks (used

effectively for identifying the genetic mechanisms underlying the evolution of variable morphology in this species, Peichel *et al.*, 2001; Colosimo *et al.*, 2004; Sharpiro *et al.*, 2004) raises the possibility of carrying out QTL mapping of the apparently heritable trait of risk-taking in sticklebacks. An additional aim of the work described in this thesis is to produce large F_2 generations from crosses between risk-taking and risk-averse sticklebacks and to carry out QTL analysis of the inheritance of this trait.

1.4 Genetic mechanisms underlying variation in body armour in sticklebacks

Although many studies documenting variation in body armour expression have been published, until recently little has been known about the precise genetic mechanisms underlying these phenotypes. Among a number of classical studies on the possible mechanisms underlying the inheritance of body armour in sticklebacks (e.g. Hagen, 1973 and Bell, 1974), work carried out by McPhail (1984), using ecologically and morphologically divergent benthic and limnetic three-spined stickleback, may be regarded as the foundation for many recent studies of the genetic basis of armour reduction.

Briefly, McPhail (1984) crossed individual three-spined stickleback from a lake in western North America containing sympatric pairs of stickleback, specialised morphologically and behaviourally for feeding on benthic and limnetic prey. The resulting hybrids (both first and second generation offspring) showed that the differences in body shape, trophic morphology and feeding behaviour are inherited between the two forms. Specifically concerning body armour (which is reduced in the benthic form, McPhail 1993), Hatfield (1997) showed that patterns of development of lateral plate (scute) number and pelvic spine length could be explained by a small number of genes with dominant and additive effects.

With the advent of new molecular tools, coupled with the construction of a genome-wide linkage map for sticklebacks (Peichel *et al.*, 2001), the groundwork for molecular genetic studies into the number and location of genes underlying the divergence of adaptive characteristics has been cemented. Peichel *et al.* (2001)

assembled the stickleback linkage map using benthic and limnetic fish from Priest Lake British Columbia, Canada. Using microsatellite markers dispersed across the genome, they produced an ordered plan of the relative gene loci on each chromosome and this year (2006) the fully sequenced genome of the stickleback has been completed.

Several recently published studies (Cole *et al.*, 2003; Shapiro *et al.*, 2004) identified a gene with a large effect on size of the pelvic girdle, *Pitx1*. This gene mapped to markers on the distal end of linkage group VII in the linkage map (see Figure 6.2, Chapter 6), with a log likelihood ratio of linkage (LOD) of 36.9 (Shapiro *et al.*, 2004). A LOD score of 3 or greater indicates that two gene loci are in close proximity to each other on a chromosome and the chances are 1000/1 in favour of those genes being inherited together. First identified in mice and humans, the *Pitx1* gene is expressed in the hind limbs only and aptly called *backfoot* (Shang *et al.*, 1997). Null mutations in this gene result in hind limb reduction and loss (Shapiro *et al.*, 2004). Two other transcription factors *Tbx4* and *Pitx2* influencing pelvic girdle size (also involved in limb reduction in mammals) map to other chromosome regions (Linkage group I and IV respectively) (Shapiro *et al.*, 2004) and have a smaller effect (Logan, 2003). It is interesting to note that asymmetry in pelvic spine length (reduction is usually greater on the right than the left side) is a consequence of *Pitx2* compensation. The gene determines laterality and, in the absence of *Pitx1*, is preferentially expressed on the left side (Marcil *et al.*, 2003). Studies on *Pitx1* have shown that the gene accounted for up to 40% of the variance in the size of the pelvic complex (Shapiro *et al.*, 2004).

A gene of large effect (*Ectodysplasin* or *Eda*) has also been identified as the basis for lateral plate expression (Colosimo *et al.*, 2006). The gene and its receptor *Ectodysplasin* receptor (*Edar*) belong to the tumour necrosis family and play an important role in the formation of skin, hair and teeth. Interestingly, a mutation in *Edar* was found to underlie scale loss in Medaka (Kondo *et al.*, 2001). The *Eda* gene maps to linkage group IV on the stickleback linkage map, and explains 75% of the observed differences in plate number. Three further minor quantitative trait loci (QTL: sections of DNA that are in close proximity to genes that underlie a trait of interest) were also detected and found to have an additive effect on plate development, increasing plate number when fish inherited alleles from a high plated parent and decreasing plate number when fish inherited alleles from a low plated

parent (Colosimo *et al.*, 2004). From an evolutionary perspective the *Eda* gene is also interesting in that it is found close to genes important in parasite defence and saline regulation. Co-selection of these closely linked genes may have been crucial to the successful invasion of freshwater by marine ancestors (Kingsley, pers. comm.).

Although QTL's for the inheritance of dorsal spines are known, the pattern of inheritance for this trait is complicated. Peichel *et al.* (2001) found QTL for spine length mapped to four different locations in the linkage map (groups I, II, VIII and XI) and recently, in a study using 3 populations, (Summers B., pers. comm.) found twelve QTL important in dorsal spine expression, suggesting a more complex basis for dorsal spine reduction than that seen in either plate or girdle complex reduction. To date, a limited amount of work on the inheritance of the anal spine has been published and little is known about the mechanisms that underlie the inheritance of this trait.

In identifying the major chromosome regions controlling armour expression (*Pitx1* underlying pelvic girdle reduction or loss and *Eda* underlying reduction of lateral plate number [Cresko *et al.*, 2004; Shapiro *et al.*, 2004; Colosimo *et al.*, 2005]), the above studies also demonstrated that the same phenotype is produced repeatedly by mutations in different populations. Reduction and loss of the pelvic complex (lateral plates, pelvic girdle and dorsal spines) has evolved numerous times within the Gasterosteidae and can be dated back to Pliocene/Miocene times (Bell, 1974, 1987, 1988; Mural, 1973). At least three genera exhibit variation in the pelvic complex, the three-spined stickleback, the nine-spined stickleback *Pungitius pungitius* (Linnaeus), and the brook stickleback *Culaea inconstans,* (Bell & Orti, 1993; Nelson, 1971; Reist, 1980a; Reist 1980b). Interestingly, of the populations so far identified that exhibit body armour reduction, almost all inhabit locations that were glaciated during the last glacial advance (Bell & Orti, 1994; Moodie & Reimchen, 1976).

Although it has been established that armour reduction in North American and Icelandic populations is a case of parallel evolution, to date no mapping studies on phenotypically similar Scottish populations have been carried out. This is of particular interest since the selective regimes responsible are different (predation versus low calcium). A major aim of this thesis is therefore, to examine the genetic mechanisms underlying armour loss in Scottish populations, to determine whether these are the same as for N. American and Icelandic populations. In terms of

molecular work, for scientific and logistic reasons, I concentrate on loss of the pelvic spines and the involvement of the *Pitx1* system. However, I also examine patterns of inheritance of the dorsal and anal spines and lateral plates in my experimental crosses.

1.5 Biodiversity and conservation

1.5.1 Scottish three-spined stickleback diversity

After the retreat of the ice-sheet 10,000 years ago, fish populations on the Outer Hebridean islands became isolated in lochs as a result of isostatic rebound and gene flow with either the ancestral population or geographically close neighbouring populations was reduced. Consequently adaptations to local environmental conditions (such as low calcium), has led to population differentiation (Campbell, 1985). In the case of fish from the Uists, this has manifested itself in a marked reduction in protective body armour in several lochs. Giles (1983) sampled 27 lochs containing armoured reduced stickleback. One location in particular though, Loch Fada on North Uist, has attracted the attention of several researchers (Giles, 1983; Cole *et al.*, 2003; Shapiro *et al.*, 2004; Coyle, 2006 unpublished) as being one of the few locations where completely armour-less stickleback are found. However, it is unknown whether the sticklebacks from this loch are genetically distinct from those in neighbouring water bodies where armoured reduction is also prevalent. As the island itself is on average no more than 17km in length and width (Beverage, 2001) and has around 180 lochs and lochans (Giles, 1983), many of which are interlinked, the expectation would be that Fada is not genetically different. Rather, fish from this loch are likely to be part of a larger metapopulation, dispersed among a number of geographically localised and interlinked lochs. If, against this expectation, populations of armour-reduced sticklebacks from the Uists were genetically distinct, this would raise questions concerning the appropriate strategy for their conservation. Should all populations be conserved or should Loch Fada, the only loch with completely armour-less fish, be afforded a higher conservation status than other nearby lochs?

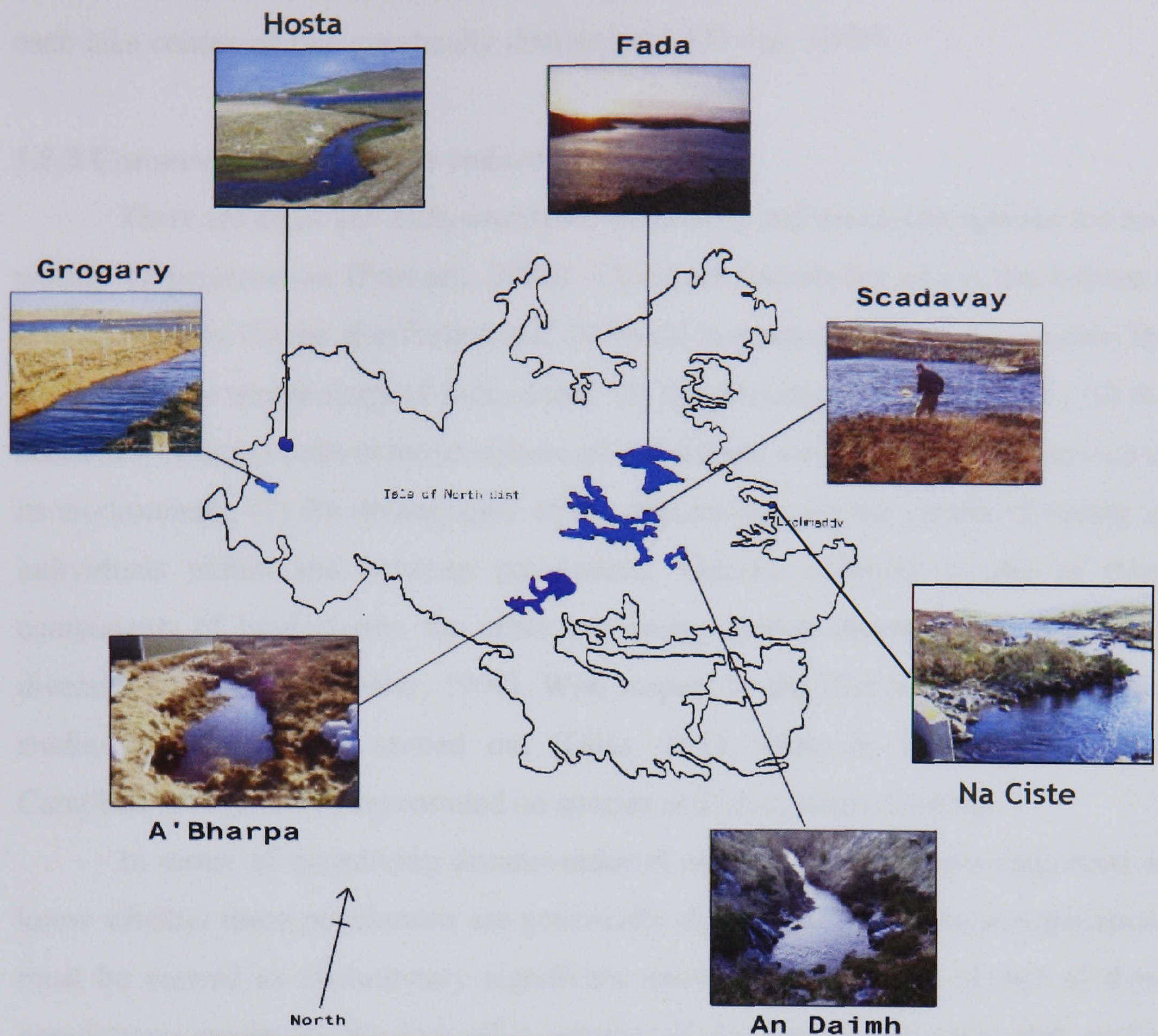

Figure 1.1 Line drawing of North Uist, Outer Hebrides, with locations and pictures of the seven study sites used on this island.

1.5.2 Assessing genetic diversity

Before these questions can be addressed, comparisons between the Uist populations, those on the mainland and on other islands should first be made. Fortunately, in tandem with environmental adaptations, genetic mutations occurring in non-coding regions of the genome also arose and these can be used to differentiate populations, even in relatively young (10,000 years) systems (Zhang & Hewitt, 2003). Such microsatellite markers (1 to 4 base-pair repeating units) have been used to explore common ancestry, geography and genetic divergence, in lake, estuarine and marine European populations of three-spined stickleback (Reusch *et al.*, 2001; Mäkinen *et al.*, 2006). The same molecular tool was utilised in assessing sympatric

benthic and limnetic stickleback pairs from two lakes, in North America, finding that each lake contained two genetically distinct pairs (Taylor, 1999).

1.5.3 Conservation of armour reduced populations

There are eight key components for protecting and managing species deemed worthy of preservation (Primack, 2006). Those are knowledge of (1) the habitat a species lives in, (2) the distribution and (3) biotic interactions of a species within the habitat, (4) the morphology of individuals, (5) the physiology of individuals, (6) the behaviour of individuals in terms of how an individuals actions permit it to survive in its environment, (7) the demography of the populations, (8) the genetic diversity of individuals within and between populations. Genetic diversity is one of three components of biodiversity, the other two being species diversity and ecosystem diversity (Redford & Richter, 1999). With respect to the Uist populations, the few studies that have been carried out (Giles, 1983; Giles & Huntingford, 1984; Campbell, 1985), have concentrated on species and ecosystems diversity.

In terms of conserving armour-reduced populations, it is now important to know whether these populations are genetically distinct. If so then these populations must be viewed as evolutionary significant units, in that the loss of one of these populations results in the loss of a portion of the genetic diversity that makes adaptation within a species possible (Waples, 1991). Fortunately, the Outer Hebrides has already been recognised as a site of global importance and has 1 World Heritage Site, 15 Special Protected Areas (SPA's), 14 Special Areas of Conservation (SAC's), 4 RAMSAR sites, 2 Biosphere Reservation and 53 Sites of Special Scientific Interest (S.S.S.I.) to name a few. However, without knowledge of the genetic diversity of fish species in this location, in particular the three-spined stickleback, appropriate management and monitoring of these potentially unique populations cannot be carried out.

Although human impact in the form of habitat destruction is unlikely in the Uists, with only 2,000 people inhabiting the island and the population in decline (SEA scoping report, 2006), an increase in fish farming, stocking of sport-fish or intentional or accidental introduction of a non-native fish species in, for example Loch Fada, may have an adverse effect on the stickleback population. For example, in Loch Lomond no less than four new fish species have been introduced and subsequently established themselves in the loch. This has had a serious impact on the

long-standing predator-prey interactions in Loch Lomond (Adams, 1994). Two further current examples and specifically concerning sticklebacks, are found in North America. The first is occurring in one of the renowned lakes containing benthic-limnetic stickleback pairs, Enos Lake, where the introduction of the Signal crayfish (*Pascifasticus lenisculus*) has coincided with the breakdown the 'species-pair' (Gow *et al.*, 2006; Taylor *et al.*, 2006). The second has seen the extinction of armour-reduced stickleback in Prator Lake, south-central Alaska, due to the introduction of an exotic fish species (Patanker *et al.*, 2006). A key aim of this thesis therefore is to examine genetic differentiation among eight populations of three-spined stickleback, using microsatellite markers. Six populations were located on the Outer Hebrides, one on the Orkney island of Stronsay and one on the Scottish mainland. It is hoped that the results of the study will inform the conservation of ecologically important armour reduced populations.

1.6 Overall aims and thesis structure

A key initial aim of this thesis was to use QTL analysis to examine the inheritance of risk-taking, a trait shown in previous studies to be repeatable in fish held in groups between trails (Huntingford, 1976). However, it became apparent early on in my study that risk-taking was not a repeatable trait in my fish, possibly because these were held in isolation between trails. As a result, I changed the emphasis of my research programme and developed a broader set of aims, while continuing to examine variation in risk-taking. These broader aims included a study of the inheritance of armour reduction in relation to calcium concentration and of genetic differentiation between stickleback populations. The final structure and output of my research programme is best understood in the light of this change in emphasis and direction. The thesis is therefore structured chronologically, with an initial chapter documenting loss of repeatability of risk-taking in fish housed in isolation, behavioural and morphological studies discussed in parallel and concluding with the discovery that all populations are genetically distinct.

With this background, the work described in this thesis was designed to answer the following 5 questions:

1. Does the calcium hypothesis hold up when one looks at a wider geological range of sites?

The general aim of **Chapter 3** was to firstly, characterise each of the study sites in terms of the ecology and chemistry found at each and secondly, to investigate the relationship between water chemistry, with particular reference to calcium concentration, and armour expression.

2. Do armoured and unarmoured fish differ in one aspect of their defensive behaviour, namely readiness to explore a novel environment?

Chapter 4 explores the variability in risk-taking, utilising the novel environment test, and attempts to relate risk-taking to armour expression both within and between populations. Variability in risk-taking has been related to predator regime and, as all sites have predators, the prediction is that armour reduced populations will be more risk-averse that armoured populations, where fish are better protect against predation.

3. Are such differences in behaviour inherited (with a subsidiary question of how repeatable this trait is) and if so, what is the genetic mechanism and can the major loci responsible be found, using QTL mapping?

The aim of **Chapter 5** was to study the inheritance of risk-taking by setting up two lines based on parental behavioural phenotype, one line using fish from the Uist populations and the other using fish from a mainland site. This study (and that described in **Chapter 4**) highlighted some unexpected complexities in quantifying risk-taking in sticklebacks using the novel environment test (in particular, inconsistent repeatability's) and these are discussed in **Chapter 2**.

4. What is the pattern of inheritance of body armour in these sites?

Pattern of inheritance in dorsal spines, pelvic complex and lateral plates, within and between different populations of three-spined stickleback, were examined in **Chapter 6**. In the same chapter, I examine whether loss of the pelvic complex in Scottish populations of three-spined stickleback is due to mutation at the *Pitx1* locus or to a mutation in an upstream regulator of *Pitx1*, by means of a QTL mapping study.

5. Are the various sites with reduced armour genetically distinct populations, and how distinct are they from nearby armoured sites?

Genetic differences between 8 differently-armoured populations in geographically distinct locations were investigated in **Chapter 6**. I used 8 microsatellite markers to differentiate populations on the basis of genetic distance, examining genetic similarity in relation to geographic distance, morphological differences and ecological differences (calcium concentration).

Chapter 2. Problems with screening risk-taking

2.1 Introduction

Individual differences in risk-taking (also termed 'boldness') have been reported in studies using several different species of fish, birds and mammals (Wilson, 1994). For example, Budaev *et al.* (1999) used Lion-head cichlids (*Steatocranus casuarius*), Dingemanse *et al.* (2002) worked with the Great tit (*Parsus major*) and Ruis *et al.* (2000) utilised the domestic pig (*Sus scrofa domesticus*). Where such differences are present, it is important to know whether these are consistent individual traits or temporary responses to current or recent circumstances. Significant repeatability of individual differences in risk-taking has been demonstrated in many studies, in that an individual's relative position remains stable across tests. For example, Dingemanse *et al.* (2002) investigated exploratory behaviour in individuals from two populations of Great tit. Birds were captured from the wild and screened within 24 hours, after which, they were released in the location from where they were taken. One fifth of these birds were recaptured and retested several months later with repeatability estimates (r) of between 0.27 and 0.66.

The three-spined stickleback (*Gasterosteus aculeatus*) is one of a number of fish species (Trinidad killifish (*Rivulus hartii*) Fraser *et al.*, 2001; Poeciliid (*Brachyraphis episcopi*) Brown & Braithwaite, 2004; Carp (*Lepomis macrochirus, Carassius carassius* and *Carassius auratus*) Yoshida *et al.*, 2005) used to test individual differences in risk-taking in a novel environment. Huntingford (1973) screened sticklebacks in groups of three to four in an unfamiliar tank with weed refuge and several weeks later repeated the test without refuge. Using Principle Components Analysis (PCA), behaviour was summarised into a single 'boldness' score, reflecting the time taken to emerge from cover and the rate fish took to explore the tank. Fish stayed in the same small groups between tests. A regression analysis showed that behavioural scores in test 1 were predictive of scores in test 2 (r^2 = 26% P = 0.001). A second study where fish were screened singly, on two separate occasions several weeks apart, and in small groups between testing, also showed consistency (r^2 = 13.3% P = 0.005; Huntingford, unpublished data). In an earlier

published study, the response to a novel environment of sticklebacks was predictive of how fish behave towards a predator several weeks later and also of response to an intruder, several months after the first tests (Huntingford, 1976). These results imply that the response of an individual to a novel environment reflects a consistent feature of the 'personality' of that fish.

A comparison of stickleback populations from high and low predation regimes showed that, differences in boldness (exhibited by individuals exposed to a model predator) were population-specific. Population differences in boldness persisted in fish reared in the laboratory without exposure to a predator (Huntingford *et al.*, 1994). This suggested a possible heritable component to the original behavioural difference, although population differences were not apparent in orphaned fish removed from their father before hatching (Tulley & Huntingford, 1987).

On the basis of the apparent consistency and heritability of differences in boldness in three-spined sticklebacks, one aim of the work described in this thesis was to investigate the underlying genetic mechanisms, using QTL mapping based on the genome-wide linkage map for sticklebacks (Peichel *et al.*, 2001). This requires accurate screening of large numbers of fish and to do this we used a standard test of exploration/activity in a novel environment (Verbeek *et al.*, 1994) to assess risk-taking (Figure 2.1). This is a well-established technique for quantifying risk-taking that is broadly predictive of some aspects of the sticklebacks' response to a predator (Huntingford, 1976; Bell & Stamps, 2004), but is easier to standardise than using a live predator or even a model predator.

To ensure that individual risk-taking was measured accurately, a number of potentially confounding variables were identified and, where possible, controlled for (see Chapter 4 for more details). Fish were deprived of food for a minimum of 20 hours prior to testing to minimise differences in hunger level, which are known to influence risk-taking (Lima & Dill, 1990). To standardise the effect of social interaction, except for the initial studies of wild caught fish where subjects were held in small groups, all other fish were individually housed for a minimum of three days prior to screening. Fish were allowed to settle for a minimum of one hour in aerated holding tanks and under low light conditions before testing, to standardise stress levels. All observations were carried out blind, ensuring unbiased screening by the observer.

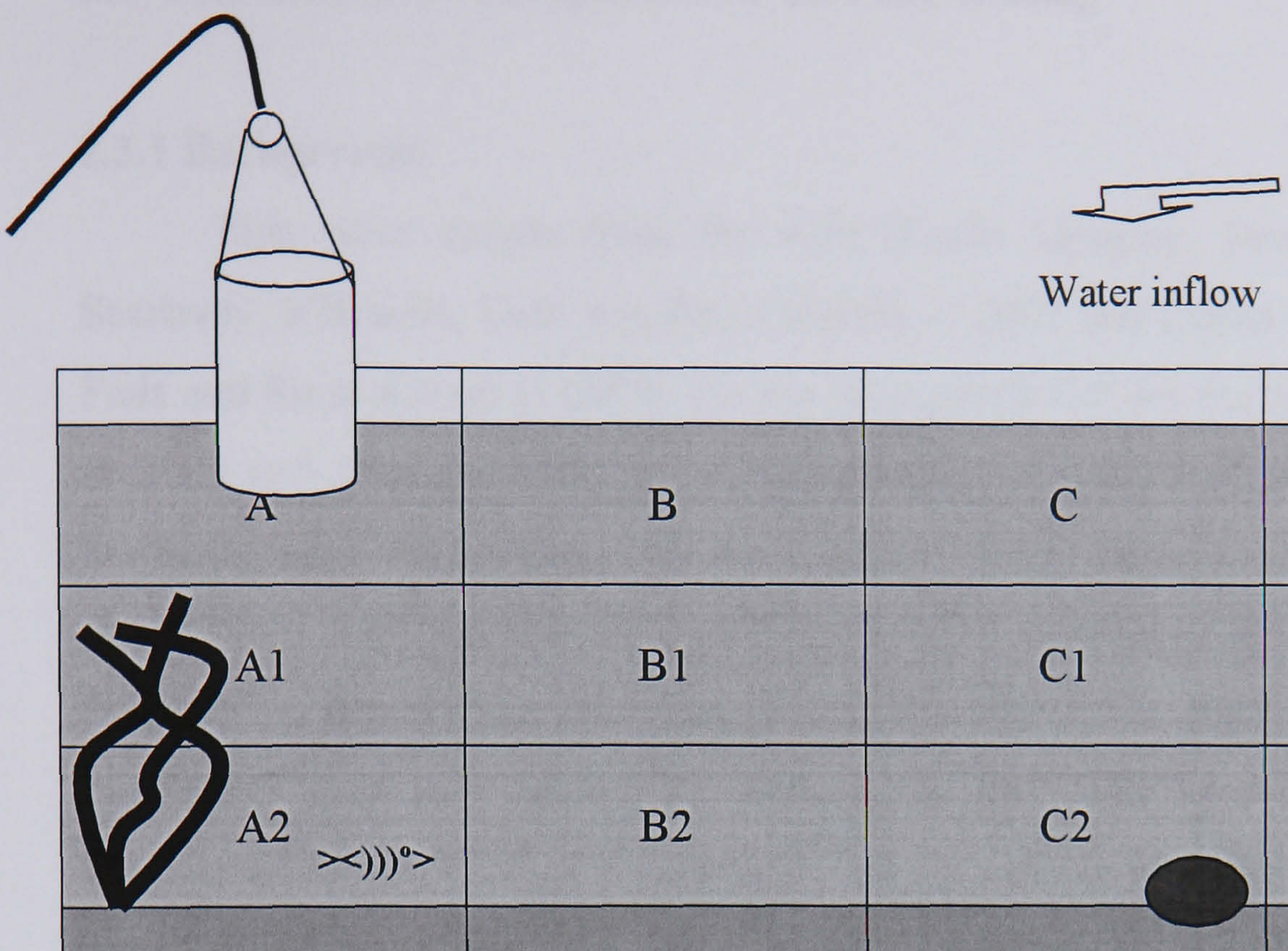

Figure 2.1 Diagram of behavioural observation tank. The tank was divided into 12 cells, top to bottom 30cm and moving from left to right 100cm (cells A, A1, A2……D, D1, D2), allowing the observor to monitor the study fish more easily.

In spite of the steps taken to ensure that I obtained "clean" scores of risk-taking, uncompromised by effects of the social and non-social environment, two problems became apparent. In the first place, it became clear that water temperature had a marked effect on behaviour and that this could not always be allowed for statistically. Secondly, when the same fish were tested on separate occasions, with a view to confirming previous findings of consistency for risk-taking by sticklebacks in a novel environment, this could not be reliably confirmed.

2.2 Aims

The aim of this chapter is therefore to provide a background for later chapters by presenting results relating to;

1. The effect of temperature on risk-taking in a novel environment.
2. Estimates of consistency, based on:
    a. Several samples of fish tested on two occasions.
    b. A sample of 25 fish tested on 5 separate occasions.

2.3 The effects of temperature on risk-taking

2.3.1 Background

Fish were caught from the wild (Lochs Grogary, Druidibeg, an Daimh, Scadavay, a'Bharpa, Fada and River Kelvin in 2003 and Lochs Grogary, a'Bharpa, Fada and River Kelvin in 2004) to serve as parental fish for the breeding programme in 2003 and 2004 (see Chapter 4). It transpired that the fish caught in 2004 were markedly more risk-averse, with the majority of fish falling into this category (see Figure 2.2). Although the tests were carried out at broadly the same time of year (Mar 14th – Apr 17th in 2003 and Feb 23rd – Mar 3rd in 2004) the temperature at which the tests were carried out differed. In 2003 fish were screened at a mean temperature of 7.1°C (Min = 5.2°C Max = 7.7°C) and in 2004 fish were screened at a mean temperature of 5.6°C (Min = 4.7°C Max = 6.6°C). This suggested that temperature was having an effect on risk-taking, even though the temperature range in the two years overlapped. A small-scale study was therefore undertaken to test this possibility and to assess the size of any detected effect of temperature on risk-taking.

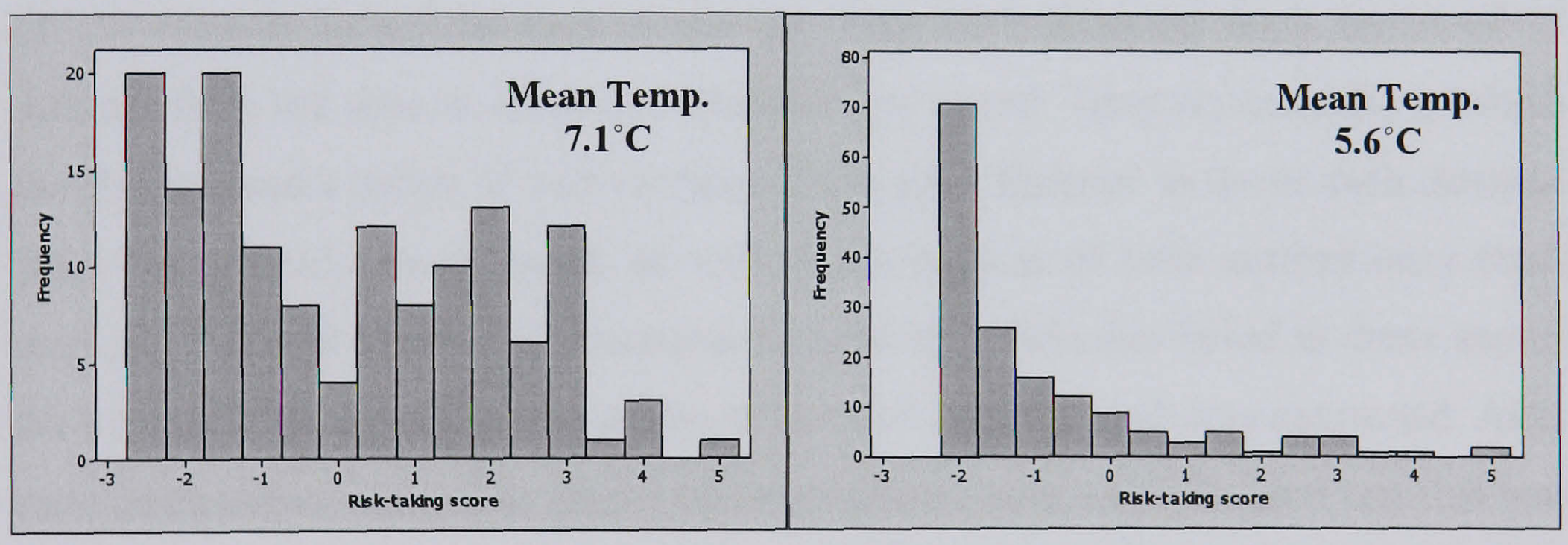

Figure 2.2 Frequency of risk-taking scores in fish caught from the wild in 2003 and screened at a mean temperature of 7.1°C (right graph) and 2004 and screened at a mean temperature of 5.6°C (left graph).

2.3.2 Materials and methods

A group of 20 fish that had been caught from the wild in 2004 (Lochs Grogary, a'Bharpa, Fada and River Kelvin) and screened once between Feb 23rd and Mar 3rd (at a mean temperature of 5.6°C) were chosen at random and moved to a separate holding tank (1m x 1m). The water temperature in the holding tank was slowly raised (over five days) to 10°C and held at this temperature for 1 week. Fish

were retested at this temperature, after which the temperature was reduced to 7°C. Fish were maintained at this temperature for one week, after which they were tested for a third time. All observations were carried at the Scottish Centre for Ecology and the Natural Environment (SCENE), Loch Lomond.

The screening method was as follows. Two to three observers seated side by side carried out observations simultaneously. Each flow-through observation tank (100cm x 40cm x 40cm, Figure 2.1) was surrounded on all sides with cardboard to conceal external movements, with a horizontal slit along the front wall for observation. The sole source of illumination was a 40watt light suspended 30cm above the tank. The bottom of the test arena was covered with sand and a 30cm high plastic plant for refuge was located in the area where the fish was released (home-base). To prevent sand being moved around while water flowed into the tank, a large stone was positioned under the in-flow pipe, 75cm from the home-base. To aid observations, a grid was drawn on the back of the tank to divide the tank into twelve equal lettered cells (25cm x 10cm). Each fish was allowed to settle in a lowered holding chamber (10cm in diameter by 50cm high) for 15 minutes prior to the start of each test. Water temperature in the tank was noted and in-flowing water was turned off 20 seconds before the start of the test. Fish were observed for a period of 15 minutes from the time at which the chamber was raised. Time (in seconds) at which the fish crossed a series of vertical lines 25cm apart (latency to move each distance from home-base) was recorded, as well as the number of cells entered once (tank use) and the total number of squares used (activity). Fish that failed to cross any of the vertical lines were given an arbitrary score of 1000 for each line uncrossed. After each observation, water was turned on to refresh the tank while the next test fish was settling. Following testing, fish were returned to their holding tanks until re testing.

Principle Component Analysis (PCA) was used to condense the behavioural data sets, summarising the measured variables into a single factor score for each fish (Huntingford, 1976 and see Chapter 4). Variables used were latency to cross lines AB, BC and CD (Figure 2.1), activity and tank use. The first principle axis (PC1) opposed latency to cross the 3 lines (negative loadings) against activity and tank use (positive loadings). This was interpreted as an index of risk-taking and is comparable to the index of boldness identified by Huntingford (1976) and Bell (2005).

2.3.3 Results and discussion

To test if risk-taking changed with temperature a Kruskal-Wallis ANOVA was carried out and found to be significant (Figure 2.3; $H = 29.74$, $DF = 2$, $P < 0.001$). *Post hoc* tests showed that the average degree of risk-taking in the first test (at a mean temperature of 5.6°C) was significantly lower (Median = -2.06) than in the tests carried out at 10°C and 7°C (10°C, Median risk-taking = 1.25; 7°C, Median risk-taking = 1.07). In addition, there was a wider distribution of risk-taking in the trials run at the higher temperature. It is quite clear from this study that there is there an effect of temperature on risk-taking, fish tested at low temperatures (Mean = 5.6°C) being significantly more risk-averse than those tested at 7°C or higher. For this reason, later tests were only carried out in the temperature range of 7 – 10°C.

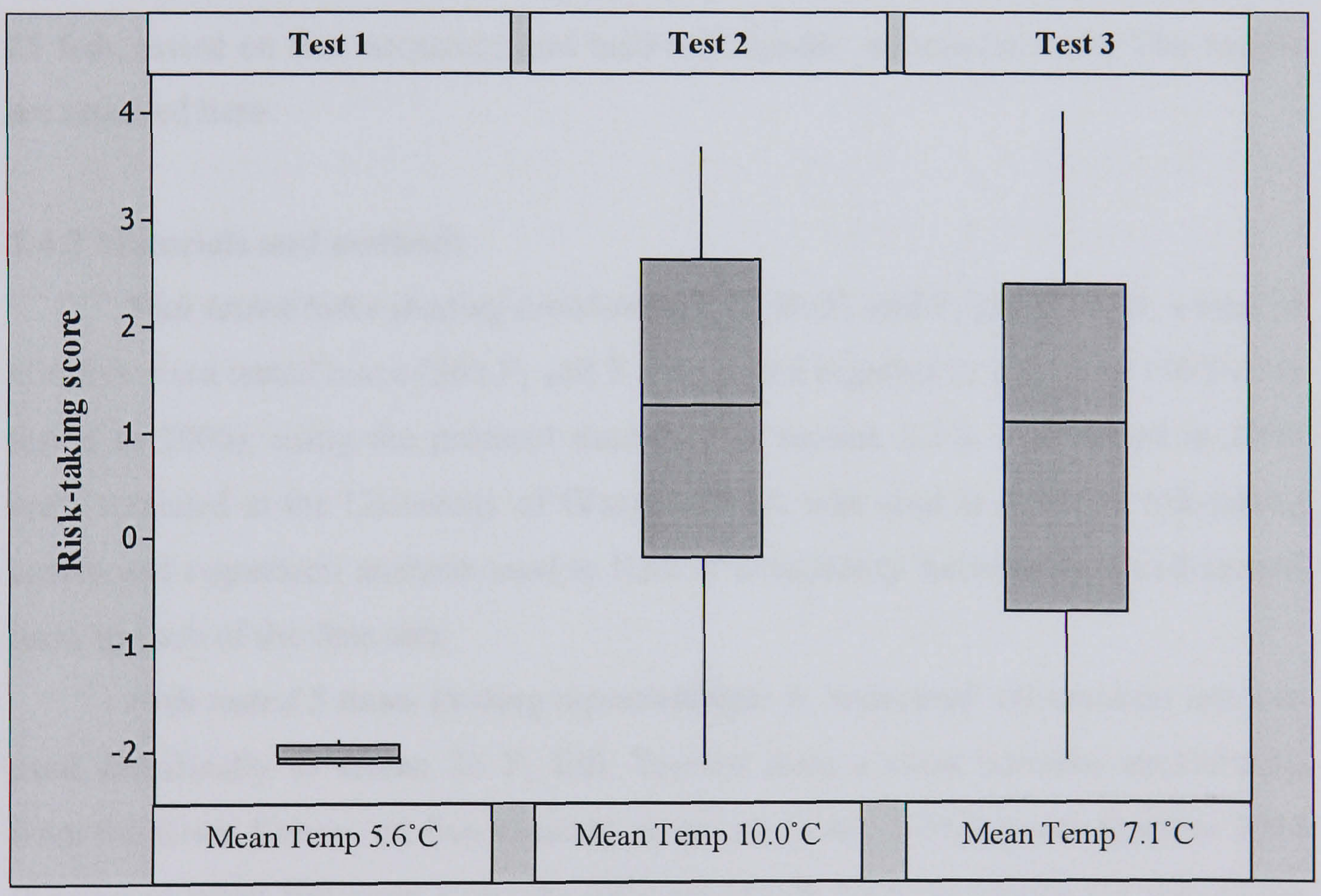

Figure 2.3 Box-plot of risk-taking scores across 3 tests at 3 different mean temperatures; Test 1 = 5.7°C, Test 2 = 10.0°C, Test 3 = 7.1°C.

2.4 Inconsistent risk-taking behaviour

2.4.1. Background

On a number of occasions and for various reasons, the same fish were tested twice (being held in isolation in between tests) and the results used to examine the consistency of risk-taking, once the effects of all uncontrolled variables, including temperature, were removed. The fish used in these tests were first and second generation progeny produced for the study of the inheritance of risk-taking (see chapter 5). The degree of consistency observed between the two tests was variable (see below). To examine this more closely, in particular to eliminate any effects of social interactions within groups (which, it was thought, might reduce consistency) and to obtain an exact measure of repeatability, a systematic study was carried out on 25 fish, tested on five occasions and held individually in between tests. The results are reported here.

2.4.2 Materials and methods

Fish tested twice (testing consistency): In the F_1 and F_2 generations, a total of 406 fish were tested twice (266 F_1 and F_2 fish tested together in 2005 and 140 F_2 fish tested in 2006), using the protocol described in section 2.3.2. Fish tested in 2006 were screened at the University of Glasgow. PCA was used to generate risk-taking scores and regression analysis used to look at consistency between first and second tests, in each of the data sets.

Fish tested 5 times (testing repeatability): A 'truncated' observation test was used specifically to screen 36 F_1 fish, derived from a cross between sticklebacks from the River Kelvin, on five separate occasions (Tests 1-5) between October 2004 (beginning when fish were 4 months old) and March 2005. Screening for risk-taking was carried out in the same manner as above, except that the test was terminated when a fish crossed the line AB. Time at which a fish crossed this line, tank use and activity (see above) were noted. Fish that failed to cross the line were observed for the full 15 minutes and given the arbitrary score of 1000. Fish were always tested in the same sequence and at the same time of day, to maximise the chances of obtaining repeatability. After testing, fish were returned to their individual holding chamber until the next behavioural screening was carried out.

Any fish exposed to an un-correctable confounding variable during screening, such as a loud noise or water flowing into the observation tank, were removed before statistical analysis was carried out (N = 11). Data were combined using PCA as described above and the PC1 scores (a "risk-taking" index accounting for 77.7% of the total variance) corrected for temperature by using residuals from the linear regression of risk-taking against temperature (r^2 = 0.298 N = 125 P = 0.001; Boylan, 2005). Temperature corrected or 'clean' risk-taking scores were then used for further analysis. Kruskal-Wallis ANOVA was used to look for significant changes in behaviour across the five tests. Scores in each pair of tests were compared for consistency using regression analysis and one-way ANOVA was used to obtain a measure of repeatability (Lessells & Boag, 1987).

2.4.3 Results and discussion

Fish tested twice: Regression analysis of risk-taking between tests for all F_1 and F_2 fish tested in 2005 showed low but significant positive relationships between the scores in the two tests (N = 266 r^2 = 12.3% P < 0.001). When fish were grouped by generation, the level of association for F_1 and F_2 fish was similar (F_1 fish, N = 213 r^2 = 11.2%, P < 0.001; F_2 fish, N = 53 r^2 = 10.6% P < 0.001) compared to F_2 fish tested in 2006 (N = 140 r^2 = 34.5% P < 0.001).

Fish tested 5 times: There was no significant difference in risk-taking behaviour across the 5 tests (Figure 2.4. Kruskal-Wallis; H = 2.52 DF = 4 P = 0.641), although there was less variability in behaviour in Test 4 and Test 5.

In our large sample of fish tested twice (held in isolation between tests) there was a significant, if weak (2005 fish), positive relationship between risk-taking scores derived from the two tests. The level of consistency was higher in F_2 fish tested in 2006. Although these fish were held in isolation and screened in the same manner as F_1 and F_2 fish tested in 2005, there was one notable difference between these two groups. Fish screened at the University of Glasgow were exposed to white noise generated by air-conditioning units within fish rooms. It is possible that fish habituated to this constant noise (Smith *et al.*, 2004) that acted as a filter blocking out intermittent noise and increased consistency between tests. However, there was no overall repeatability in risk-taking across tests in the smaller sample of fish tested on 5 occasions and held in isolation in between. This is in contrast to published

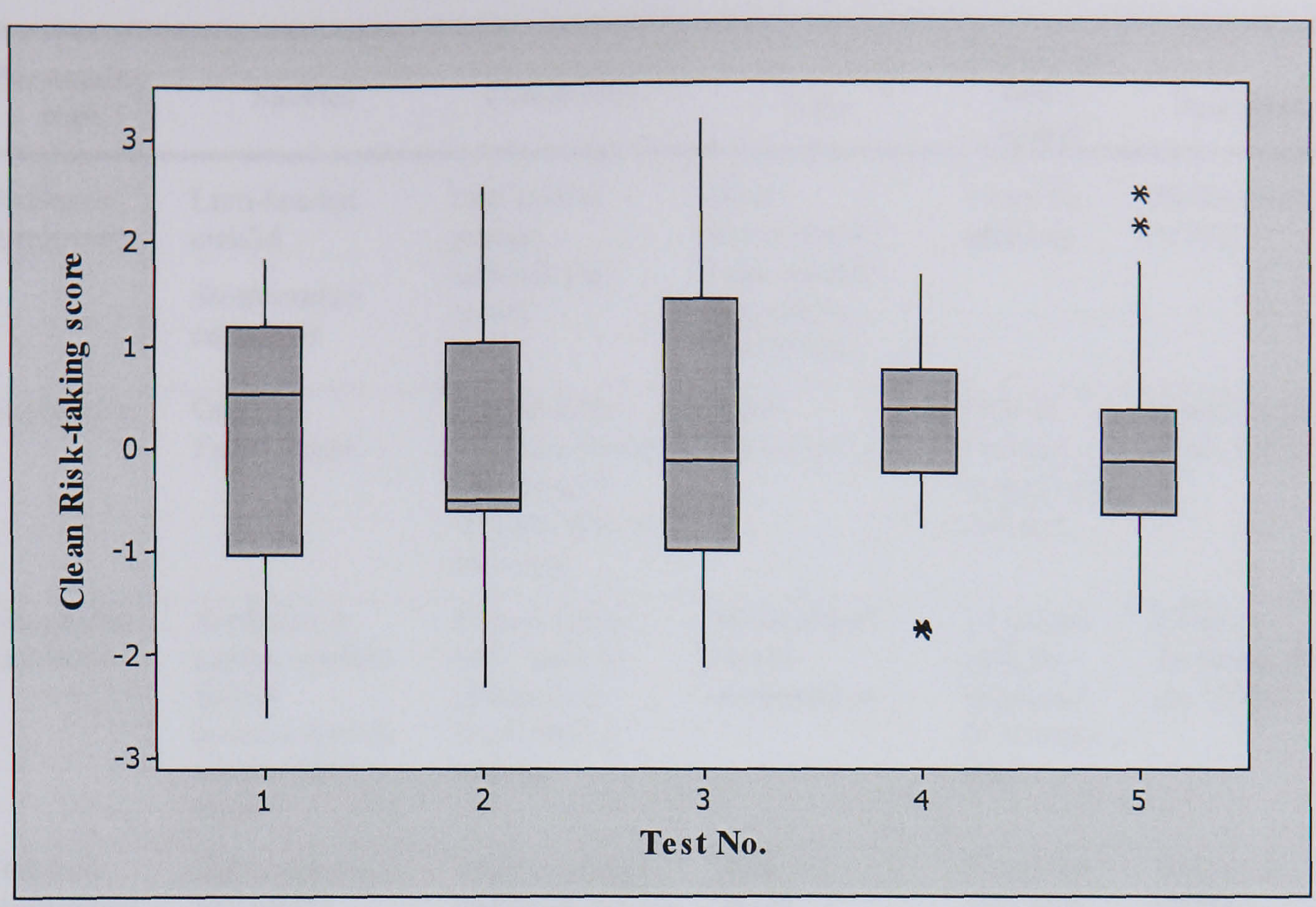

Figure 2.4 Representation of the median and range of rank risk-taking scores of 25 individual fish tested 5 times over a period of 6 months. Test 1: 19&20th Oct; Test 2: 17&18th Nov; Test 3: 15&16 Dec; Test 4: 13&14 Jan; Test 5: 06&07 Mar.

studies summarised in Table 2.1, were tests were carried out on two occasions only. It is also in contrast to Huntingford's earlier studies of sticklebacks (1973, 1976). However, it does agree with Bell & Stamps (2004), who did not find a significant correlation between individual levels of activity in a novel environment in the same sticklebacks tested as juveniles and (c. 9 weeks later) as sub-adults (held in groups between trials).

Table 2.2 shows the results of comparisons between all pairs of tests. Although there were marginally significant positive relationships between Tests 1 & 2 and Tests1 & 4, overall there was very little relationship between the risk-taking scores of the same fish in successive tests. In line with this, the value for repeatability (r) was very low ($r = 0.013$).

Personality trait	Species	Conditions	Tests	Number of times tested	Reference
Boldness Aggression	Lion-headed cichlid *Steatocranus casuarius*	Fish held in groups. Individually tested.	Novel environment. Inter-specific inspection. Mirror test.	Twice for each test.	Budaev et al., (1999)
Exploration	Great tit *Parus major*	Caught from wild and tested individually within 24hrs of catching.	Novel environment.	Fifth of birds re-caught and retested.	Dingemanse et al., (2002).
Neophobia Boldness	Sardinian & garden warbler *Sylvia melanocephala momus & S. borin*	Caught from wild, held in groups one week before testing.	Novel object. Novel environment.	Twice for each test. Repeated 10 months later.	Mettke-Hofmann et al., (2005)
Boldness Aggression Feeding competition	Domestic pig *Sus scrofa domesticus*	Kept in groups. Tested in groups and individually.	Back test. Novel environment. Feeding competition test.	Twice for each test.	Ruis et al., (2000)

Table 2.1 Selection of papers on personality traits, syndromes or coping strategies, species used, conditions animals were held under (before, during and after testing), the type and number of times the test was used and the paper reference.

	Dependent variable			
Independent variable	Test 2	Test 3	Test 4	Test 5
Test 1	$r^2 = 8.9, p = 0.08$	$r^2 = 0.0, p = 0.50$	$r^2 = 9.1, p = 0.08$	$r^2 = 0.0, p = 0.41$
Test 2		$r^2 = 7.7, p = 0.10$	$r^2 = 1.3, p = 0.26$	$r^2 = 2.1, p = 0.23$
Test 3			$r^2 = 0.0, p = 0.92$	$r^2 = 0.0, p = 0.77$
Test 4				$r^2 = 0.0, p = 0.85$

Table 2.2 Comparisons of risk-taking scores, using regression analysis, between all pairs of tests; Tests 1 & 2, Tests 2 & 3, Tests 3 & 4 and Tests 4 & 5 with r^2 and P values.

There are a number of possible reasons for our failure to find significant repeatability in our longer-scale tests. Casting around for possible explanations against which the data could be tested, temperature and habituation appeared likely candidates. The 5 tests were carried out over a period of declining water temperature.

If all our fish became increasingly reluctant to leave cover at low temperatures, loss of variability in later tests might explain the lack of relationship between successive tests. Another possibility is that over the 5 tests, the fish might well have habituated to the initially novel environment (Brown, 2001). Such an effect might cause all fish to emerge from cover quickly, gaining high risk-taking scores. Here again, loss of variability in later tests might explain the lack of relationship between successive tests. Unfortunately, since temperature and test number were strongly associated (Spearman rank order correlation; $R_s = 0.247$ N = 5 P = 0.005), we could not separate their effects statistically. However, as Figure 2.4 shows, neither the median nor the range of risk-taking scores changed over the study period, so we believe that we can rule out these two possible explanations for the lack of individual repeatability in our tests.

A third possibility is that our failure to find individual repeatability in risk-taking where other studies have done so, arises from the fact that our subjects were held in isolation between successive tests (partly to allow fish to be identified, but also to rule out possible influences of social interactions within groups). Perhaps individual differences in risk-taking are somehow reinforced by experience within groups and change when fish are separated from the group. That interactions within groups can influence risk-taking is demonstrated by Magnhagen & Staffan (2005), who found that in young of the year perch (*Perca fluviatilis*) time to emerge from cover (reflecting a shy-bold continuum) was affected by group composition. When a shy individual was placed in a group consisting of bold fish only it became bolder, spending more time in open water, and when a bold fish was placed in a shy group, it became shyer, conversely spending more time hiding.

Wilson *et al.* (1993) found that differences between sunfish (*Lepomis gibbosus*) classified as shy or bold in a field test were stable so long as fish remained in the field, but broke down after a period of social separation in the laboratory. It is possible then that differences in risk-taking in the three-spined stickleback are produced and maintained by social forces. Removal of these forces, for example holding fish in social isolation, allows risk-averse individuals to become risk-takers and *visa versa*. Quite what such social forces might be requires further research.

2.5 Conclusions

The results presented in this chapter suggest that rate of exploration of a novel environment test (a standard, robust, easy to control and repeatable test that has been used to identify risk-taking in stickleback and other animals and so suitable for the purpose of providing data for a QTL mapping) was, in retrospect, not suitable in the present context for two reasons. Firstly, temperature has a strong effect on behaviour; although within a small range this can be corrected for statistically, low temperatures produce too many risk-averse (or non-emerging) individuals for this to work. Secondly, although in some circumstances and with large sample sizes there is significant consistency, this effect is at best weak. This is interesting in itself, but in the light of the above results QTL mapping of risk-taking was not carried out.

Chapter 3. Reduction of body armour in three-spined stickleback: the role of calcium

3.1 Introduction

3.1.1 Three-spined stickleback as a research species

Evolution induced by local selection regimes has resulted in behavioural, physiological and morphological changes in numerous populations of three-spined stickleback (*Gasterosteus aculeatus* L.). In fact, such is the great variation in morphology seen in this species that for many years it was classified as over 40 different species. Equally diverse are the many behavioural adaptations that have also been studied over many years. It is this great variability that makes this fish of interest to evolutionary biologists. In addition, the stickleback is widely distributed, is small, easy to keep and rear in captivity, tolerates handling well and has a relatively short generation time making it an ideal research species. Although reproductive barriers can occur between populations, in most cases crosses between them are possible in the laboratory. This, coupled with the advent of new molecular tools, including the construction of a genome wide linkage map for sticklebacks, has now made it possible for molecular genetic studies into the number and location of genes underlying the divergence of adaptive characteristics (Peichel *et al.*, 2001). For example, recent studies have identified major chromosome regions controlling armour expression (e.g. *Pitx1* was identified as a gene of large effect for pelvic size and *Eda* alleles play an important role in the reduction of lateral plate number) and demonstrated that the same regions are repeatedly used in different populations to produce the same phenotype (Cresko *et al.*, 2004; Shapiro *et al.*, 2004; Colosimo *et al.*, 2005).

3.1.2 Morphological adaptation to predation

Three-spined stickleback have three dorsal spines (after which they are named), two pelvic spines attached to a robust pelvic girdle and bony lateral plates that protect them against predation by invertebrates, fish, birds and mammals. However, development of protective armour is very variable and in a small number

of populations, absent. Reduction and loss of bony armour in North American populations has mainly been linked to predator regime (Reimchen, 1980). In the 'predation hypothesis' proposed by Reimchen (1980), the presence of predatory fish selected against stickleback with few or no spines, these fish being preyed upon more successfully that normally armoured fish. In the absence of such predators, which also feed on invertebrates, there is an increase in piscivorous insects. Sticklebacks with normal body armour are now selected against, as invertebrates such as dragonfly naiads can capture and hold onto normally armoured fish more easily. A reduction in skeletal armour lessens the chances of capture, as invertebrates have fewer places to grip. Alternatively, Giles (1983) proposed that in Scottish populations, the reduction and loss of spines and plates is associated with and possibly a response to reduced calcium (Ca^{2+}) concentration. In an associated breeding experiment he found that differences in armour were inherited, in that they persisted when offspring of spine reduced fish were reared in calcium rich water.

Although these hypotheses suggest that both biotic and abiotic factors play an important role in the loss of protective body armour, neither fully explains armour reduction. Un-armoured fish are sometimes found at sites with high predation pressure (Giles & Huntingford, 1984) and the correlation between reduced armour and low Ca^{2+} concentration is not perfect, since normally armoured fish are found at low calcium sites. As Giles (1983) study was based on populations that were geographically very close and possibly not genetically distinct, a study into the relationship between armour reduction and water chemistry in a larger sample of populations, with equivalent predation regimes and from a wider geographic area, is required. This is the main aim of the study reported in this chapter.

3.1.3 Characterising the study sites

The 11 lochs used in this study (Lochs Quien [Isle of Bute], Druidibeg [S. Uist], na Ciste [N. Uist], an Daimh [N. Uist], Fada [N. Uist], a'Bharpa [N. Uist], Scadavay [N. Uist], Grogary [N. Uist], Hosta [N. Uist], Charrasan [Isle of Lewis] and Meikle Burn [Stronsay, Orkney]) are all located on islands off the Western and Northern coast of Scotland. Only one site, the River Kelvin, is located on the mainland and is the only riverine site (see Figure 3.1 and Figure 1.1, Chapter 1). Although three-spined stickleback from a number of these water bodies have been used in several studies (Giles, 1983; Giles & Huntingford, 1984; Campbell 1979,

Cole *et al.*, 2003; Shapiro *et al.*, 2004), few sites have been categorised in terms of both their ecology and water chemistry. Giles (1983) measured Ca^{2+} levels at Lochs an Daimh, Fada, a'Bharpa, Scadavay, Grogary and Hosta and noted fish and bird predators. Giles & Huntingford (1984) detailed predators at Lochs Fada, an Daimh and a'Bharpa. However, little information on the remaining 6 water bodies is available. Therefore in order to achieve the initial aim of this study, characterisation of each location, primarily on terms of water chemistry but also in terms of predator regime, was carried out.

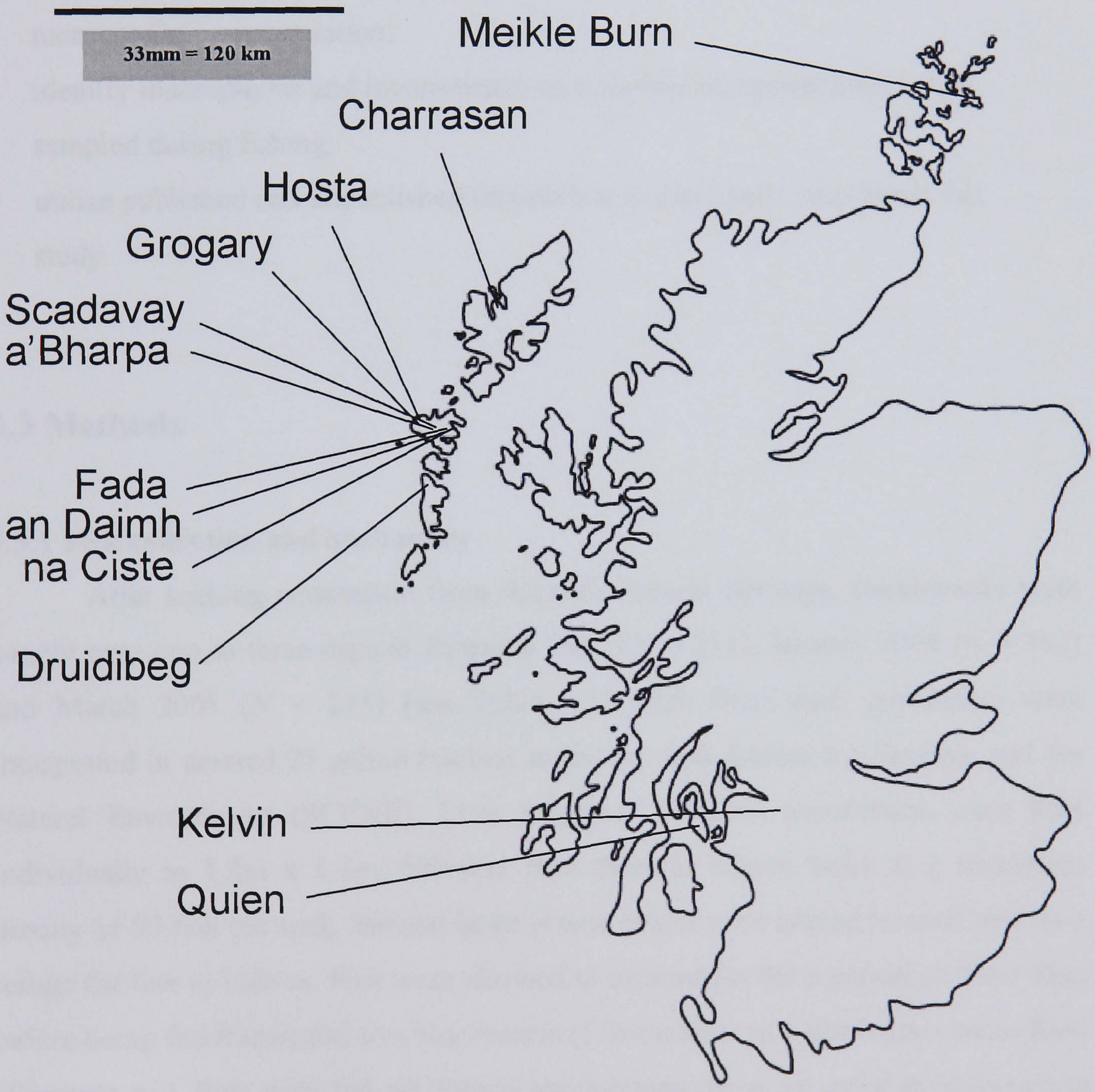

Figure 3.1 Outline drawing of Scotland showing the location of the study sites.

3.2 Aims

With this background, the specific aim of the study described here was to investigate variability in body armour and how this relates to water chemistry, in particular Ca^{2+} concentration. A second aim was to characterise the study sites by carrying out a quantitative measure of water chemistry at all sites (excluding Loch Quien and Meikle Burn), and a qualitative survey of the plants and invertebrates at three sites on North Uist. In particular, my objective was, for each of the sites to:

- assess the level of productivity at each of the sites by measuring phosphorous.
- measure pH.
- measure Ca^{2+} concentration.
- identify macrophytes and invertebrates in a number of representative sites sampled during fishing.
- utilize published and unpublished literature to expand and compliment this study.

3.3 Methods

3.3.1 Fish collection and husbandry

After seeking permission from Scottish Natural Heritage, sticklebacks were caught over two to three days in February 2003 (N = 211), January 2004 (N = 342) and March 2005 (N = 235) (see Table 3.1). Fish from each population were transported in aerated 25 gallon buckets to the Scottish Centre for Ecology and the Natural Environment (SCENE), Loch Lomond. Sampled populations were held individually in 1.3m x 1.3m, 500-litre flow-through indoor tanks at a maximum density of 50 fish per tank. Several large plastic plants were placed in each tank as a refuge for fish to hide in. Fish were allowed to acclimatise for a period of three days before being fed frozen and live bloodworm (*Chironomus* sp.) and frozen water fleas (*Daphnia* sp.). Fish were fed *ad libitum* and maintained on an ambient photoperiod and loch water temperature (6 ± 2° Celsius).

Location (Grid ref.)	Site size (L x W)	Site description	Capture method	No. of fish caught
Kelvin NS570674	20mx2m Max depth 40cm	Slow flowing, shallow river approximately 8m wide. Sand and stone substrate. Many macrophytes. Overhanging vegetation.	Hand net	2003 = ***20*** 2004 = ***28***
Grogary NF717709	25mx1m Max depth 40cm	Channelled stream into the loch. Mud substrate. No macrophytes. High earth banks with no overhanging vegetation.	Hand net Drag net	2003 = ***42*** 2004 = ***50*** 2005 = ***35***
Hosta NF723727	40mx5m Max depth 60cm	Slow flowing stream from loch to sea. Sand substrate and little organic matter. Many macrophytes, no overhanging vegetation.	Hand net	2005 = ***17***
Druidibeg NF788384	25mx5m Max depth 80cm	Small bay in large loch. Rock and sand substrate. Many macrophytes. No overhanging vegetation.	Hand net Drag net	2003 = ***31*** 2005 = ***28***
an Daimh NF888679	15mx50cm Max depth 40cm	Ditch beside loch. Mud substrate. Many macrophytes. Grass growing thickly along waters edge.	Hand net	2003 = ***17*** 2005 = ***35***
Charrasan NF19886-	30mx1m Max depth 1m	Small bay within the loch. Peat substrate and no macrophytes.	Hand net	2004 = ***50***
na Ciste NF 906683	5mx2m Max depth 50cm	Small bay with sand and rock substrate. Macrophytes and overhanging vegetation.	Hand net	2005 = ***25***
Meikle Burn HY659254	Unknown	Small shallow stream. Poor quality, eutrophic water.	Hand net	2004 = ***30***
Quien NF06798-	50mx2m Max depth 50cm	Stone substrate. Many macrophytes and large reed banks.	Hand net	2004 = ***50***
Scadavay NF871672	150mx1m Max depth 1m	Large deep loch. Sampled around circumference of water body. Rock substrate, few macrophytes. No overhanging vegetation.	Hand net Electro-fishing	2003 = ***31*** 2005 = ***35***
a'Bharpa NF833656	5mx2m Max depth 80cm	Small river running out of loch. Fish farm located next to study site. Rock/sand substrate with few macrophytes. Overhanging vegetation.	Hand net	2003 = ***48*** 2004 = ***75*** 2005 = ***30***
Fada NF891706	7mx10m Max depth 1m	Shallow bay in large loch. Sand/rock substrate. Many macrophytes. Grass at water edge, no overhanging vegetation.	Hand net Electro-fishing	2003 = ***23*** 2004 = ***61*** 2005 = ***30***

Table 3.1 Table of study sites with each Lochs map co-ordinate, a description of the sampling site, method of fish capture and number of fish caught in a particular year.

Fish were not marked and to keep track of individual identity and to allow comparison of behavioural and morphological phenotypes, after behavioural observations (see Chapter 4), they were held in individual chambers. The chambers (120mm x 100mm, with mesh windows and a mesh bottom) were held in an outdoor flow-through flume (outer circumference 19.8m, inner circumference 15.8, width 60cm, depth 60cm) under natural light conditions and ambient loch water temperature (6.6 ± 1.1° Celsius). A 2cm² piece of black plastic was placed in each cell providing a hiding place. Chambers were cleaned daily to remove uneaten food and faeces.

Fish from Meikle Burn and Loch Quien were donated to this study by Dr C. Bean and Dr. A. Bell (Scottish Natural Heritage and University of Illinois, respectively) and have been included because of their interesting morphology. Fish from Meikle Burn were also utilised in the study of population differences at the genetic level, reported in chapter seven.

3.3.2 Literature, surveys and local information

Several peer-reviewed papers were available on the ecology and water chemistry of a number of lochs on the Outer Hebrides (Berry, 1979; Campbell, 1979; Giles, 1983; 1987). Information on large-scale ecological surveys is limited to a small number of lochs with high conservation status, including Mointeach Scadabhaigh SPA (an area mainly concerned with Loch Scadavay but also touching on Fada, a'Bharpa and an Daimh), North Uist Machair (Lochs Grogary and Hosta) and Loch Druidibeg. Most of this information was provided by Scottish Natural Heritage (SNH), but other useful reports were available on the internet from National Biodiversity Network Gateway (NBN Gateway), United Nations Education, Scientific and Cultural Organisation Man & the Biosphere (UNESCO-MAB) Biosphere Reserves Directory, Joint Nature Conservation Committee (JNCC) and the Royal Society for the Protection of Birds (RSPB). Local information from anglers provided additional unpublished information on the species of fish found at the study sites (G. MacDonald, North Uist Estates) and individuals from SNH made available supplementary information on conservation status and ecology of lochs (Dr. C. Bean and Dr. A. Stevenson).

3.3.3 On-site surveys of invertebrates and macrophytes cover

In April 2004, Lochs Fada, a'Bharpa and Grogary were re-visited (see below). On this occasion invertebrate and macrophyte samples were taken but water was not sampled. A representative selection of invertebrates caught while fishing for stickleback were preserved in 100% ethanol and identified down to either family or species (Croft, 1986; Olsen *et al.*, 2001). Macrophytes were first drawn and then a detailed description of the sampling site was made. Plants were kept in loch water and identified down to the family or species level immediately after returning to the university (Haslam *et al.*, 1975).

3.3.4 Water sampling

Water sampling was carried out at seven sites in February 2003; the River Kelvin and Lochs Druidibeg, Grogary, a'Bharpa, Scadavay, Fada and an Daimh. In January 2004 water was sampled again from the River Kelvin and Lochs Grogary, a'Bharpa and Fada. In addition a water sample was also taken at Loch Charrasan. The final sampling trip was undertaken in March 2005. All original sites sampled in 2003 were revisited plus two new sites, Lochs Hosta and na Ciste were also sampled. Water chemistry was not tested at Meikle Burn and Loch Quien and as a result no conclusions will be drawn from the expression of armour in these populations in relation to Ca^{2+} concentration.

All plastic bottles and glassware were soaked over-night in DECON 90 (phosphate free detergent), rinsed in pirite (phosphate free) water and dried in a drying cabinet. A 500ml sample was taken at each site and the pH measured with a Jenway Model 3150 pH meter. Samples were stored in a cool box. At the end of the fishing day the water was divided into two 250ml bottles. One 250ml sample was filtered using Whatman glass microfibre filters 12.5cm GF/A, and acidified with 10% nitric acid to pH2. This was used to measure Ca^{2+} concentration. The second 250ml sample was also acidified with 10% nitric acid but not filtered and used to measure total phosphorus. All samples were refrigerated until analyse was carried out.

3.3.5 Water chemistry analysis: Total phosphorous

Before analysis all glassware and equipment was soaked in DECON 90 overnight, rinsed with pirite water, soaked for half an hour in 30%H_2SO_4, rinsed again with pirite water and dried in a drying cabinet.

A 25ml sample of loch water was pipetted into a 100ml screw cap bottle and 0.5ml 30%H_2SO_4 plus 0.25g $K_2S_2O_8$ added. Samples were heated for 30mins at 98 to 137 KPa in an autoclave. After cooling the water samples were added to 50ml volumetric flasks and made up to 50ml with pirite water. This process was repeated for the blanks (deionised water filtered through microfibre filter paper).

Three working standards 0µgP/ml, 100µgP/ml and 250µgP/ml were made. Solution A = Phosphate P 1000µg P/ml and Solution B = 1ml of Sol. A made up to 100ml.

Standard 1. - 0 µg/L → 0ml Sol. B + 1ml 30% H_2SO_4 + 0.3g K_2SO_4 made up to 250ml with pirite water.

Standard 2. - 100 µg/L → 1ml Sol. B + 1ml 30% H_2SO_4 + 0.3g K_2SO_4 made up to 250ml with pirite water.

Standard 3. - 250 µg/L → 2.5ml Sol. B + 1ml 30% H_2SO_4 + 0.3g K_2SO_4 made up to 250ml with pirite water.

Total phosphorus was determined by ion chromatography using the Technicon method. This method is based on a phosphomolybdate complex formation, using antimony to speed up the development of the lightly coloured product. Ascorbic acid was then used to reduce the product to give a more intense blue colour that is measured at 880nm.

3.3.6 Water chemistry analysis: Ca^{2+} concentration

A crude measure of Ca^{2+} concentration was carried out to roughly determine the Ca^{2+} level in each of the water samples. This was done by adding 5ml of potassium chloride (KCl) to a 1000ml volumetric flask and making up to 1000ml with pirite water. 0.5ml of this stock solution was then added to a 100ml flask and made up to the 100ml mark with pirite water. All water samples were then measured against the standard, using a Perkin-Elmer 1100B AA Spectrophotometer and the appropriate dilutions made.

Between 2ml and 45ml water sample was added to a (X2) 50ml volumetric flask + 5ml stock solution (25gKCl in 250ml deionised water) + deionised water (if needed).

Two working standards 0ppm and 2.5ppm calcium were made.

0ppm Standard (X2) → 10ml KCl (stock solution) + 90ml deionised water.
5ppm Standard (X2) → 10ml KCl (stock solution) + 0.25ml Calcium + 89.75ml deionised water.

Ca^{2+} concentration was determined by atomic absorption spectrometry. In this method, a sample is aspirated into a flame and atomized (Greenberg *et al.*, 1992). Two samples from each loch and two samples of the working standards were used to measure calcium. The average of the two results was taken and the appropriate multiplications made to find the real amount of calcium in the loch samples.

3.3.7 Morphological screening

All fish caught over the three-year period were used for morphological analysis of population differences in armour expression. First and second dorsal spine and pelvic spine length was measured from the base of the front of the spine to the tip. Body depth was taken at the widest section of the fish, in front of the first dorsal spine and in front of the pelvic girdle. All measurements were taken with callipers to the nearest 0.1mm. Counts of lateral plate number were made under binocular microscope (X10). Number of lateral plates was used to explore another facet of armour protection. Having first checked that the most fish were symmetrical, (left versus right, Mann-Whitney U test $P = 0.846$), left plate number only was used.

3.3.8 Analysis of morphological data

To quantify armour expression and to get a measure of the difficulty a gape limited predator faces when trying to swallow a stickleback (designated a 'mouthful' measure), the longest dorsal spine (usually the second), body depth at the pectoral fin and left and right pelvic spine lengths were added together. In individuals lacking the second dorsal spine, the first dorsal spine length was used instead. Left lateral plate number was used as a measure of 'plate protection'. Between-populations comparisons of 'mouthful' and 'plate protection' were made using a non-parametric Kruskal-Wallis test. Spearmans Rank Order Correlation tests were used to look at the relationship between armour protection and Ca^{2+} concentration.

3.4 Results

3.4.1 General description of study sites

The River Kelvin

The River Kelvin rises in the Kilsyth Hills, North East of Glasgow and flows over a combination of igneous and sedimentary rocks for 33.5km (21 miles) though rural and urban sites before enter the River Clyde, 3km (2 miles) west of Glasgow city centre (Ordinance survey, 1979). For many years water quality in the Kelvin has been poor due to sewage pollution, agricultural/urban run-off and ferruginous mining-discharges. However, in the last ten years water quality has improved considerably, and salmon have returned to the river for the first time in 100 years (SEPA, 2006). A number of predators of sticklebacks and their eggs are found at this river. Avian predators include grey heron (*Ardea cinerea*), kingfisher (*Alcedo atthis*) and cormorants (*Phalacrocorax carbo*). Piscine predators include brown trout (*Salmo trutta*) and eel (*Anguilla anguilla*).

Loch Grogary, North Uist

Situated on the Western side of the island, Grogary is a long narrow shallow loch bisected by a road and approximately one kilometre in length. In contrast to many of the other study sites it is a biologically productive, alkaline, Ca^{2+} rich loch. The water body has a high conservation status and has been designated as a Site of Special Scientific Research (SSSI), a candidate Special Areas of Conservation (cSAC) and a special protection area (SPA). Lewisean gneiss underlies the loch (this type of bedrock constitutes all the islands of the Outer Hebrides) and the substrate is Atlantic shell rich sand soil (Machair). Heron, mergansers (e.g. *Mergus serrator*), gulls (e.g. *Larus laridae*) and terns (e.g. *Sterna paradisaea* and *Sterna hirundo*) are found at this loch as well as Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*), brown trout and eel.

Loch Hosta, North Uist

Also situated on the West of the island, the loch is similar in size and depth, ecology and water chemistry to Grogary. However in contrast to Grogary, the loch is connected to the sea via a small river. Plants and invertebrates were not sampled at

this site. Avian predators include heron, merganser, gulls and terns. Piscine predators are brown trout and rainbow trout (*Oncorhynchus mykiss*).

Loch Druidibeg, South Uist

Loch Druidibeg is a National Nature Reserve owned and managed by Scottish Natural Heritage. At its widest point the loch is around three kilometres. It has a high conservation status (SSSI, cSAC, SPA). On bedrock of Lewisean gneiss, the loch sits on peaty moor-land and is classified as an oligotrophic loch. Numerous avian predators are found at this site including heron, divers, gulls and terns. Brown trout, eel and nine-spined stickleback (*Pungitius pungitius*) are also present.

Loch an Daimh, North Uist

Situated on the south-eastern side of North Uist, an Daimh is a small oligotrophic loch with few macrophytes. However, samples were taken from a small stream running parallel to the main water body and connected to the loch via a tunnel. The loch has a conservation designation of cSAC and SPA. Many species of avian predators are present including, heron, grebes, gulls, terns, divers and merganser. Two piscine predators are present; brown trout and eel.

Loch Charrasan, Lewis

The loch is centrally located and is similar in character to the oligotrophic peaty lochs on North Uist. No published information concerning this site is available. The status of avian predators is unknown and arctic charr (*Salvelinus alpinus*) are the only recorded piscine predator.

Loch na Ciste, North Uist

Loch na Ciste is the most easterly of all the lochs on North Uist. It is connected to the sea and inundated daily with seawater. It is a small lochan, approximately 10m at the widest point with a conservation designation of SAC. Macrophytes and invertebrates were not sampled. Gulls and terns are present as are salmon, sea trout, brown trout and eel.

Meikle Burn, Stronsay, Orkney Isles

Meikle Burn is a small shallow stream, flowing out of a standing water body, Meikel Water. Water quality is poor and the burn is eutrophic. Little information is available for this site and fish used in this study were caught by Dr. Colin Bean. The status of avian predators is unknown but brown trout are present.

Loch Quien, Isle of Bute

Loch Quien is a small loch, around a kilometre in length, situated in the South West of the Isle of Bute. The loch sits on the Highland Boundary Fault, is underlain with metamorphic and sedimentary rock and surrounded by arable grazing land. Plants and invertebrates were not sampled at this site and water chemistry was not tested. Avian predators at this site include grey heron, red-breasted merganser and gulls. The loch is stocked with brown trout but other piscine predators are unknown.

Loch Scadavay, North Uist

Centrally located and constituting a significant part of Mointeach Scadabhaigh (large area of lochs and lochans on peatland with a high conservation status SSSI, cSAC, SPA), this loch is the largest on the island of North Uist and at its widest point is approximately four and a half kilometres. Situated on peaty moor-land, it is oligotrophic with 'tea-stained' water. Plants and invertebrates were not sampled at this loch. Several avian predators are present including heron, red-throated loon (*Gavia stellata*) and black-throated divers (*Gavia arctica*), gulls and terns. Three fish predators are also present, brown trout, arctic charr and eel.

Loch a'Bharpa, North Uist

Located in the centre of the island at its widest point the loch is approximately one and a half kilometres and has similar water chemistry to Scadavay. The loch has not been designated but its western and southern shores constitute part of the margin with Mointeach Scadabhaigh. Avian predators include heron, merganser, divers, grebes, gulls and terns. Brown trout, eel and charr are also present.

Loch Fada, North Uist

To the east of Scadavay and slightly smaller, approximately three kilometres in length, is Loch Fada. The loch is an oligotrophic, peat moor-land water body with 'tea-stained' water. The loch has not been designated but its northern and eastern shores are part of the boundary of Mointeach Scadabhaigh. Heron, grebes, gulls, terns, divers and merganser are all found here, as are, trout, eel and charr.

3.4.2 Invertebrate sampling

8 species of invertebrates were found at Lochs Grogary and a'Bharpa and 7 species at Loch Fada. In terms of abundance, Loch a'Bharpa had the highest numbers of invertebrates, Loch Grogary had the second highest and Loch Fada, the lowest.

Loch Grogary	Loch a'Bharpa	Loch Fada
Dragonfly nymphs Caddis fly larva Mayfly nymph Great Diving Beetle larva (*Dytiscus marginalis*) Scavenger beetle (*Hydrophilus aterrimus*) Stonefly nymph (*Taeniopteryx nebulosa*) Water boatman (*Cymatia sp.*) Common wandering pond snail (*Lymnaea peregra*).	Dragonfly nymphs Mayfly nymph Freshwater snails Freshwater shrimp Alder fly larva (*Sialis* sp.) Water boatman (*Cymatia sp.*) Great Diving Beetle larva (*Dytiscus marginalis*) Scavenger beetle (*Hydrophilus aterrimus*)	Dragonfly nymphs Water boatman Damsel fly larva Caddis fly larva Spider (unidentified) Scavenger beetle Alder fly larva (*Sialis* sp.)

Table 3.2 Invertebrates caught at three study sites and identified to species when possible.

3.4.3 Macrophyte sampling

Of the three lochs sampled Grogary was the most abundant in plant life. The rare Slender naiad (*Najad flexilis*) is found at this site (although not sampled in this survey) and as a result this loch is protected under domestic and international legislation (Wingfield 2004). Shetland pond week (*Potamogeton Rutilus*) is also found in the loch (G. MacDonald pers. comm.) but again not sampled in this survey. Loch a'Bharpa was also abundant in plant life with large clumps of macrophytes in

the middle and sides of the study stream. Again Loch Fada had the least abundant macrophytes in comparison to the above two sites.

Loch Grogary	Loch a'Bharpa	Loch Fada
Yellow iris (*Iris pseudacorus*) Yellow water lily (*Nuphar lutea*) Jointed rush (*Juncus articulatus*) Nuttalls pondweed (*Elodea nuttallii*)	Yellow iris (*Iris pseudacorus*) Water lobelia (*Lobelia dortmanna*) Yellow water lily (*Nuphar lutea*) Jointed rush (*Juncus articulatus*)	Bulbous rush (*Juncus bulbosus*) Water milfoil (*Myriophyllum* sp.) Pondweed (*Potamogeton* sp.)

Table 3.3 Macrophytes sampled at three study sites and identified to species when possible.

3.4.4 Ca^{2+} concentration, phosphorous and pH level

Levels of Ca^{2+} concentration clearly differed between study sites (see Table 3.4). Water bodies could be divided into two distinct categories 'high' Ca^{2+} sites where concentration was >25mgCa/L and 'low' Ca^{2+} sites where concentration was <5mgCa/L. Sites high in calcium were also alkaline, whereas low Ca^{2+} sites tended to be more acidic. Where lochs were tested on more than one occasion, both Ca^{2+} concentration and pH were broadly consistent across years (see Tables 3.4 and 3.5). Conversely, phosphorous level fluctuated across years (Table 3.6). Phosphorous is used as an indicator of nutrient enrichment, but is also used to determine the trophic status of a water body (SEPA, 2002). As Machair lochs (eutrophic, biologically rich and alkaline) Hosta and Grogary have higher concentrations of phosphorous. Conversely, peaty moor-land sites (all remaining lochs) are biological poor, oligotrophic and acidic. Most lochs conform to this classification with the exception of two sites sampled in 2005, Lochs Fada

Site	Calcium 2003	Calcium 2004	Calcium 2005
Kelvin	Not sampled	35.8	34.25
Grogary	44.8	34	50.5
Hosta	Not sampled	Not sampled	27.37
Druidibeg	4.3	Not sampled	4.7
an Daimh	2.69	Not sampled	2.3
Charrasan	Not sampled	1.2	Not sampled
na Ciste	Not sampled	Not sampled	2.71
Scadavay	1.89	Not sampled	2.69
a'Bharpa	1.71	1.58	2.12
Fada	2.29	1.56	2.29

Table 3.4 Calcium concentration (measured in mg/L) at ten study sites measured on one or more occasions from 2003 to 2005. NS signifies that the site was not sampled in that year.

Site	PH 2003	pH 2004	pH 2005
Kelvin	Not sampled	7.45	7.8
Grogary	7.5	7.63	7.05
Hosta	Not sampled	Not sampled	7.22
Druidibeg	6.1	Not sampled	6.09
an Daimh	6.68	Not sampled	6.3
Charrasan	Not sampled	5.93	Not sampled
na Ciste	Not sampled	Not sampled	5.4
Scadavay	5.9	Not sampled	5.53
a'Bharpa	6.4	6.48	5.64
Fada	6.26	6.13	6.01

Table 3.5 pH at ten study sites measured on one or more occasions from 2003 to 2005. NS signifies that the site was not sampled in that year.

Site	Phosphorous 2003	Phosphorous 2004	Phosphorous 2005
Kelvin	Not sampled	709.3	32
Grogary	90	20.98	38
Hosta	Not sampled	Not sampled	114
Druidibeg	12	Not sampled	18
an Daimh	18	Not sampled	16
Charrasan	Not sampled	6.88	Not sampled
na Ciste	Not sampled	Not sampled	110
Scadavay	20	Not sampled	8
a'Bharpa	18	6.54	26
Fada	1	4.92	68

Table 3.6 Phosphorous concentration (measured in ug/L) at ten study sites measured on one or more occasions from 2003 to 2005. NS signifies that the site was not sampled in that year.

and na Ciste. The latter has a fish farm in close proximity to the sample site and this may be the cause of an increase in the nutrient load. The highest concentration of phosphorous was recorded at the River Kelvin (709.3ug/L). Although this figure may appear high, the concentration of total phosphorous may vary over a year by as much as an order of magnitude (SEPA, 2002).

3.4.5 Variation in protective armour within and between populations

Tables 3.7 and 3.8, summarise the morphological status of sticklebacks from all study sites, combined over all years. Of the twelve populations sampled, seven comprised fish that had the normal full complement of three dorsal spines and two pelvic spines (see Table 3.7). Three of these populations were from high Ca^{2+} sites. Almost all fish at Loch Druidibeg had three dorsal spines, with the exception of one that had four spines. High frequencies of fish with two dorsal spines, the majority missing the second dorsal spine, were found at Lochs Scadavay and a'Bharpa. However, at Loch Fada, more than ninety percent of the fish had less than three spines and a third of fish had no spines at all. Individuals from Loch na Ciste also exhibited a high frequency of spine reduction, with most fish having two spines or fewer. Reduction in dorsal spine number was usually accompanied by a reduction or loss of the pelvic spines, except for a single fish from Druidibeg with no ventral spines but a full compliment of dorsal spines.

There was a significant difference between populations for 'mouthful' score (Kruskal-Wallis; $H = 644.61$, $DF = 11$, $P < 0.001$). Fish from the River Kelvin and Lochs Hosta, Quien, Grogary, Meikle Burn and an Daimh were generally better protected than fish from Lochs Druidibeg, Charrasan, na Ciste, a'Bharpa, Scadavay and Fada, although there was a considerable amount of within population variation (see Figure 3.2). Notably fish from Loch na Ciste where the 'mouthful' score ranged from a low of 2.7 (lowest score = 1.4 at Loch Scadavay) and to a high of 22.3 (highest score = 24.4 at River Kelvin).

		No. Dorsal spines (%)				Pelvic spines (%)
	N	(3)	(2)	(1)	(0)	(2)
Kelvin	47	100	0	0	0	100
Grogary	127	100	0	0	0	100
Hosta	17	100	0	0	0	100
Druidibeg	*59	98.3	0	0	0	98.3
an Daimh	51	100	0	0	0	100
Charrasan	50	92	6	2	0	20
na Ciste	25	20	40	36	4	24
Meikle	30	100	0	0	0	100
Quien	50	100	0	0	0	100
Scadavay	66	1.5	92.4	6.1	0	4.5
Bharpa	151	35.7	64.3	0	0	0
Fada	114	6	31.5	32.5	30	0

Table 3.7 Percentage frequency of individual fish (N = 787) with dorsal and pelvic spines at all study sites sampled in all years. High Ca^{2+} concentration sites are shade. One fish at Loch Druidibeg (*) had four dorsal spines.

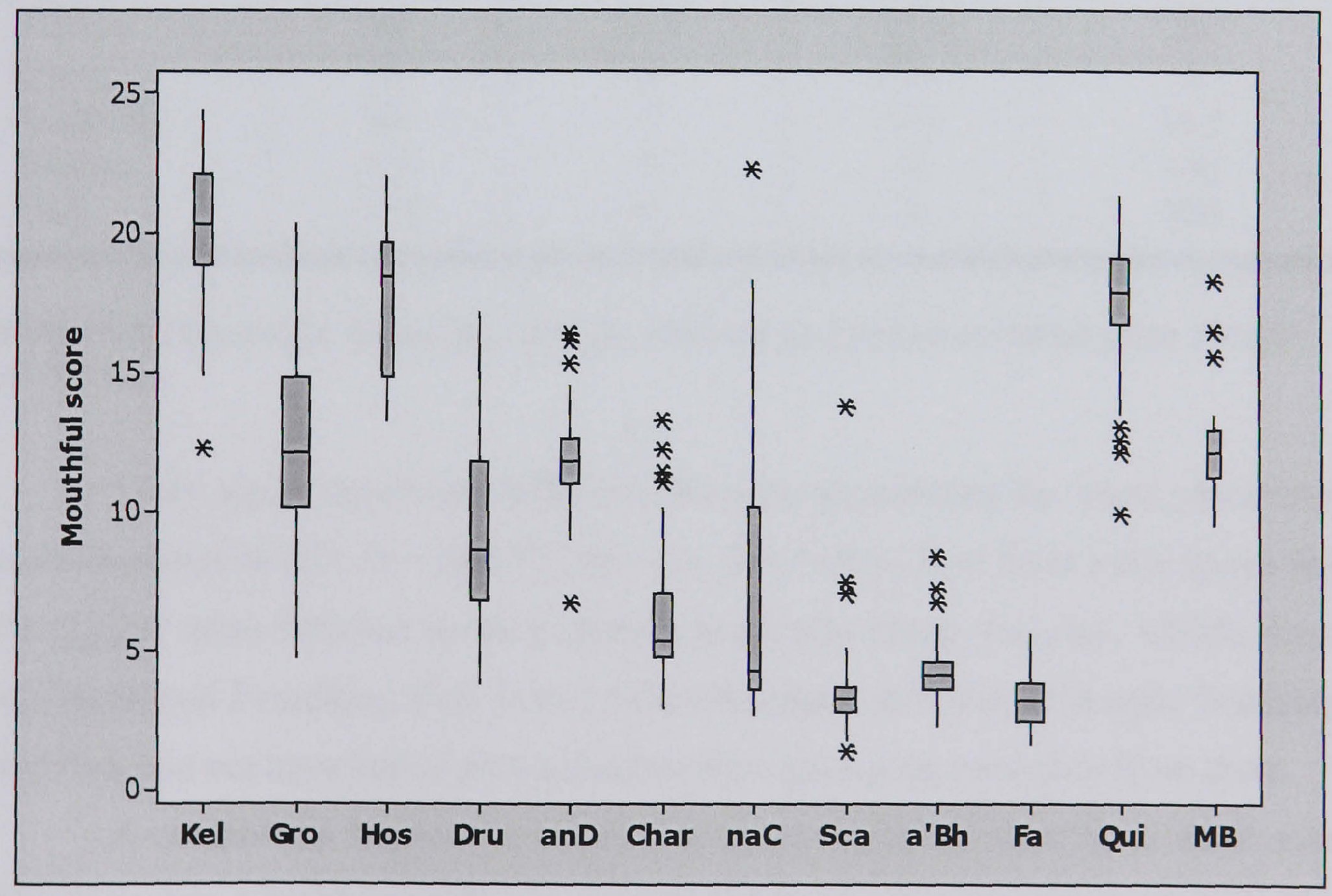

Figure 3.2 Boxplot of 'mouthful' score for the 12 study populations, with box width proportional to the population size.

Lateral plate number was also highly variable (see Table 3.8). Four populations had fish with more than eight plates and some of these fish also had a keel (bony plates along the caudal peduncle normally associated with heavily armoured marine stickleback). The highest plate number (24) was recorded at Meikle Burn, with high plate numbers also being found at Lochs na Ciste and Quien and the River Kelvin (lateral plate number: 23, 22 and 15 respectively). Most fish had between one and eight plates, with seven populations composed of more than fifty percent of this form. However, in five populations the most common form was plateless, constituting more than seventy-five percent of all fish. In three populations, Charrasan, a'Bharpa and Fada only the plateless morph was present.

	N	> 8 Plates (%)	1-8 Plates (%)	0 Plates (%)
Kelvin	47	8.5	91.5	0
Grogary	127	0	98.4	1.6
Hosta	17	0	100	0
Druidibeg	59	0	79.6	20.4
an Daimh	51	0	100	0
Charrasan	50	0	0	100
na Ciste	25	4	20	76
Meikle	30	16.6	53.4	30
Quien	50	30	70	0
Scadavay	66	0	1.5	98.5
Bharpa	151	0	0	100
Fada	114	0	0	100

Table 3.8 Percentage frequency of high, medium and plateless lateral plate morphs in 787 fish.

There was a significant difference between populations for 'plate protection' score (Kruskal-Wallis; $H = 680.77$, $DF = 11$, $P = 0.000$). Fish from Loch Quien had the highest score followed by River Kelvin and Lochs Hosta, Grogary, Meikle Burn, an Daimh and Druidibeg. Fish from Lochs Charrasan, na Ciste, a'Bharpa, Scadavay and Fada did not have lateral plates and therefore gained no protection from them.

A comparison between 'mouthful' score and 'plate protection' score showed that there was a highly significant correlation between both measures of protection (Spearmans Rank Order Correlation; $N = 787$, $R_s = 0.863$, $P < 0.001$).

3.4.6 Variable armour morphology between populations in relation to Ca^{2+} concentration

There was a significant positive correlation between armour protection (excluding fish from Lochs Quien and Meikle Burn) and Ca^{2+} concentration (Spearmans Rank Order Correlation for 'mouthful' score; N = 707, R_s = 0.629, P < 0.001: Spearmans Rank Order Correlation for 'plate protection' score; N = 707, R_s = 0.822, P < 0.001). As armour protection increased, Ca^{2+} concentration also increased.

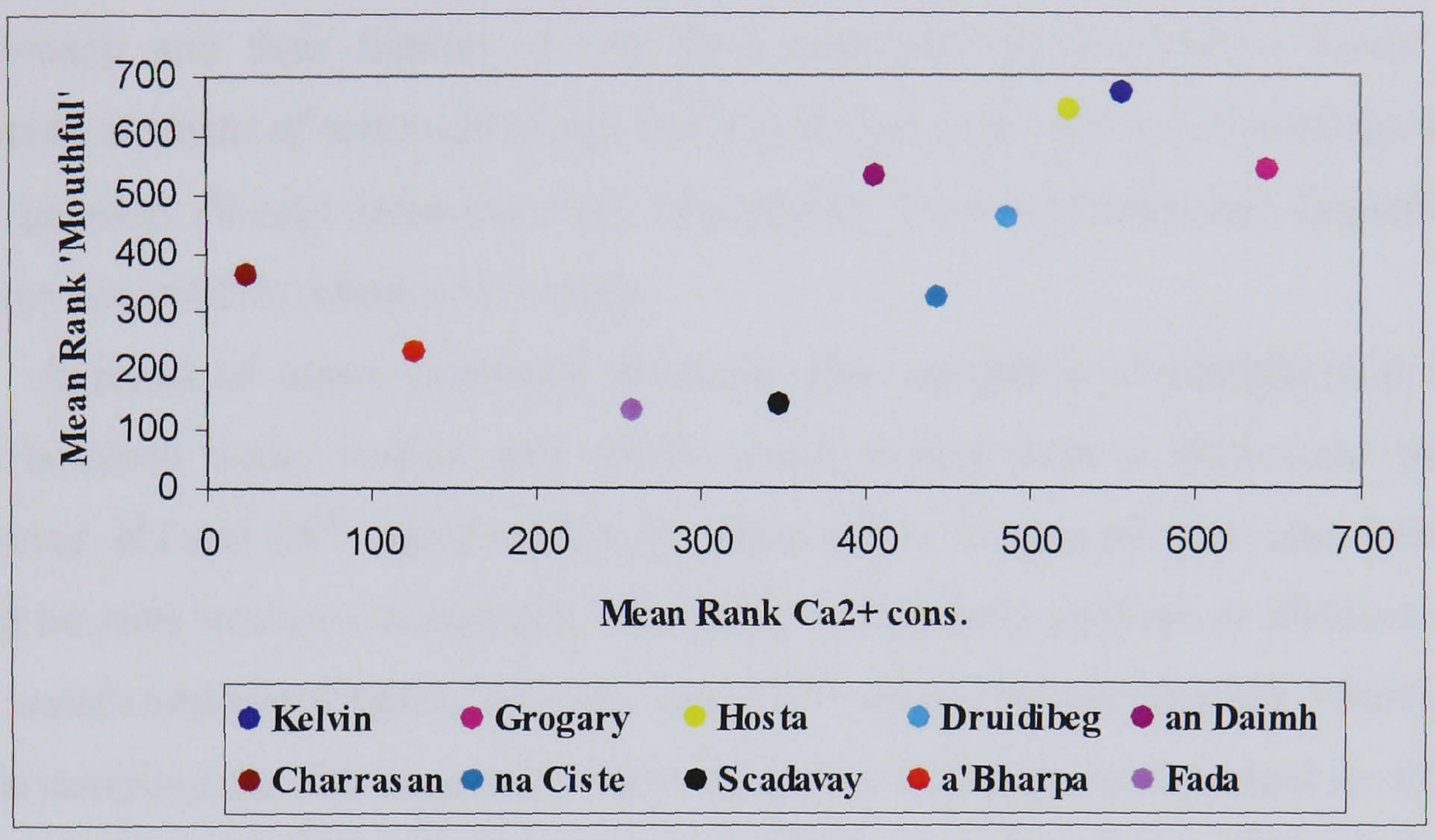

Figure 3.3 Scatterplot of mean rank 'mouthful' for each population plotted against mean rank Ca^{2+} concentration.

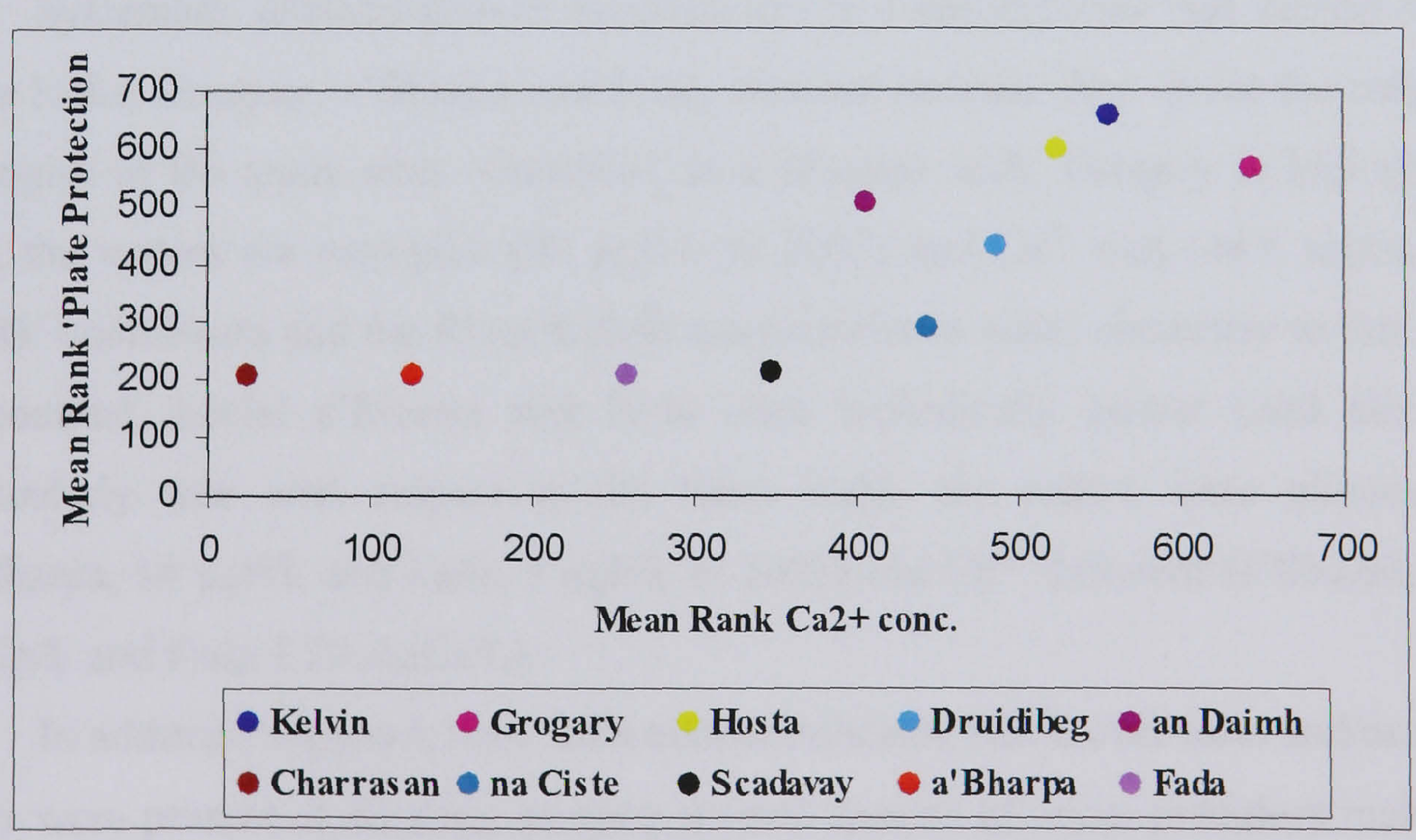

Figure 3.4 Scatterplot of mean rank 'plate protection' for each population plotted against mean rank Ca^{2+} concentration.

3.5 Discussion

The primary aim of this study was to explore further the suggestion that loss of protective armour among Scottish populations of three-spined sticklebacks is an adaptation to reduced Ca^{2+} concentration, rather than to local predation regimes, by looking at sites from a wider geographic range than that used by Giles (1983). However, in order to fully understand the relationship between three-spined stickleback and their habitat, it was first necessary to characterise study sites primarily in terms of water chemistry but also in terms of ecology. In addition to the data gathered directly from the sites, information from published and unpublished sources was used to enhance this study.

Analysis of water chemistry illustrates the variability of phosphorous level, both between water bodies and within water bodies over a three-year period. However, pH and Ca^{2+} concentration remained stable. In general then, sites could be could be split into two categories; eutrophic, biologically productive alkaline, Ca^{2+} rich waters and oligotrophic, low pH, low Ca^{2+} waters. Non-systematic observation while sampling for fish suggested that similar invertebrates were present at all sites although generally less numerous at the peaty moor-land sites. Some of these species, for example, diving beetle larvae and dragonfly larvae prey on stickleback.

Systematic identification of invertebrates and macrophytes was carried out at three lochs, Grogary, a'Bharpa and Fada, selected because they cover the range of ecologies at the study sites. Classified as a Machair loch, Grogary is biologically rich, the waters are eutrophic (90 µgP/L in 2003) and Ca^{2+} rich (44.8 mgCa/L in 2003). Loch Hosta and the River Kelvin were similar in water chemistry to this loch. In contrast, Lochs a'Bharpa and Fada were biologically poorer (and this was particularly true with respect to the latter loch), the waters were oligotrophic (a'Bharpa, 18 µgP/L and Fada, 1 µgP/L in 2003) and Ca^{2+} deficient (a'Bharpa, 1.71 mgCa/L and Fada 2.29 mgCa/L).

In addition, supplementary information indicated that brown trout and/or arctic charr were present at all sites, as were several species of avian predators including heron, gulls, grebes, divers and terns. Although precise predator number is unknown, predation may be regarded as high as at least two of the three major stickleback predator types are present at all study sites. However, as macrophytes are present at

almost all sites, sticklebacks can reduce the chance of predation by hiding in the plants and in the case of the peaty sites may gain some protection from the 'tea-stained' water from bird predation.

At each of the study sites three-spined stickleback are faced with invertebrate, fish and bird predators and at some sites, with very low levels of calcium. Armour loss has been attributed to predation regime and calcium availability (Reimchen 1980; Giles, 1983). The low Ca^{2+} sites sit on peaty moor-land, are nutrient poor and acidic. The peat substrate has a high affinity for cations, which are preferentially adsorbed, displacing hydrogen ions and increasing the acidity of the water (Sjörs & Gunnarsson, 2002). In contrast, the high Ca^{2+} sites are nutrient rich and alkaline. Lochs Grogary and Hosta are in close proximity to the sea and oceanic spray coupled with the shell-rich sand substrate of the lochs, account for the high Ca^{2+} concentration. In the River Kelvin a combination of geology, metal concentration of the soil, water pH and organic matter determine the calcium. Ca^{2+} concentration is reflective of the variation in armour expression observed across the study sites, in that loss of armour only occurs at low calcium sites. The data collected provide partial support for the calcium availability theory, in that fish with reduced armour were only found at sites with low Ca^{2+} concentration. The support is partial, in the sense that not all sticklebacks from low-calcium sites showed reduced numbers of protective spines and lateral plates.

It is possible that at sites were armour expression and Ca^{2+} concentration are low (for example Lochs Fada and a'Bharpa) there may be a cost to expressing the normal compliment of body armour. As freshwater fish obtain most of calcium requirements by excreting and absorbing Ca^{2+} into or from the surrounding water (Simkiss, 1974), a reduction in non-essential bony body armour may reduce this cost. Secondly, even in fish with spines these may be of such poor quality spines as to be of no use against attack (a weakness of this study was that no account was taken of spine strength) and so it may be better to have no spines at all. The trade-off for fish at low Ca^{2+} sites may be to reduce or lose non-essential anti-predator armour (Giles, 1983) in favour of improved hydrodynamics and increased speed of the startle-response (Bergstrom, 2002). Individuals may also utilise the tea-stained waters (characteristic of peaty moor-land lochs) as a form of protect against predators. This may be particularly true with respect to avian predation where sight is important in prey detection.

In conclusion, I found variability in body armour within and between study sites and a significant positive correlation between armour development and calcium concentration.

Chapter 4. Reduction of body armour: implications for risk-taking

4.1 Introduction

Sticklebacks vary in strength of an array of behavioural adaptations that serve to reduce the chances of capture by predators. This variability too may be related to predator regime. For example, populations living sympatrically with piscine predators show better escape responses than those from habitats without predators (Giles & Huntingford, 1984). Within-population differences are also apparent, with for example, some fish being willing to take greater risks in a variety of circumstances, including direct encounters with a predator and rate of exploration of a novel environment (Bell, 2005). Such differences in risk-taking are likely to have implications for individual survival. Risk-takers are for example, more willing to forage in new and potentially dangerous environments (Wilson *et al.*, 1993) and to indulge in the risky business of predator inspection (Murphy & Pitcher, 1997) than risk-averse individuals.

However, such differences may be counter-balanced by other anti-predator defences that reduce the success rate of the predator. One such counter-balancing defence might be the development of body armour (Gross, 1978 and see Chapter 3), since the defensive spines of sticklebacks increase the time required by piscivorous predators to handle and manipulate capture prey, which in turn increase the chances of escape (Reimchen, 2000). There is also a trade-off between armour and escape speed. For example, in brook stickleback (*Culaea inconstans*) fish lacking a pelvic girdle and pelvic spines had a faster escape response and bent deeper in the early phase of the response than fish with a fully developed pelvic complex (Andraso, 1997). Thus the notion then that un-armoured fish are more vulnerable than armoured fish may be an over simplification and the study reported in this chapter was designed to relate variability in risk-taking to variability in degree of development of body armour, at the level of both populations and individuals.

4.2 Aims

With this background, the specific aim of the study described here was to investigate variability in risk-taking behaviour and whether this is related to armour expression, within and between populations.

4.3 Materials and methods

Seven populations, River Kelvin and Lochs Grogary, Druidibeg, an Daimh, Scadavay, a'Bharpa and Fada, were used to explore variable risk-taking and the relationship between risk-taking and armour development. Details of study sites and sampling regimes are given in Table 3.1, Chapter 3.

4.3.1 Behavioural screening

Risk-taking was assessed using a standard test of exploration/activity in a novel environment (Verbeek *et al.*, 1994). This method of quantifying risk-taking was chosen because it is a well-established technique that is broadly predictive of some aspects of the sticklebacks' response to a predator (Huntingford, 1976; Bell & Stamps, 2004). It also has the advantage of being easier to standardise than using either a live predator behind glass or a model.

To ensure that individual risk-taking was measured accurately a number of potentially confounding variables were identified and, where possible, controlled for. Tests were all carried out within a limited temperature range (7 ± 0.5). Fish were deprived of food 20 hours prior to testing to minimise differences in hunger level, which are known to influence risk-taking (Lima & Dill, 1990). To standardise the effect of social interaction, in 2003 fish were held in small groups (two to four fish) and were transferred from their home tanks to smaller holding tanks in the observation room on the day screening was carried out. In 2004, all fish were individually housed three to twelve days prior to screening thereby removing all possible effects of social interactions. There was no significant difference in the level of risk-taking between years, suggesting that fish held in low densities in large tanks prior to testing behave in a similar fashion to fish held in isolation. To standardise stress levels, fish were allowed to settle for a minimum of one hour in the aerated

holding tanks and under low light conditions before testing. One fish from each population was tested alternately, in one of two observation tanks, to allow for the possible effect of the tank and the time of day.

In 2003 a total of 142 fish were screened from seven populations over a period of two months and in two blocks, March 14th to 27th and April 7th to 17th. In 2004 a further 158 fish from four populations (Kelvin, Grogary, Fada and a'Bharpa) were tested in one block between February 23rd and March 3rd. For a full explanation of behavioural observation procedures see chapter 2. After testing, fish were returned to their individual holding chamber for subsequent morphological screening (see Chapter 3).

4.3.2 Development of behavioural techniques

After the first screening of risk-taking behaviour on wild fish caught in 2003, a further total of 1770 wild caught and lab-reared fish were screened over a 3 year period. Of these study fish, 809 were tested two or more times, a minimum of one week after the first screening ended. Preliminary analysis of 2004 wild caught fish data, showed that there were many more risk-averse fish in test one than in test two, skewing the distribution of risk-taking scores (see Figure 4.1a).

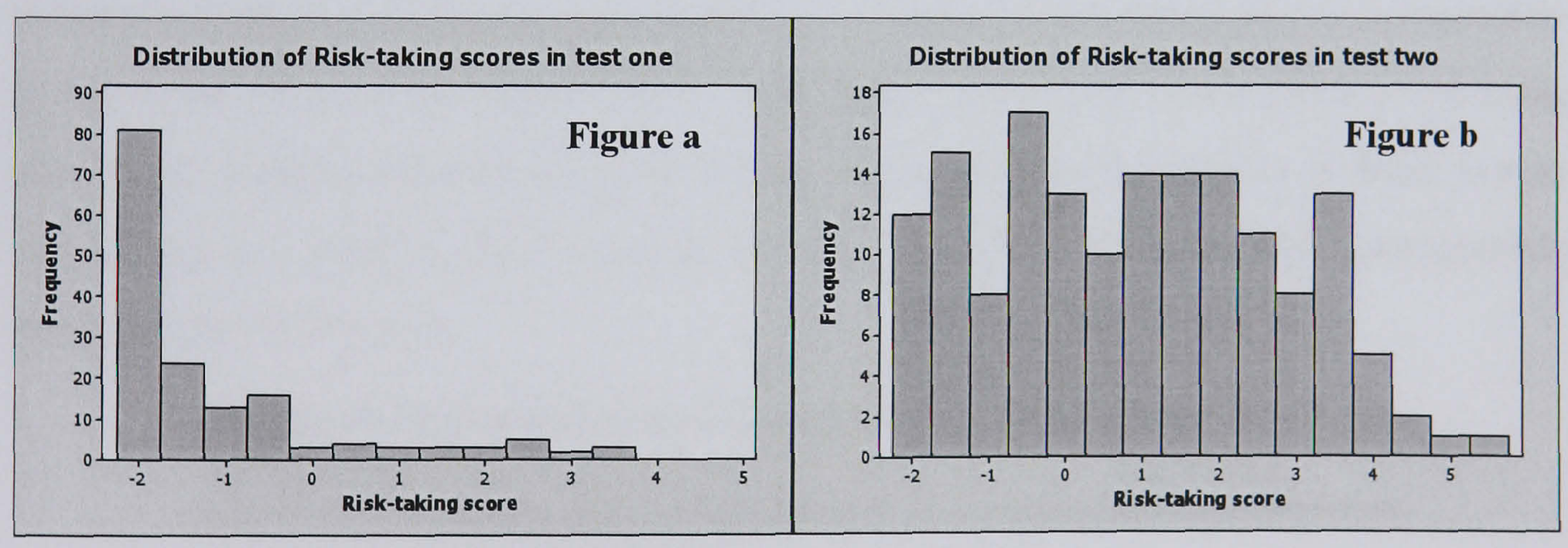

Figure 4.1a and b Distribution of risk-taking in wild caught 2004 fish tested twice. Higher numbers of risk-averse fish are present in test one (Figure a) than in test two (Figure b).

Further investigation revealed that fish in test one were screened at a lower temperature than were those in test two. However, wild caught fish tested once in 2003 were tested at a mean temperature of 7°C (+/- 0.5) and had a wide distribution of behavioural phenotypes, similar to that see in Figure 4.1b. Temperature then may have been an important variable effecting behaviour (for a full discussion, see

Chapter 2). For this reason, only data from test two were used for comparisons within and between populations, even though fish were no longer completely naïve with regards to the novel environment test and were on average bolder in the second test.

4.4 Data analysis

4.4.1 Analysis of behavioural data

All data were check for normality with an Anderson Darling test. Data not normally distributed were analysed with non-parametric tests. Principle Components Analysis (PCA) was used to condense the behavioural data set from all fish tested in 2003 and 2004, summarising the measured variables into a single factor score for each fish (Huntingford, 1976). Variables used were latency to cross lines AB, BC and/or CD (Figure 2.1, Chapter 2), activity and tank use. The analysis was carried out on unranked data, having first established that scores derived from ranked and unranked data were equivalent (r_2 = 98.6%, $P < 0.001$). Table 4.1 shows the first principle component (PC1), which accounted for 74% of the total variance and opposed latency to cross the 3 lines (negative loadings) against activity and tank use (positive loadings). This was interpreted as an index of risk-taking and is comparable to the index of boldness identified by Huntingford (1976) and Bell (2005). Utilizing the scores derived from the PCA, fish were categorised as Risk-taker or Risk-averse depending on whether they were in the top or bottom (respectively) interquartile range of the PC1 scores.

Behaviour	**Loading**
All observations and populations 2003 & 2004	
Latency AB	-0.430
Latency BC	-0.479
Latency CD	-0.469
Activity	0.389
Tank use	0.463
Variation explained (%)	**74.0**

Table 4.1 Principle components analysis (PCA) opposed latency to explore against movement, giving an index of risk-taking. Variables used were latency to explore (Latency, AB,BC,CD) and movement (Activity and Tank use). The Eigenvalue explained 74% of the variation in the test.

Table 4.2 summarises the results of analyses aimed at identifying and where necessary allowing for any effects on risk-taking of the controlled and uncontrolled variables recorded during behavioural screening. Spearmans rank order correlation (SRC) was used to identify variables effecting behaviour at the level of the individual. There was a significant association between the time of day that an individual was tested and behaviour of the fish ($R_s = 0.2$, $P = 0.012$), fish becoming somewhat bolder as the day progressed, possibly because they were hungrier in the afternoon. To correct for this effect, scores were expressed as residuals from the linear regression of risk-taking against time of day (Boylan, 2005).

Variable	**Effect of variable on behaviour between individuals**			
	2003		2004	
	R_s	P	R_s	P
Temperature	-0.060	0.476	-0.13	0.098
Date	-0.098	0.248	0.06	0.414
SL	0.029	0.741	0.02	0.893
Time tested	0.211	**0.012**	0.07	0.384
Test position	-0.050	0.557	0.02	0.799

Table 4.2 Table of statistical analysis of controlled and uncontrolled variables potentially effecting risk-taking at the level of the individual. In column one are the variables that may have an effect on behaviour. The test statistics (Spearmans Rank Order Correlation) for variables effecting individual risk-taking in fish tested in 2003, are found in columns two and three. In columns four and five, are the test statistics for variables effecting individual risk-taking in fish tested in 2004.

A Chi-square test of association was used to explore the distribution of males to females within populations. The test revealed a marginally non-significant difference between populations caught in 2003 ($Chi^2 = 10.66$, $DF = 6$, $P = 0.099$). Samples from Kelvin, Scadavay and a'Bharpa were slightly female-biased, while the sample from Loch Fada was slightly male-biased. However, although there was an uneven distribution of males and females further analysis using a Mann-Whitney U test of risk-taking in males compared to risk-taking in females showed that there was no significant difference in behaviour between the sexes ($W = 9682.5$, $P = 0.9417$). Chi-square analysis also showed that there was no effect of tank number on risk-

taking between populations in either 2003 ($Chi^2 = 1.330$, DF = 6, P = 0.970) or 2004 ($Chi^2 = 0.099$, DF = 3, P = 0.992).

Kruskal-Wallis (K-W) test of temperature, date, SL, time tested and test position identified a number of variables that differed between populations (see Table 4.3). Dates on which fish were tested and the point in time a fish was observed in relation to the beginning of the test (i.e. test position 1 equates to the first fish tested on day one of screening, test position 2 equates to the second fish tested on one, as so on) differed significantly between populations tested in 2003 (K-W test of populations and date, $P < 0.001$; K-W test of populations and test position, $P < 0.001$). Fish were tested in two blocks. All Kelvin fish and most Druidibeg fish were tested in the second block and as a consequence, had a higher test position number. SL also differed significantly between populations in 2003 and in 2004 (K-W test of SL and population; $P < 0.001$ for both years). Fish from the River Kelvin were longer (see Table 4.4) than fish from any other site (all Fada fish and many Grogary and a'Bharpa fish were missing from the 2004 analysis. In an unfortunate incident, a juvenile mink ate many of the fish observed in 2004 and so few morphological (or gender) data for these fish were recorded).

Variable	**Effect of variable on behaviour between populations**			
	2003		2004	
	H	P	H	P
Temperature	4.93	0.553	3.32	0.509
Date	29.49	**<0.001**	6.63	0.085
SL	78.28	**<0.001**	21.06	**<0.001**
Time tested	3.85	0.697	2.75	0.433
Test position	29.09	**<0.001**	5.28	0.152

Table 4.3 Table of statistical analysis of controlled and uncontrolled variables potentially effecting risk-taking between populations. In column one are the variables that may have an effect on behaviour. The test statistics (Spearmans Rank Order Correlation) for variables effecting individual risk-taking in fish tested in 2003, are found in columns two and three. In columns four and five, are the test statistics for variables effecting individual risk-taking in fish tested in 2004.

However, although significant differences of various possibly confounding variables were found between populations, in fact none had a significant effect on

behaviour (K-W test of risk-taking score and population in 2003; H = 12.60, DF = 6, P = 0.050: K-W test of risk-taking score and population in 2004; H = 6.63, DF = 3, P = 0.095).

Site	N	Mean Standard Length	SE Mean
Kelvin	19	47.93	0.887
Grogary	19	39.16	0.857
Druidibeg	18	32.76	1.12
an Daimh	16	35.26	1.15
Scadavay	20	31.26	1.16
a'Bharpa	20	34.56	0.846
Fada	20	30.68	0.762

Table 4.4 Mean standard length with standard error of fish used in observation screening carried out in 2003. The longest fish are found in the River Kelvin and the shortest in Loch Fada.

The four populations tested in 2003 and in 2004 were re-analysed with a PCA to investigate effects of year of testing on behaviour (N = 238). A comparison of risk-taking scores between all fish tested in 2003 against all fish tested in 2004 showed that there was no significant difference in behaviour between years (Mann-Whitney U test of rank risk-taking score fish tested 2003 and rank risk-taking score fish tested 2004; W = 9676.5, P = 0.8171) indicating that behaviour was stable between years.

4.5 Results

4.5.1 Variable risk-taking behaviour between populations

The distribution of risk-taking was markedly variable within populations tested in 2003 (see Figure 4.2). Individuals from Lochs an Daimh, a'Bharpa and Scadavay tended to have relatively low scores (risk-averse fish in populations; an Daimh = 15/17, a'Bharpa = 11/20, Scadavay = 14/22). In contrast, fish from Lochs Druidibeg and Grogary tended to have relatively high scores (risk-takers; Druidibeg = 14/23, Grogary = 13/20). However, no highly significant differences in risk-taking were detected between populations tested in either 2003 or in 2004 (K-W test of risk-

taking and population; P = 0.050 and P = 0.095 respectively). In 2003, the marginally non-significant difference was due to Loch an Daimh, where fish tended to have the lowest risk-taking scores and in 2004 where fish from Loch Grogary tended to have higher risk-taking scores.

Figure 4.2 Population distributions of risk-taking scores for each of the 7 study populations (1=River Kelvin, 2=Loch Grogary, 3=Loch Druidibeg, 4=Loch an Daimh, 5=Loch Scadavay, 6=Loch a'Bharpa, 7=Loch Fada). Risk-averse fish have negative scores and risk-taking fish have positive scores.

4.5.2 Behaviour in relation to body armour within and between populations

Comparing risk-taking with armour development at the level of population averages (Figure 4.3a and b), although fish from Loch an Daimh were slightly more risk-averse (with marginal significance, see above) than those from all other populations, this population was in the mid-range for both measures of protection (mouthful and plate protection). At the individual level (all populations combined), there was no correlation between risk-taking score and protection (Spearmans Rank Order Correlation behaviour and mouthful, N = 131 R_s = 0.091 P = 0.303; Spearmans Rank Order Correlation behaviour and plate protection, N = 131 R_s = 0.112 P = 0.201). Within population comparisons showed a significant correlation between mouthful and risk taking only among fish from Loch Grogary (Spearmans Rank Order Correlation behaviour and mouthful, N = 19 R_s =-0.574 P = 0.01), fish with high risk-taking scores being less well protected than those with lower scores. For fish from the River Kelvin only, there was a weakly positive association between plate number and risk-taking (Spearmans Rank Order Correlation behaviour and plate protection, N = 19 R_s =0.392 P = 0.097), risk-takers tending to have more plates than risk-averse individuals.

4.6 Discussion

A main aim of this chapter was to characterise individual variability in risk-taking in three-spined sticklebacks and its relationship to variable body armour, both within and between populations. Within most of the study populations there was a spread of behavioural phenotypes ranging from very risk-prone to very risk-averse. This axis of behaviour (also termed the bold-shy continuum) has been observed in other populations of stickleback (Bell & Stamps, 2004) and other species of animals (Gosling, 2001; Dingemanse *et al.*, 2003; Sih *et al.*, 2004).

Candidate explanations for the evolution and maintenance of such behavioural variation are variable selection regimes such as food availability and the outcome of the competition for food, parasites (Wilson *et al.*, 1993; Sih *et al.*, 2004) and risk of predation (Huntingford, 1982). In addition the risk-taking phenotypes

Figure a.

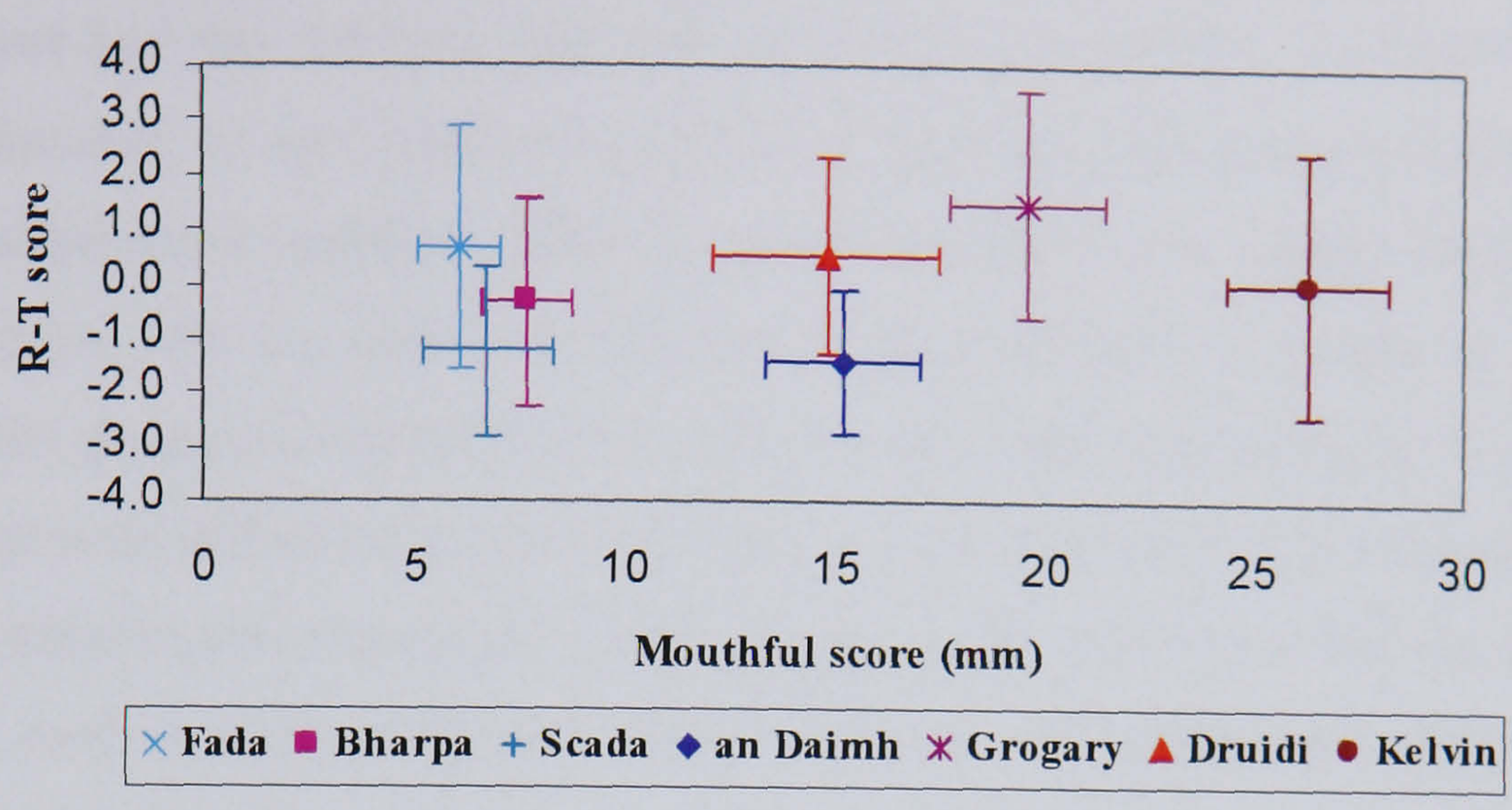

Figure b.

Figure 4.3 a. Population medians, with standard deviation, of mouthful measure (1st or 2nd DS length + depth + length of two PS) plotted against median risk-taking scores. **b.** Population medians, with standard deviation, of left lateral plate number plotted against median risk-taking score.

may be under frequency- and/or density-dependent selection. For example, to explore the possibility of density dependent selection, Wilson *et al.* (1994) used the scenario of placing individual fish sequentially into a new environment. This environment has two habitats, safe and dangerous and initially fish enter the safe habitat. However, as the number of individuals increases, the common food resource in the safe habitat is depleted. Although predation risk is low, energy return is also low. As more fish are added, it becomes more beneficial to forage in the less-crowded but dangerous habitat, than in the over-crowed but safe habitat. Such fitness payoffs between different foraging strategies can result in the stable coexistence of differing behavioural phenotypes. Interestingly at both the River Kelvin and Loch Fada (the most and least armoured fish), there is a wide distribution of behavioural phenotypes but few intermediates. In these populations there may be a cost to being intermediate. For example, intermediates may be less efficient foragers or have less well developed anti-predator behaviours and so are preferentially preyed upon. Further ecological studies are required to elucidate this finding. However, the apparently bimodal distribution at these sites may be an artefact of the cut-off point for risk-averse fish, in that there is a maximum score for individuals who remain stationary during the whole observation period but no maximum score for risk-takers.

Levels of risk-taking did not differ between the study populations. A possible explanation may be that, as all populations are under similar predation pressures, all have similar distributions of behavioural phenotypes. Alternatively, populations might be different in certain aspects of behaviour but our chosen test, exploration of a novel environment (used to identify risk-prone and risk-averse fish for a larger study), did not pick these up. Giles & Huntingford (1984) utilised a model heron and a live pike to elucidate population differences in anti-predator behaviour and perhaps this approach coupled with exploration in a novel environment may ultimately have been more informative.

There was no trace of a correlation at the population level between risk-taking and the degree of morphological protection from body armour. At the individual level, in two populations only was there a weak relationship between risk-taking and protection, as measured by both the mouthful score and plate protection score. Long spines and lateral armour plates provide structural defences against attack and reduce the probability of being eaten. Previous studies suggest that

different degrees of development of such armour may be associated with differences in behaviour. For example, well-armoured three-spined sticklebacks take greater risks in a novel and potentially dangerous environment than do the relatively poorly armoured nine-spined sticklebacks (Benzie, 1965). One might expect then, a positive correlation between boldness and armour expression in our study populations. However, I found no trace of such a relationship. Indeed at one site (Loch Grogary), a negative correlation between risk-taking and mouthful was found. Although this effect was weak and may possible be a statistical artefact.

I also found a range of behavioural phenotypes within populations, but no significant difference in behaviour between populations. No correlation between risk-taking and body armour was found. Extensive ecological surveys are now necessary to fully understand the complicated relationships between site, risk-taking and anti-predator armour.

Chapter 5. A study of the inheritance of risk-taking behaviour within and between populations of three-spined stickleback

5.1 Introduction

Individuals of numerous species differ consistently in response to new and potentially dangerous situations (Wilson *et al.*, 1994; Koolhaas *et al.*, 1999; Gosling, 2001). Such differences often take the form of a continuum from extreme shyness to extreme boldness (Wilson *et al.*, 1994). Boldness in this context can be defined simply as a propensity to take risks and shyness the propensity to avoid risk. In several cases differences in risk-taking have been shown to be repeatable, in the sense that the relative boldness or shyness are consistent when the same individuals are tested in the same context on different occasions (Verbeek *et al.*, 1994). Differences in boldness or risk-taking are often reflected in a range of functionally-distinct contexts; in other words, they are organised into behavioural syndromes (Sih *et al.*, 2004). Thus, compared to risk-avoiders, risk-takers may be more active in a novel environment (Bell & Stamp, 2004), more likely to take novel food items (Wilson *et al.*, 1993), more likely to approach and inspect a predator (Huntingford, 1976) and more ready to instigate fights with conspecifics, even if a predator is present (Brick & Jakobsson, 2002). Differences in risk-taking may also be associated with differences in behavioural plasticity (timid individuals are more likely than bold ones to develop flexible routines) and in physiological responses to a variety of stressors; such suites of behavioural and physiological responses to challenge are often referred to as different coping strategies (Koolhaas *et al.*, 1999; Korte *et al.*, 2005).

There is a growing literature on the adaptive consequences of variable risk-taking and on the evolutionary processes that maintain such variation over time. For example, being more willing to forage in novel and potentially unsafe environments may well expose the animal to possible predation. On the other hand, it potentially allows individuals to exploit new, uncontested food resource that risk-averse

individuals may avoid (Wilson *et al.*, 1993). Just how well the different phenotypes fare depends on environmental conditions, including the distribution of resources (Dingemanse, 2004) and predators (Brick & Jakobsson, 2002). Whether such differences in performance translate into changes in frequency of the phenotypes over time depends on whether the behaviour situation is inherited (Drent *et al.*, 2003). This had been shown to be the case for a number of well-documented examples of variable risk-taking/coping strategies, including mice and rats (Koolhaas *et al.*, 1999) and great tits (Dingemanse *et al.*, 2002; Drent *et al.*, 2002).

The three-spined stickleback has become something of a model species for this kind of study. The species exhibits great variation in risk-taking both within (Huntingford, 1976; Bell, 2005) and between populations (Giles and Huntingford 1984). Bell (2005) showed that variable risk-taking (at least as reflected in tendency to approach and inspect a predator) has a significant heritable component. And aggression, with which it is correlated, is also an inherited trait (Bakker, 1988). In addition, a genome-wide linkage map for sticklebacks has recently been developed (Peichel *et al.*, 2001). This has been used effectively for quantitative trait loci (QTL) analysis for the genetic mechanisms underlying the evolution of variable morphology in this species (Colosimo *et al.*, 2004; Sharpiro *et al.*, 2004). Given the apparent heritability of risk-taking in sticklebacks, it was deemed worthwhile to attempt a similar QTL analysis for this behavioural trait.

5.2 Aims

With this background, the main aim of this study was to generate a large F_2 generation, to screen these fish for risk-taking and to conduct a QTL analysis. I used as my assay the behaviour of sticklebacks while exploring a novel environment, since this is more easily controlled than direct response to a predator, but is predictive of an individuals risk-taking response to a predator or conspecific (Huntingford, 1976; Bell, 2005). A subsidiary aim was to confirm the heritability of this trait. In the event, I found no evidence that this aspect of variable risk-taking has a heritable component, so the full molecular analysis was not carried out. I present here the data on which this conclusion was based and discuss possible explanations.

5.3 Materials and methods

5.3.1 Study and sampling sites

Fish from three locations were used for this study. Two sites, Lochs Fada and Grogary, were situated on the Outer Hebridean island of North Uist, off the West coast of Scotland. These sites represented two typical habitats on this island, namely high and low calcium sites. Loch Fada is located on the Eastern side of N. Uist, approximately three kilometres in length and was a typical peaty moor-land water body. The loch is oligotrophic, acidic and calcium poor, with 'tea-stained' water. Predation risk is high. Heron (*Ardea cinerea*), merganser (*Mergus serrator*) and grebe are present, as are trout (*Salmo trutta*) and charr (*Salvelinus alpinus*). Conversely, Loch Grogary is located on the Western side of N. Uist, is a long narrow shallow loch bisected by a road and approximately one kilometre in length. It is a biologically productive, mesotrophic loch, alkaline and rich in calcium. Classified a Machair loch, the substrate is Atlantic shell rich sand soil. Predation risk is high here too, avian predators including heron, mergansers, gulls and terns. Piscine predators present are Atlantic salmon (*Salmo salar*) and trout. In contrast to the two island sites was the third study site, the River Kelvin. Rising in the Kilsyth Hills, North East of Glasgow the river flows though rural and urban sites before entering the River Clyde, 3km (2 miles) west of Glasgow city centre. A number of predators of sticklebacks are found at this river and including kingfisher (*Alcedo atthis*) heron, gulls, divers and brown trout.

The sampling site at Loch Fada (NF891706) was a shallow bay, approximately 7m x 10m, with a maximum depth of 1m. The substrate here is mainly sand and large rocks with soft organic matter at the loch edge. Several macrophytes including bulbous rush (*Juncus bulbosum*), water milfoil (*Myriophyllum* sp.) and pondweed (*Potamogeton* sp.) are present, but no over-hanging vegetation at the loch side. At Loch Grogary (NF717709) fish were caught in a channelled stream, with high earth banks and an organic/mud substrate approximately 25m x 1m and 40cm deep. There is little vegetation over-hanging the bank sides, but numerous macrophytes are found there, including jointed rush (*Juncus articulatus*), yellow iris (*Iris pseudacorus*), yellow water lily (*Nuphar lutes*) and Nuttalls pondweed (*Elodea nuttallii*). The latter is a highly invasive introduced species and great care was taken

to wash all equipment in salt water before moving onto another site. The final sample site, the River Kelvin (NS570674) was a shallow, slow flowing mill run (20m x 2m), situated parallel to river. The substrate is mud and cobble, with clumps of macrophytes within and along the channel. After gaining permission from Scottish Natural Heritage, bank-side collections of stickleback were made in February 2003 at Lochs Grogary and Fada, netting 23 and 42 fish respectively. Fish from the River Kelvin were hand-netted in 2004 (N = 28).

Fish from each site differed in the amount of protective armour they possessed (Table 5.1). Those from Loch Fada had reduced armour, with some individuals having no dorsal spines, pelvic spines or lateral plates, while fish from Loch Grogary possessed the full complement of three dorsal spines, two pelvic spines and between 0 to 6 lateral plates (fish sampled over 3 years, see Chapter 3). Individuals from both sites were crossed as part of a study designed to examine the inheritance of protective armour. These lineages were also used for the analysis of the possible inheritance of behaviour. Fish from the River Kelvin were more heavily armoured, with some having as many as 15 lateral plates and were regarded in this study as the standard for body armour (fish sampled over 2 years, see Chapter 3). Individuals were used in the analysis of the inheritance of body armour and, as they showed a marked range of risk-taking at the individual level, were also used to investigate the possible inheritance of behaviour.

Pop.	N	3 DS	2 DS	1 DS	O DS	PS	Mean Plates (+/- SD)
Fada	23	3	8	6	6	0	0
Grogary	42	42	0	0	0	42	3.47 (0.89)
Kelvin	28	28	0	0	0	28	4.64 (1.61)

Table 5.1 Table of amour morphology seen in the 3 populations used in this study. In columns one and two, population name and the number of fish sampled from each population. In the next four columns is the number of fish with 3 dorsal spines (DS), 2DS, 1 DS and 0 DS. Column seven is the number of fish with pelvic spines (PS) and the final column contains the mean lateral plate number with standard deviation (+/- SD) for each population.

5.3.2 Fish husbandry

Fish were transported to the Scottish Centre for Ecology and the Natural Environment (SCENE) Field Station, Loch Lomond, in aerated 25 gallon buckets. Sampled populations were held individually in 1.3m x 1.3m, 500-litre flow-through indoor tanks at a maximum density of 50 fish per tank. Large, homemade plastic plants were placed within the tanks as refuges. After an acclimation period of three days, fish were fed frozen and live bloodworm (*Chironomus* sp.) and frozen water fleas (*Daphnia* sp.). Fish were fed *ad libitum* and maintained on an ambient photoperiod and loch water temperature (6 ± 2°C). Fish were not marked and, to keep track of individual identity, after behavioural observations, fish were held individually in chambers. The chambers (120mm x 100mm, with mesh windows and a mesh bottom) were held in an outdoor flow-through flume (outer circumference 19.8m, inner circumference 15.8, width 60cm, depth 60cm) under natural light conditions and ambient loch water temperature (6.6 ± 1.1°C). To provide a hiding place a $2cm^2$ piece of black plastic was placed in each cell. Uneaten food and faeces were removed daily.

5.3.3 Behavioural screening

Risk-taking was assessed using a standard test of exploration/activity in a novel environment (Verbeek, *et al.*, 1994). For a full discussion of behavioural screening see chapter 4. To ensure that individual risk-taking was measured accurately, a number of potentially confounding variables were identified and, where possible, controlled for. Fish were deprived of food for a minimum of 20 hours prior to testing to minimise differences in hunger level, which are known to influence risk-taking (Lima & Dill, 1990). To standardise the effect of social interaction, 2003 parental fish were held in small groups (two to four fish) and were transferred from their home tank to smaller holding tanks in the observation room on the day screening was carried out. There-after all fish were individually housed for a minimum of three days prior to screening, thereby minimising the immediate effects of social interactions and maximising the chances of picking up inherited behavioural differences.

To standardise stress levels, fish were allowed to settle for a minimum of one hour in aerated holding tanks and under low light conditions before testing. In the parental generation (see below for details of breeding programme), one fish from

each population was tested alternately (2003 fish were tested with five other populations and 2004 fish were tested with three other populations), in one of two observation tanks. The first filial (F_1) cross, generated in 2004 (see below) was screened in one of three observation tanks. The observations were carried out blind, ensuring unbiased screening by the observer. An assistant randomly netted fish from their home tank and placed each in an individually numbered chamber. The identity of each fish's parental phenotype remained unknown until the end of the experiment. All second filial (F_2) progeny were tested in one of two observation tanks, the fish being isolated as mentioned above. Two F_1 families were generated in 2003 and tested at different times. Therefore the parental phenotype of the progeny was known and so blind tests could not be carried out. Where fish were tested twice, behavioural scores from test one only were used. After testing, fish returned to their individual holding chamber for subsequent behavioural crosses.

Principle Component Analysis (PCA) was used to condense the behavioural data sets from Grogary and Fada fish (Lineage U) and Kelvin fish (Lineage K), summarising the measured variables into a single factor score for each fish (Huntingford, 1976). The analysis was carried out on unranked data, having first established that scores derived from ranked and unranked data were equivalent (r^2 = 94.2% and r^2 = 0.98%, P = <0.001 for the two lineages described below). Table 5.2 shows the first principle axis (PC1), which accounted for 76.4% and 81% (respectively) of the total variance and opposed latency to cross the 3 lines (negative loadings) against activity and tank use (positive loadings). This was interpreted as an index of risk-taking and is comparable to the index of boldness identified by Huntingford (1976) and Bell (2005). On the basis of their risk-taking scores, fish were classified as risk-takers or risk-avoiders if they were positioned in the upper or lower interquartile range of PC1 scores respectively. All other fish were classified as risk-intermediate.

Behaviour	Loading
All observations of Fada and Grogary P1, F1 and F2's	
Latency AB	-0.445
Latency BC	-0.470
Latency CD	-0.427
Activity	0.430
Tank use	0.463
Variation explained (%)	**76.4**
All observations of Kelvin P1, F1 and F2's	
Latency AB	-0.459
Latency BC	-0.474
Latency CD	-0.457
Activity	0.386
Tank use	0.455
Variation explained (%)	**81.0**

Table 5.2 Principle components analysis (PCA) opposed latency to explore against movement, giving an index of risk-taking. Variables used were latency to explore (Latency, AB,BC,CD) and movement (Activity and Tank use). The Eigenvalue explained 76.4% of the variation in the combined tests in lineage U (top half of table) and in lineage K (bottom half of table) the Eigenvalue explained 81.0% of the variation in the combined tests.

5.3.4 Breeding regimes

Two lineages were established, one using fish from the River Kelvin as parents and the other using fish from Lochs Fada and Grogary. Two parental crosses were carried out in 2003, hereafter referred to as Line U (see Figure 5.1) and always having the maternal phenotype first. One intrapopulation cross, (FF), between Fada risk-takers resulted in a family of 70 F_1 offspring. One interpopulation cross (GF) between a Grogary risk-taking female and a Fada risk-averse male resulted in one family of 80 F_1 offspring. Six GF F_1 fish were intercrossed (GF1-1 risk-averse x risk-averse; GF1-2 risk-averse x risk-taker; GF1-3 risk-taker x risk-taker) resulting in 115 F_2 progeny between the three families (see Figure 5.2 and Table 5.3).

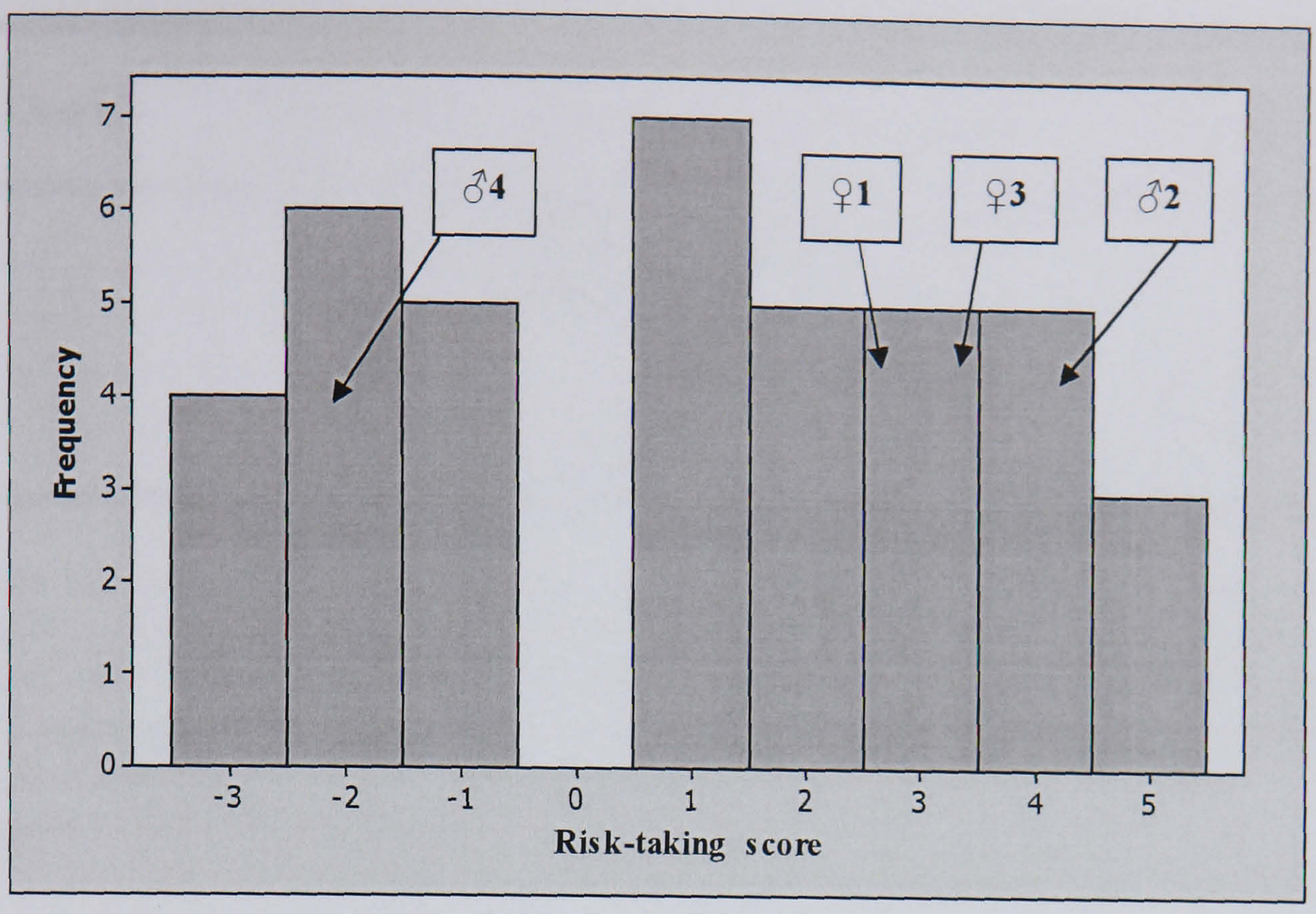

Figure 5.1 Behavioural distribution of risk-taking scores in the combined Lochs Fada and Grogary wild caught fish. Numbers in boxes relate to the numbers assigned to parents used to produce the F1 generation (Line U; FF = ♀1 x ♂2 and GF = ♀3 x ♂4). The arrows point to individual Risk-taking scores derived from a principle component analysis.

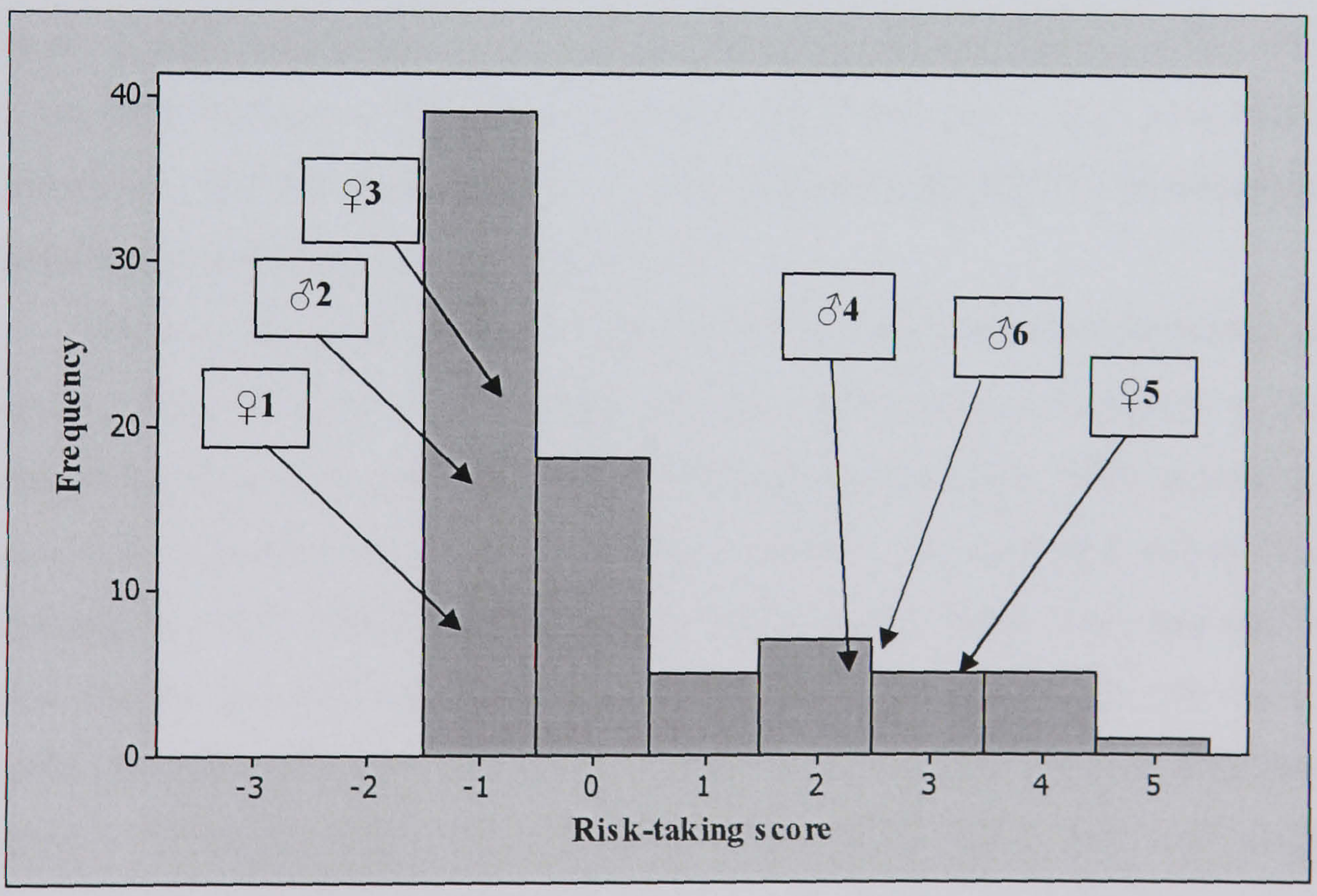

Figure 5.2 Behavioural distribution of risk-taking scores in the inter-population GF cross (risk-taker x risk-averse). Numbers in boxes relate to the numbers assigned to parents used to produce the F2 generation (Line U; GF1 = ♀1 x ♂2, GF2 = ♀3 x ♂4 and GF3 = ♀5 x ♂6). The arrows point to individual R-T scores derived from a principle component analysis.

Line U	Phenotype	Median score	F_1 progeny	F_2 Progeny
FF	T x T	-1.146 *	N = 70	
GF	T x A	-0.5910	N = 80	
GF1	A x A	0.1322		N = 43
GF2	A x T	-1.0112		N = 26
GF3	T x T	-0.5049		N = 46

Table 5.3 Table of the Line U lineage. Column two shows the parental phenotype with T signifying a risk-taker (or 'bold' fish) and A signifying a risk-averse fish (or 'timid' fish). Column three shows the median risk-taking score for that family and the * signifying that a family was significantly different and the arrow shows which family it differed from. The final two columns show the number of F_1 and F_2 progeny each family produced.

Eight fish of differing behavioural phenotypes were chosen from wild caught sticklebacks from the River Kelvin screened in 2004 and four parental crosses carried out (se Figure 5.4), hereafter referred to as Line K; These were KK1 (risk-taker x risk-taker), KK2 (risk-averse x risk-averse), KK3 (risk-averse x risk-averse) and KK4 (risk-taker x risk-averse). These crosses produced a total of 280 F1 progeny. Eight fish from family KK4 were inter-crossed, resulting in 137 F2 progeny between four families, KK4-1 (intermediate x intermediate), KK4-2 (risk-averse x intermediate), KK4-3 (intermediate x intermediate) and KK4-4 (intermediate x intermediate)(see Figure 5.5 and Table 5.4).

Males in breeding condition were moved to sandy-bottomed breeding tanks (45cm × 27cm × 15cm) with nesting material. After nest construction, a gravid female was placed in the tank and the pair left to breed naturally. After spawning, the female was removed and the fertilised eggs placed in an incubator and artificially oxygenated at 16°C. Males rebuilt a nest within one to three days and the same gravid female returned to the tank to breed again. Post hatching, fry were fed Liquifry No1 (INTERPET) for one week and there after maintained on a mixture of enriched AF high-grade *Artemia* (INVE AQUACULTURE nv) and chopped bloodworm. At 8 weeks fry were moved to small holding tanks (25cm × 20cm × 45cm) and kept in family groups of 10 to 15 fish until selected for behavioural screening.

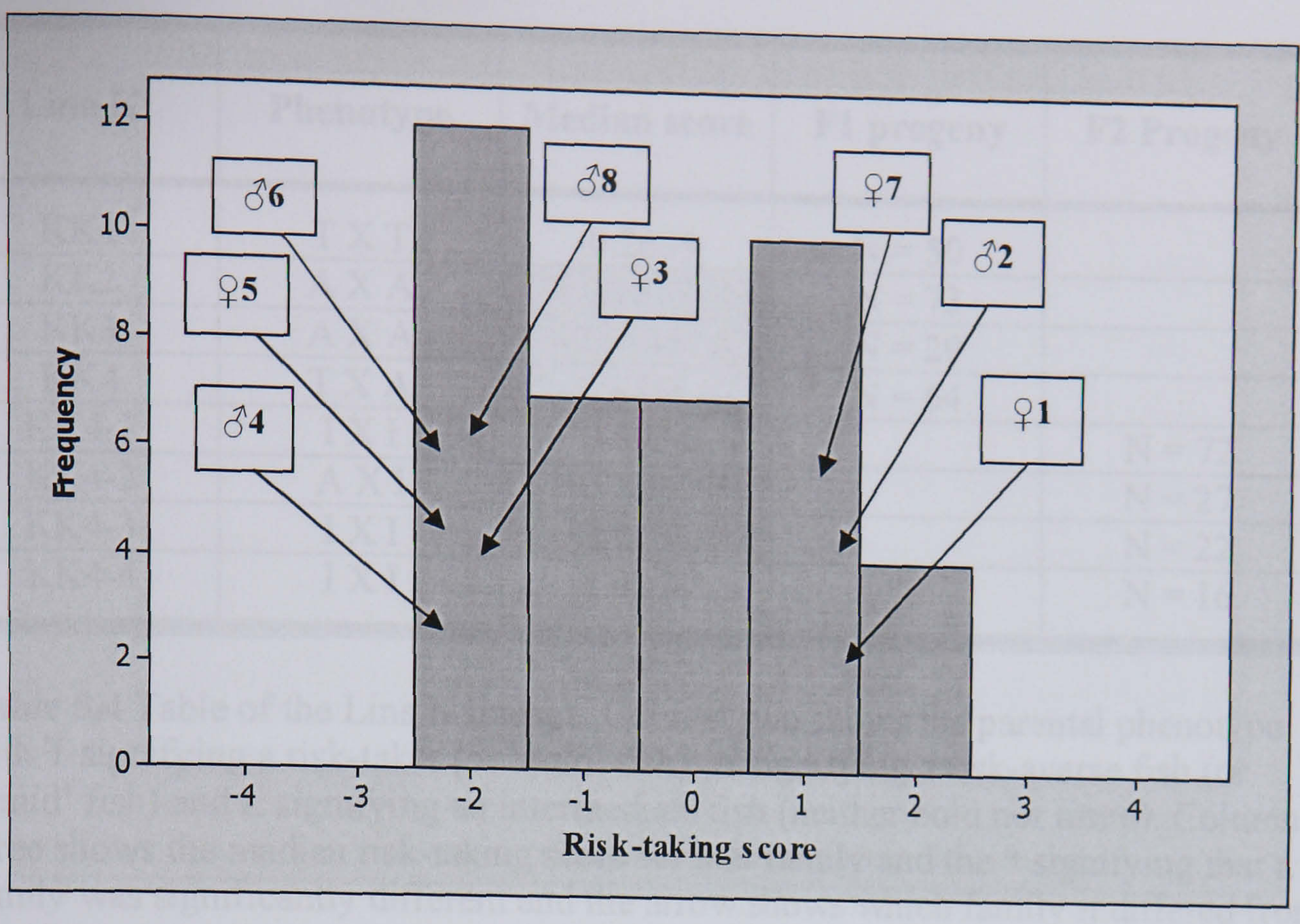

Figure 5.3 Behavioural distribution of risk-taking scores in wild caught River Kelvin fish. Numbers in boxes relate to the numbers assigned to parents used to produce the F1 generation (Line K; KK1 = ♀1 x ♂2, KK2 = ♀3 x ♂4, KK3 = ♀5 x ♂6 and KK4 = ♀7 x ♂8). The arrows point to individual R-T scores derived from a principle component analysis.

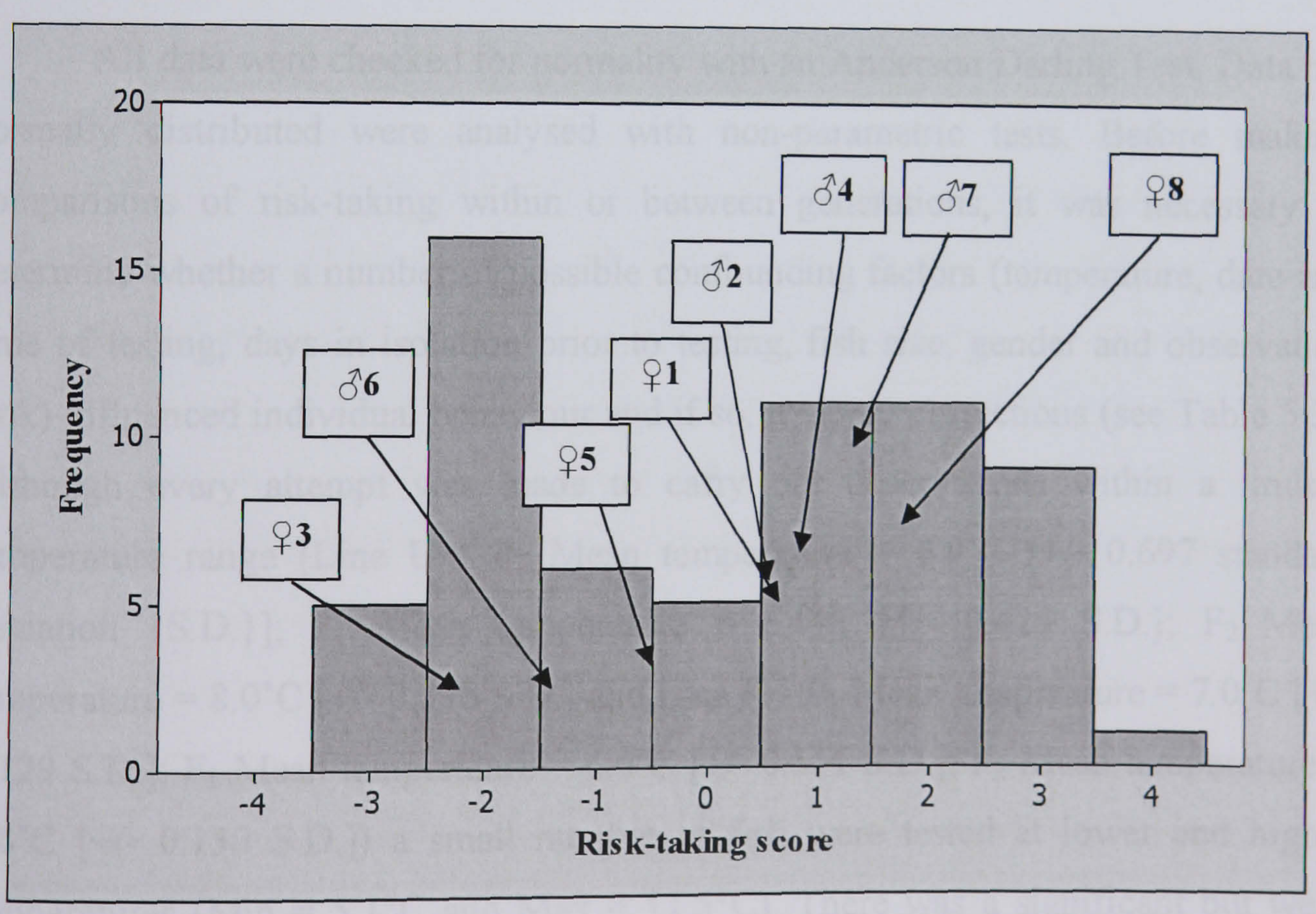

Figure 5.4 Behavioural distribution of risk-taking scores the KK4 cross (risk-taker x risk-averse). Numbers in boxes relate to the numbers assigned to parents used to produce the F2 generation (Line K; KK4-1 = ♀1 x ♂2, KK4-2 = ♀3 x ♂4, KK4-3 = ♀5 x ♂6 and KK4-4 = ♂7 x ♀8). The arrows point to individual R-T scores derived from a principle component analysis.

Line K	Phenotype	Median score	F1 progeny	F2 Progeny
KK1	T X T	-0.2699	N = 50	
KK2	A X A	0.1817	N = 73	
KK3	A X A	-2.3145 *	N = 29	
KK4	T X A	0.4456	N = 64	
KK4-1	I X I	0.8934		N = 72
KK4-2	A X I	0.1803 *		N = 27
KK4-3	I X I	1.2254		N = 22
KK4-4	I X I	1.0278		N = 16

Table 5.4 Table of the Line K lineage. Column two shows the parental phenotype with T signifying a risk-taker (or 'bold' fish), A signifying a risk-averse fish (or 'timid' fish) and I, signifying an intermediate fish (neither bold nor timid). Column three shows the median risk-taking score for that family and the * signifying that a family was significantly different and the arrow shows which family it differed from. The final two columns show the number of F_1 and F_2 progeny each family produced.

5.4 Data analysis

All data were checked for normality with an Anderson Darling Test. Data not normally distributed were analysed with non-parametric tests. Before making comparisons of risk-taking within or between generations, it was necessary to determine whether a number of possible confounding factors (temperature, date and time of testing, days in isolation prior to testing, fish size, gender and observation tank) influenced individual behaviour and if so, to apply corrections (see Table 5.3). Although every attempt was made to carry out observations within a limited temperature range (Line U - P_1 Mean temperature = 6.9°C [+/- 0.697 standard deviation {S.D.}]; F_1 Mean temperature = 6.6°C [+/- 0.428 S.D.]; F_2 Mean temperature = 8.0°C [+/- 0.396 S.D.] and Line K - P_1 Mean temperature = 7.0°C [+/- 0.129 S.D.]; F_1 Mean temperature = 6.6°C [+/- 0.561 S.D.]; F_2 Mean temperature = 8.8°C [+/- 0.130 S.D.]) a small number of fish were tested at lower and higher temperatures (Min = 5.1°C and Max = 11.5°C). There was a significant but weak association between temperature and behaviour of the fish (Spearman Rank Correlations; Line U - $R_s = 0.156$, $P = 0.006$; Line K - $R_s = 0.297$, $P < 0.001$), with levels of risk-taking increasing somewhat with the temperature (see Chapter two). To

correct for variables effecting behaviour, scores were expressed as residuals from the linear regression of risk-taking against temperature (Boylan, 2005).

Behavioural observations began on different dates and spanned a five-month period in Line U (P_1 = Mar-Apr 03; F_1 = Feb & Mar 04; F_2 = Dec-Jan 05/06) and a six-month period in Line K (P_1 = Mar 03; F_1 = Oct-Feb 04/05; F_2 = Jan-Feb 06). However, there was no effect of test date on the behaviour of the fish and no corrections were made. This was also true of the time a fish was tested. Although the start and end time of observations differed from generation to generation (Line U – P_1 = 10:15 to 14:55; F_1 = 09:30 to 16:14; F_2 = 08:30 to 16:15; Line K – P_1 = 09.25 to 17:46; F_1 = 08:00 to 17:19; F_2 = 07:58 to 16:46) there was no effect of time tested on behaviour at the level of the individual.

The number of days that an individual fish was held in isolation in its chamber (Max Time In Chamber) varied greatly within and between generations (Line U – P_1 = 0 days; F_1 = 75-147 days; F_2 = 13-48 days; Line K – P_1 = 7-16; F_1 = 9-72; F_2 = 6 days) and, had a significant but weak effect on behaviour (Spearmans Rank Correlation; Line U - R_s = -0.239, $P < 0.001$; Line K - R_s = 0.121, $P = 0.016$). In Line U, the longer fish were held in isolation the more timid their behaviour. Conversely for Line K, the longer fish were in isolation, the bolder they became. With regards to fish size, there were differences in mean standard length between generations (Line U – P_1 = 34.81mm; F_1 = 26.52mm; F_2 = 30.81mm and Line K – P_1 = 44.11mm; F_1 = 42.68mm; F_2 = 38.17mm) but no significant effect of S.L. on individual behaviour (see Table 5.5). Although age of the fish was known in the F_1 and F_2 generations it was not used in the investigation of factors affecting behaviour, as this variable was correlated with standard length. There was an even distribution of males and females between generations and between observation tanks and gender had no effect on behaviour. Although there was an effect of tank on behaviour in Line K (K-W of risk-taking and tank No., $P = 0.048$, fish tested in observation tank 2 being bolder than those tested in tank 1), generations and families were evenly distributed between the two tanks (P_1 and F_2 fish screened in two tanks - Chi^2 = 3.124, DF = 4, $P = 0.537$; F_1 fish screened in three tanks - Chi^2 = 3.572, DF = 6, P = 0.734). This indicated that P_1 fish, F_1 and F_2 families were tested randomly between observation tanks and therefore any potential behavioural differences between families were unaffected by tank number. Clean risk-taking score could now be used to look at the differences in behaviour between generations and families.

Variable	Effect of variable on behaviour between individuals			
	Line U		*Line K*	
	R_s	P	R_s	P
Temperature	0.156	**0.006**	0.297	**<0.001**
Date	-0.032	0.580	-0.003	0.954
Time tested	0.046	0.424	0.019	0.709
Max. TIC	-0.239	**<0.001**	0.121	**0.016**
SL	0.041	0.510	-0.093	0.108

Table 5.5 Statistical analysis of controlled and uncontrolled variables. In column one are the variables that may have an effect on behaviour. The temperature observations were carried out at, the date on which the observation was carried out, the time an observation was carried out at, the maximum amount of time fish were in chambers (Max. TIC) and the standard length of fish (SL). In the second and third columns are the test statistics for variables effecting individual risk-taking in all Line U fish tested over a period of three years and the following two columns test statistics for all fish in Line K.

5.5 Results

5.5.1 Lines derived from sticklebacks from North Uist (Line U)

Comparing all fish within each generation (Figure 5.5), there was a marginally-significant difference in risk-taking between generations (Kruskal-Wallis test of generation and risk-taking; $P = 0.076$). Post-hoc tests revealed that the parental generation and F_2 fish were the most different from each other, F_2 fish tending to be more risk-averse. Fish in the F_1 generation had a smaller behavioural distribution than either the parental or F_2 fish, but had higher numbers of risk-intermediate individuals.

Comparing families within generations in F_1 fish (Figure 5.6), there was a significant difference in risk-taking between FF (offspring of two risk-takers) and GF (offspring of risk-taker x risk-averse) families (Mann-Whitney test of F_1 families and risk-taking; $W = 0.4287$, $P = 0.002$). Interestingly, family FF was more risk-averse than family GF, despite the fact that the former cross was between two risk-averse individuals. There were no significant differences in risk-taking between F_2 families

even though these families were selected to be risk-prone, risk-intermediate and risk-averse.

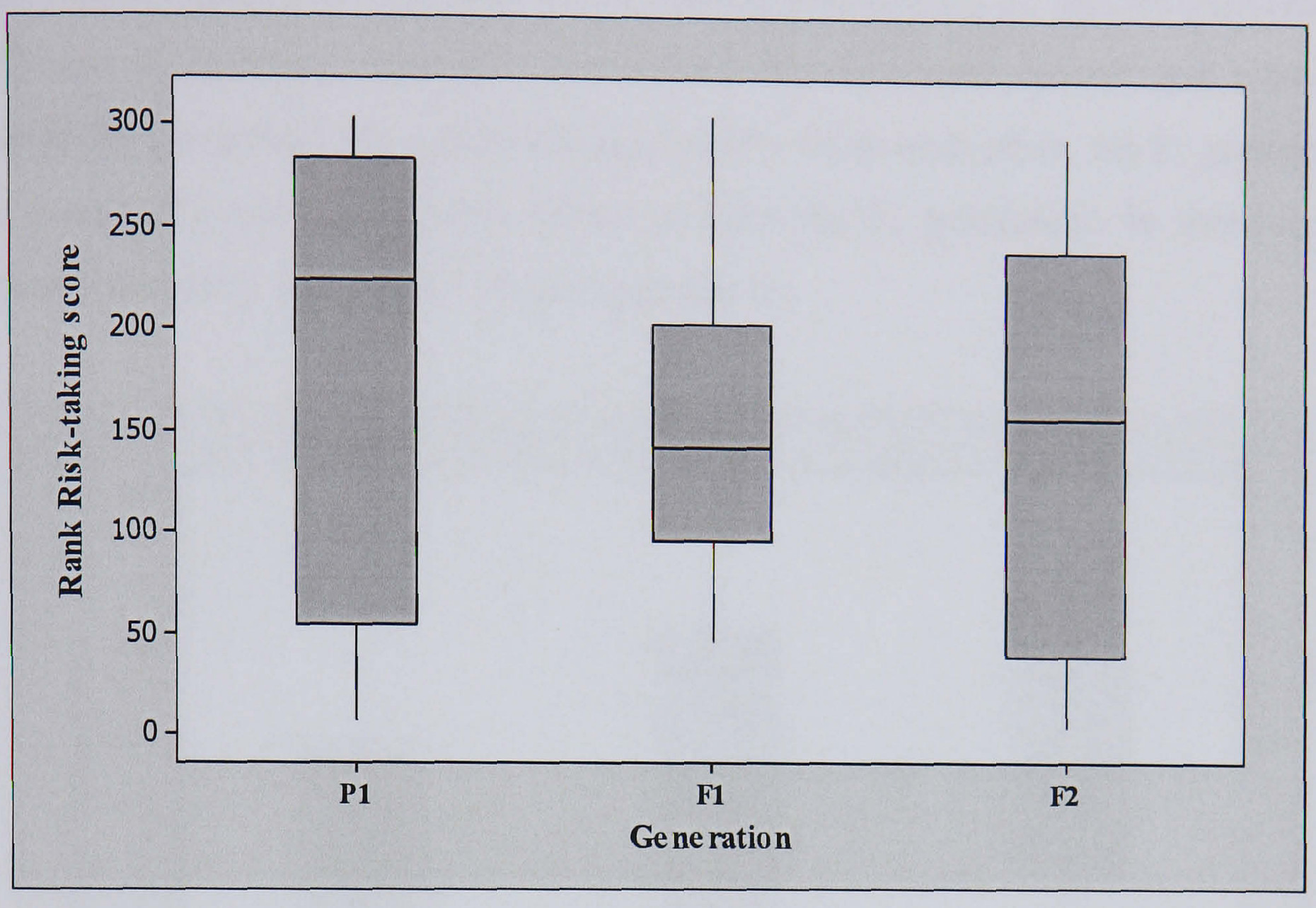

Figure 5.5 Plot of rank risk-taking behaviour across generations in Line U. Median risk-taking score, derived from a Kruskal-Wallis test, was higher and the range of risk-taking behaviour largest in the P_1 generation.

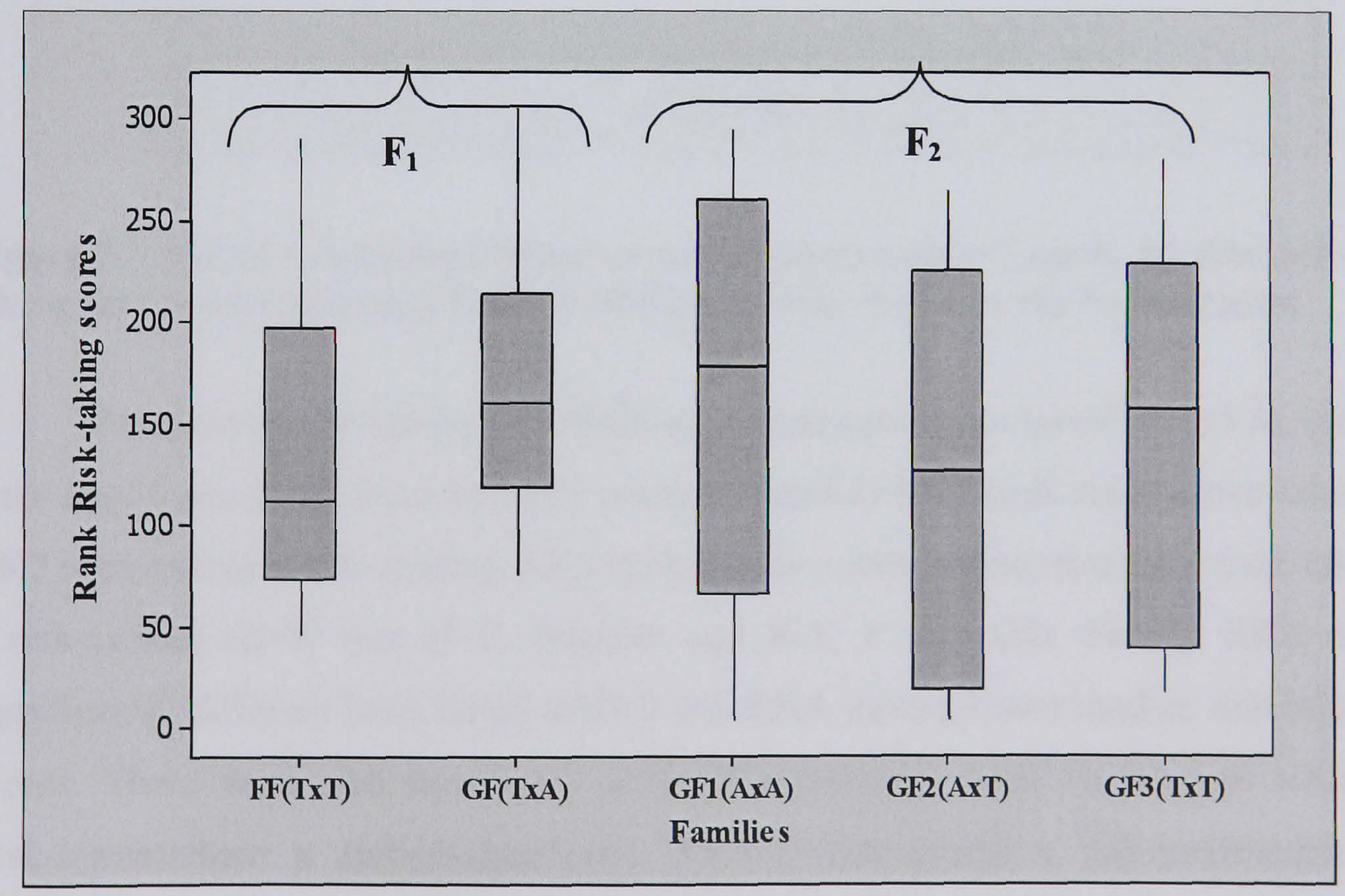

Figure 5.6 Plot of rank risk-taking behaviour score between families and across generations in Line U. Median risk-taking score, derived from a Kruskal-Wallis test showed that the families differed significantly. Further analysis (Mann-Whitney U test) showed a significant difference between F1 families only.

5.5.2 Lines derived from sticklebacks from the River Kelvin (Line K)

Comparing all fish within each generation (Figure 5.7), in this lineage too there was a significant difference in risk-taking between P_1, F_1 and F_2 generations (K-W test of generation and R-T, $P = 0.042$). Post-hoc tests showed that although none of the generation pairs differed significantly from each other, the F_2 generation was marginally non-significantly different from the F_1 generation. In this lineage median risk-taking increased with each generation.

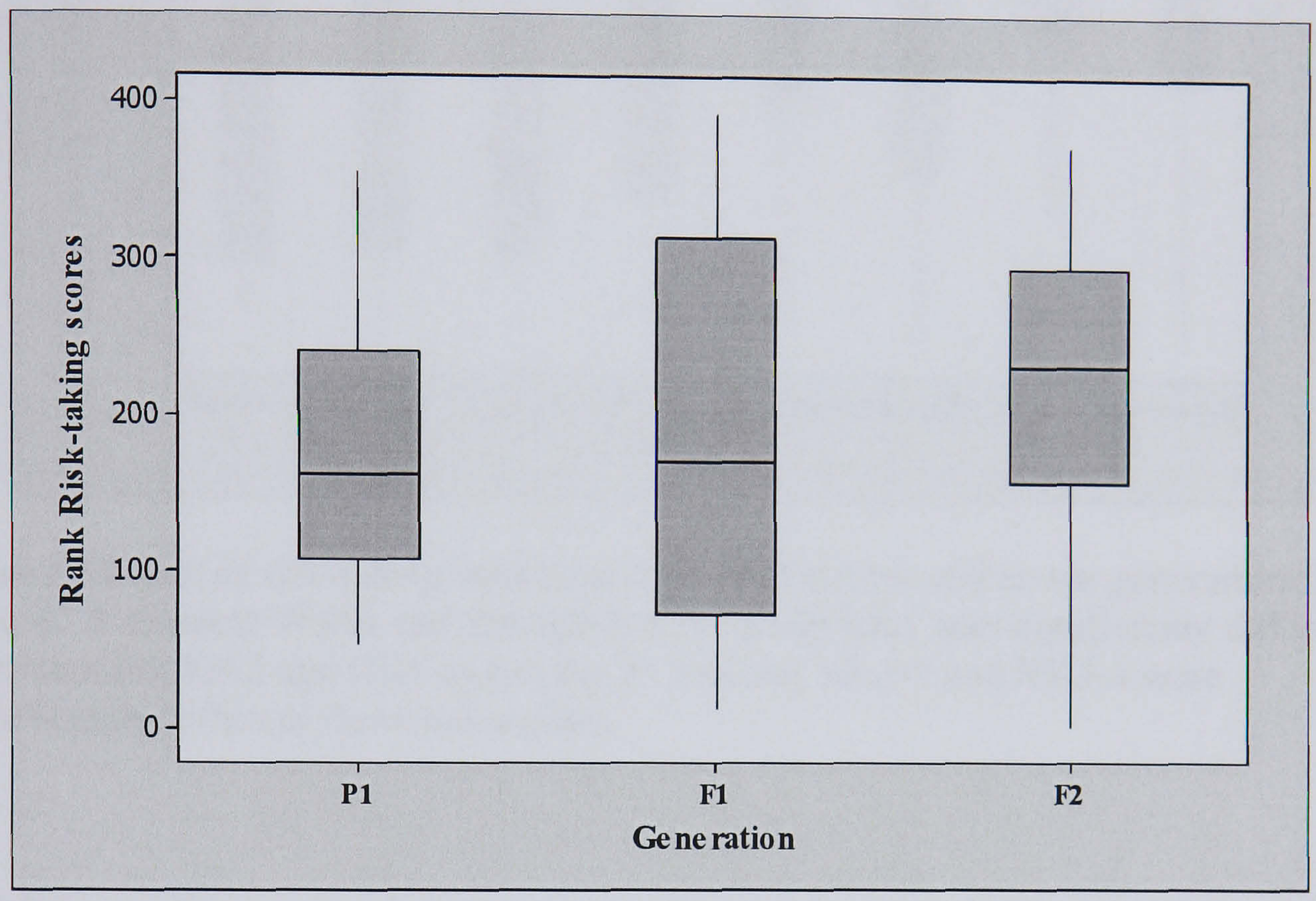

Figure 5.7 Plot of risk-taking behaviour across generations in Line K. Median risk-taking score, derived from a Kruskal-Wallis test, was higher in the F_2 generation.

Comparing risk-taking with families in each generation (see Figure 5.8), there were significant differences between some F_1 families KK1 (risk-taker x risk-taker), KK2 (risk-averse x risk-averse), KK3 (risk-averse x risk-averse) and KK4 (risk-taker x risk-averse) (K-W test of F_1 families and R-T, $P = 0.024$). Family KK3 was significantly different from families KK2 and KK4, having lower median risk-taking scores. There were also significant differences between some F_2 families KK4-1 (risk-intermediate x risk-intermediate), KK4-2 (risk-averse x risk-intermediate), KK4-3 (risk-intermediate x risk-intermediate) and KK4-4 (risk-intermediate x risk-intermediate) (K-W test of F_2 families and R-T, $P = 0.007$). Analysis identified KK4-

2 and KK4-3 as being significantly different from one another, the former being more risk-averse than the latter.

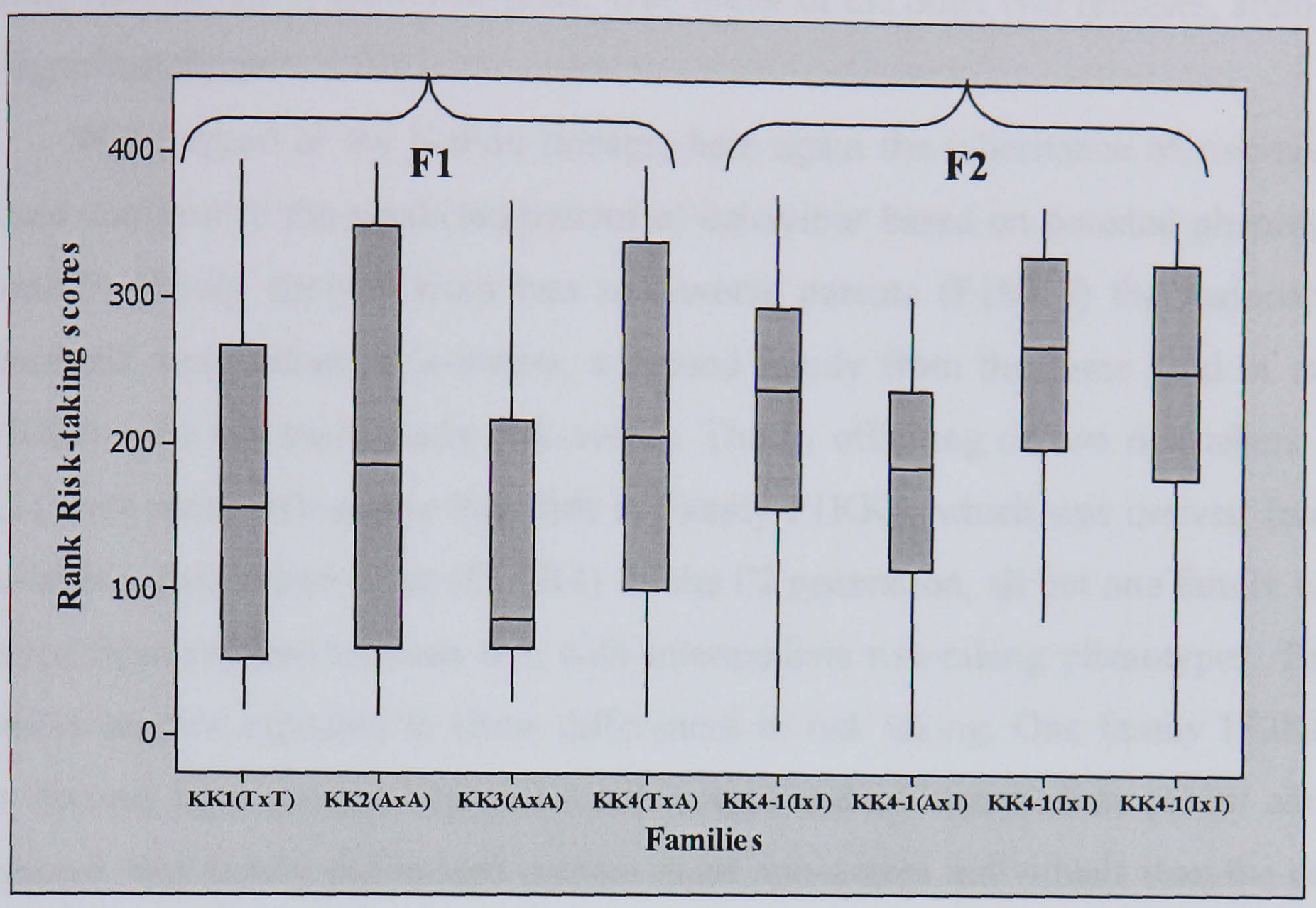

Figure 5.8 Plot of risk-taking behaviour between families and across generations in Line K. A Kruskal-Wallis test showed that F_1 family KK3 was significantly different from families KK2 and KK4 and in the F_2 families; KK4-2 and KK4-3 were significantly different from one another.

5.6 Discussion

There were trends in risk-taking across the generations, but as these were in opposite directions in the two lineages, the biological significance (if any) is not clear. There were also some significant family differences in both the F_1 and F2 generations in both lineages, but offspring phenotype showed little relationship with the parental phenotype.

In the Uist lineage the expectation in the F_1 generation would be that offspring of crosses between risk takers (Family F1FF), would be more risk-prone than the offspring of the cross between the risk-taking and risk-averse fish (Family F1GF) cross. In fact, the opposite was the case, with F1FF fish being the more risk-averse. In the F_2 generation, the prediction was that offspring of two risk-takers (Family F2GF3) would be more risk-prone than those of the cross between a risk-

taker and a risk-avoider (Family F2GF 2). This prediction was confirmed, but individuals in another family derived from a cross between two risk-averse fish (Family number GF1) more risk-prone than either of the other two families, although not significantly so.

With regard to the Kelvin lineage, here again the inheritance of risk-taking did not conform to the predicted pattern of behaviour based on parental phenotype. In one F_1 family derived from two risk-averse parents (F1KK3) the majority of individuals were indeed risk-averse, a second family from the same kind of cross (F1KK2) were not particularly risk-averse. The F_1 offspring of two risk-takers (F1 KK1) were more risk-averse than fish in Family F1KK4, which was derived from a risk-taker x risk-averse cross (F1KK4). In the F2 generation, all but one family were derived from crosses between fish with intermediate risk-taking phenotypes. These families are not expected to show differences in risk taking. One family (F2KK4) was derived from a cross between a risk averse and an intermediate parent and as predicted, this family did indeed contain more risk-averse individuals than the other families in this lineage/generation.

Overall, therefore, although I found some significant differences in risk-taking between families in both generations and in both lineages, it is clear then that risk-taking is not inherited in any simple way. This then begs the question of what the underlying cause of the differences between families might be. One possible explanation is that risk-taking is influenced by social interactions within rearing groups and that family-level differences arise because the fish were reared in groups of different sizes. Another possibility is that the different risk-taking phenotypes experience differential mortality and that family differences arise because the different families experienced different levels of mortality. A third possibility is that the mothers of the broods experienced different degrees of stress during courtship and mating, so that variable amounts of cortisol were transferred to the eggs, influencing the behaviour of the offspring (Leatherland, 1999).

Table 5.6 summarises data on clutch sizes, hatching success, mortality and rearing group size for all the families in our study, together with their median risk taking score. There is no relationship between family-level risk-taking and size of rearing group. While this does not rule out the possibility that early social interactions modulate later risk-taking, it does make a simple version, at least, unlikely. Nor is there any relationship between mortality rate and family-level risk

taking. This rules-out the suggestion that differential mortality by behavioural phenotype generates family differences, at least in its simple form. So perhaps effects of maternal stress caused the differences in risk-taking between families that I have described here.

The above explanations may be further complicated by inconsistent risk-taking behaviour. Whatever the case, the marked individual differences in risk-taking that I describe in this study are clearly not inherited in any simple sense. For this reason, QTL analysis for this trait was not carried out.

Family	Phenotype	Median score	Egg No. (~)	No. of clutches	No. of fry (post hatching)	% Fry mortality	No of fry (post moving)	No. of holding tanks	No of fry per tank
F1-FF	T X T	-1.146	100	4	92	23	70	4	17 & 18
F1-GF	T X A	-0.5910	100	4	94	14	80	4	20
F2-GF1	A X A	0.1322	60	2	51	14	44	2	22
F2-GF2	A X T	-1.0112	30	1	27	0	27	1	27
F2-GF3	T X T	-0.5049	80	3	68	31	47	2	23 & 24
F1-KK1	T X T	-0.2699	80	2	79	0	79	4	19 & 20
F1-KK2	A X A	0.1817	140	2	133	3	129	4	32 & 33
F1-KK3	A X A	-2.145	120	1	113	31	35	1	35
F1-KK4	T X A	0.4456	230	2	137	50 (from 1 clutch)	69	5	13
F2-KK1	I X I	0.8934	600	8	95	24	72	8	9 & 10
F2-KK2	A X I	0.1803	130	2	35	20	27	3	9
F2-KK3	I X I	1.2254	450	7	80	72	22	3	7 & 8
F2-KK4	I X I	1.0278	220	4	20	20	16	2	8

Table 5.6 Family number, behavioural phenotype cross and median risk-taking score, together with clutch sizes, hatching success, mortality and rearing group size.

Chapter 6. Armour reduction and loss in Scottish populations of three-spined stickleback (*Gasterosteus aculeatus*)

6.1 Introduction

6.1.1 Study species

The three-spined stickleback is a small, euryhaline teolost fish found in a large variety of habitats across the Northern Hemisphere (Wootton, 1984). It is easy to catch, can be held in large numbers in a relatively small amount of space, tolerates captivity well and produces large numbers of offspring. Following the pioneering research carried out by Niko Tinbergen in the 1950's into the reproductive behaviour of sticklebacks, the fish has been used as a model species to answer diverse questions in behaviour, physiology and morphology. More recently, the three-spined stickleback has been taken up by evolutionary biologists and molecular geneticists and used to help unlock the secrets of vertebrate phenotypic diversity.

6.1.2 Stickleback phenotypic diversity

Such is the great variation in morphology seen in the three-spined stickleback that for many years it was classified as over 40 different species and it is for precisely this reason that the fish is of interest to evolutionary biologists. Parallel evolution of armour reduction has occurred in a small number of independent locations ranging from the Northwest coast of Scotland to Iceland, North America and the NW coast of Canada (Campbell, 1979; Reimchen, 1980; Kristjánsson *et al.*, 2002). On an evolutionary scale, the divergence of morphological features has been rapid. Around 10,000 years after the retreat of the ice-sheet, freshwater habitats were colonised by heavily armoured marine three-spined sticklebacks. These fish had (and still have today) three long, barbed dorsal spines, a robust pelvic girdle and spines (pelvic complex), up to 35 lateral plates running along the lateral sides from the front of the pectoral fin to the tail and a keeled (plated) caudal peduncle (Wootton, 1976) (see Figure 6.1).

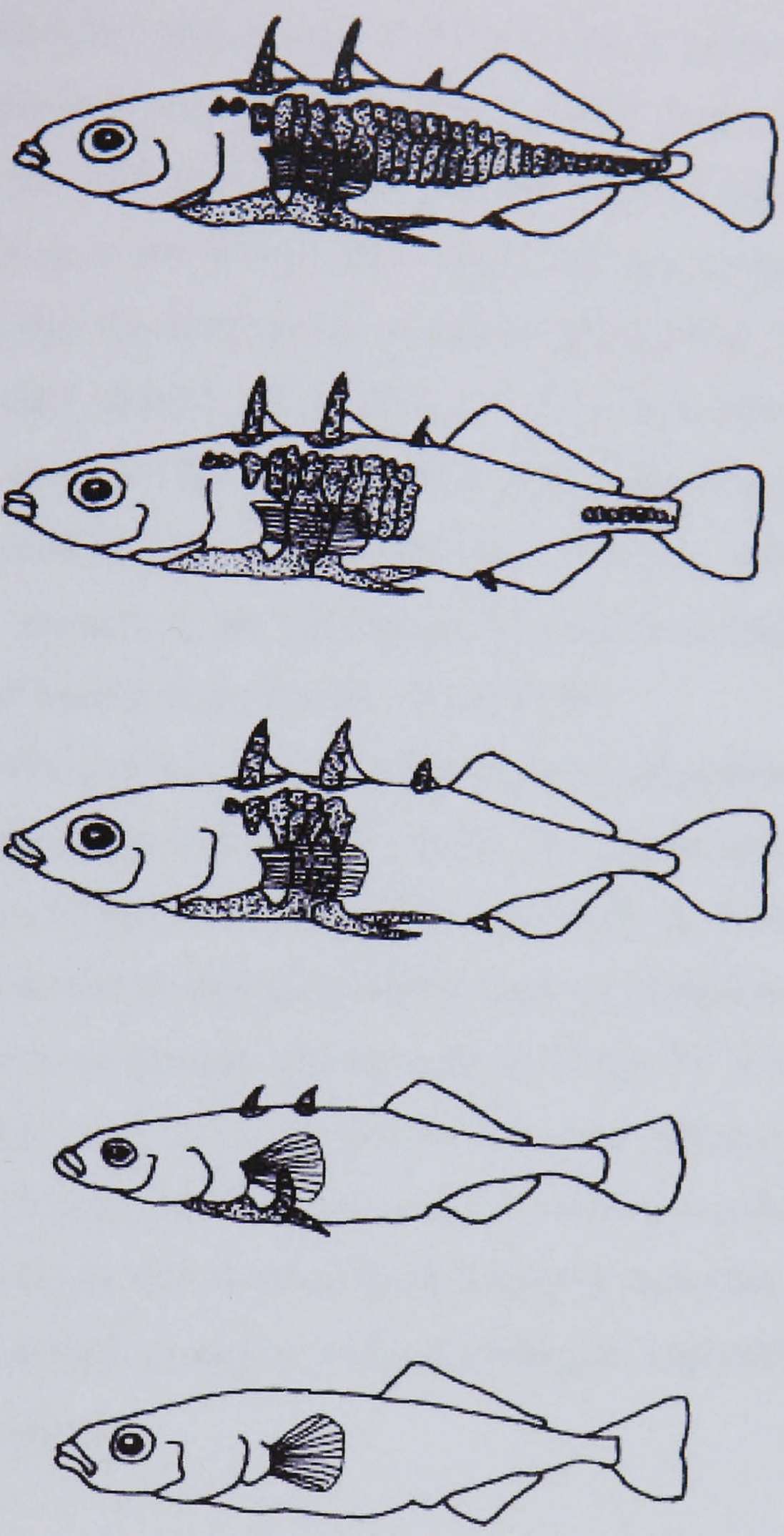

Figure 6.1 Line drawing of the morphological forms of *Gasterosteus aculeatus*. Top is the fully armoured morph, next is armoured reduced, below is low plated. The final two morphs are plateless and spine-deficient.

In less than 10,000 generations, a significant reduction in protective body armour occurred in the freshwater descendents of these marine colonists. Dorsal spines became shorter, reduced in number or absent, the pelvic complex became smaller or (occasionally) absent, the keel disappeared and typically lateral plate number decreased to between 0 and 7 (Schluter & Nagel, 1995). Predominantly driven by natural selection, parallel evolution (Bell, 2001) of armour reduction and/or loss is a likely response to local selection regimes in North American populations (Gross, 1978). For example, in the presence of predatory fish, long

dorsal and pelvic spines and an increase in lateral plate number are selected for. Fish gain protection from gape limited fish by having longer spines and plates protect against toothed predators (Gross, 1978; Reimchen, 1995). In the presence of avian or invertebrate predators plate and spine number are selected against. Lateral plates provide less protection from avian attack, were fish are compressed, and spines provide a grasping site for invertebrate predators (Bergstrom, 2002; Reimchen & Nosil, 2002). The total absence of predatory fish has also been implicated in the reduction and loss of spines but all lakes where spine-less morphs are found also have avian predators (Reimchen, 1980). Therefore, the type of predator rather than simple presence or absence of any predation risk may be a major driving force in reduction and loss of body armour (Cresko *et al.*, 2004).

In contrast, Giles (1983) proposed that calcium concentration is the selective agent in the reduction of protective body armour. Using armour reduced populations from North Uist, one of the Outer Hebridean islands off the West coast of Scotland (UK), he found that low armoured populations were only found in low calcium lochs (<5 mg Ca^{2+}/L) but never in high calcium lochs (>25 mg Ca^{2+}/L). He suggested that there may an energy cost of having armour in a low calcium environment. For example, reduction of non-essential bony armour results in a reduction of the energy required to absorb calcium for essential body formation from the surrounding water. This then implies that both biological and non-biological important factors in the loss of protective body armour.

6.1.3 Genetic studies

There have been several classical studies on the possible mechanisms underlying the inheritance of body armour, for example, inheritance of plate number (Hagen, 1973) and pelvic girdle (Bell, 1974). However, with the advent of new molecular tools coupled with the construction of a genome-wide map for sticklebacks (Peichel *et al.*, 2001), the groundwork for molecular genetic studies into the number and location of genes underlying the divergence of adaptive characteristics has been laid. Peichel *et al.* (2001) assembled the stickleback map, using microsatellite markers dispersed across the genome, into an ordered plan of the relative gene loci on each chromosome (see Figure 6.2).

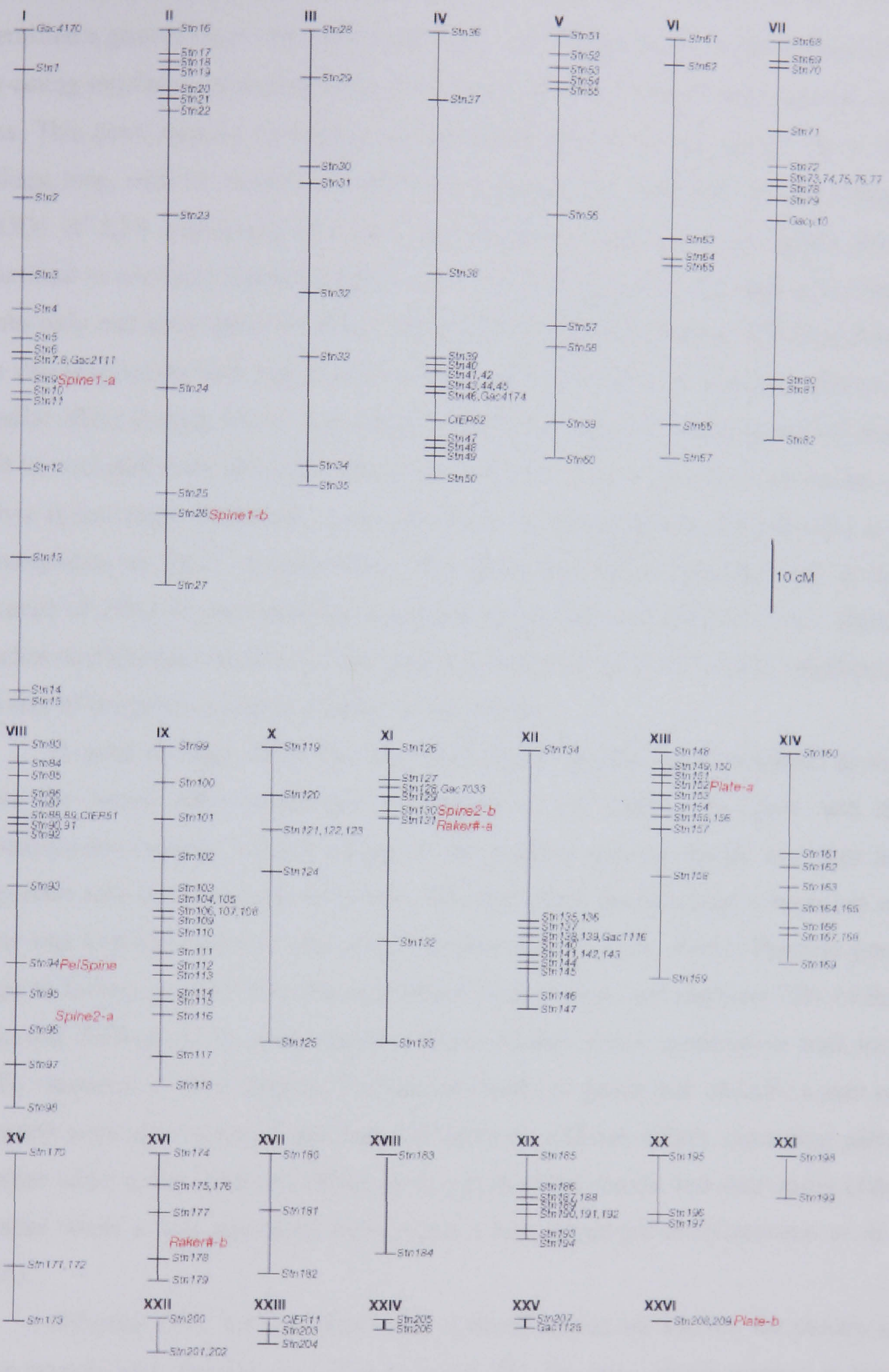

Figure 6.2 Genetic linkage map of the three-spined stickleback constructed by Peichel *et al.* (2001) taken from the journal Nature Vol. 414 p902. A roman numeral (I to XXV) has been given to each linkage group in sequence of genetic length. The prefix *Stn* denotes those microsatellites identified at Stanford University and QTL's affecting skeletal armour and feeding morphology are in red.

Several recently published studies (Cole *et al.*, 2003; Shapiro *et al.*, 2004) identified a gene of large effect for pelvic size, *Pitx1*. Shapiro *et al.* (2004) found that *cis*-acting regulatory mutations at the *Pitx1* gene resulted in hind limb reduction and loss. This gene mapped to markers on the distal end of linkage group VII in the linkage map, with for example, a pelvic spine length log likelihood ratio of linkage (LOD) of 82.8 explaining 65.3% of the variance (Shapiro *et al.*, 2004). First identified in mice and humans (Shang *et al.*, 1997) this gene is expressed in the hind limbs only and aptly called *backfoot*. Two other transcription factors *Tbx4* and *Pitx2* are also involved in limb reduction but map to other chromosome regions and have a smaller effect (Logan, 2003). Since these genes do not map to linkage group VII they will be excluded from this study. However, it is interesting to note that asymmetry in pelvic spine length (reduction is usually greater on the right than the left side) is a consequence of *Pitx2* compensation. The gene determines laterality and in the absence of *Pitx1* is preferentially expressed on the left side (Marcil *et al.*, 2003). Studies on *Pitx1* have shown that the gene accounted for up to 40% of the variance in the size of the pelvic complex (Shapiro *et al.*, 2004).

A gene of large effect *Ectodysplasin* (*Eda*) has also been identified as the basis for lateral plate expression (Colosimo *et al.*, 2005). The gene and its *Ectodysplasin* receptor (*Edar*) belong to the tumour necrosis family and play an important role in the formation of skin, hair and teeth. Interestingly, a mutation in *Edar* was found to underlie scale loss in Medaka (Kondo *et al.*, 2001). The *Eda* gene maps to linkage group IV on the stickleback linkage map, and explains 75% of the observed differences in plate number. Three further minor quantitative trait loci (QTL: sections of DNA that are in close proximity to genes that underlie a trait of interest) were also detected and found to have an additive effect, increasing plate number when a fish inherited alleles from a high plated parent and decreasing plate number when a fish inherited alleles from a low plated parent (Colosimo *et al.*, 2004).

Although QTL for the inheritance of dorsal spines are known, the pattern is more complicated. Peichel *et al.* (2001) found QTL for spine length mapped to four different locations in the linkage map (groups I, II, VIII and XI) and recently, in a study using 3 North American populations, (Summers B, pers. comm.) found twelve QTL important in dorsal spine expression. This suggests a more flexible basis for dorsal spine reduction than that seen in either plate or girdle complex reduction

where inheritance shows more Mendelian basis. To date a limited amount of work on the inheritance of the anal spine has been published and little is known about the mechanisms that underlie the inheritance of this trait.

In this study fish from Loch Fada (Grid reference: NF891706) and the River Kelvin (Grid reference: NS570674) were used to investigate the evolution of spine loss in Scottish three-spined stickleback. Loch Fada is located on the Hebridean island of North Uist off the North West coast of Scotland (see Figure 6.3). The island has an area of 86,856 acres and is speckled with upwards of 180 lochs and lochans (small lakes) (Beveridge, 2001). The loch itself has a low pH, low calcium level and is oligotrophic (Giles, 1983). Stickleback predators include brown trout (*Salmo trutta*), slavonian grebe (*Podiceps auratus*) and larval and adult *Dytiscus* (Coleoptera) (pers. obs.). Fish at this site have very high levels of armour reduction with approximately 26% of the population being completely armour-less. None of the fish express a pelvic complex and only 13% have the normal three dorsal spines after which the species is named. In this system armour reduction may be a response to low calcium levels rather than predation regime (all predators being present). Reduction of body armour may limit the amount of Ca^{2+} ions lost from the body to the environment and decrease the overall amount of calcium required for the formation of a fish's bony components (Giles, 1983). In comparison, fish from most inland sites including our second study site (River Kelvin, NS570674) are robustly armoured and have the full compliment of spines, plates and girdle. The spines of the Kelvin fish are heavily barbed and a small number of individuals have a keel, normally associated with marine/anadromous stickleback.

QTL mapping of armour inheritance was restricted to the pelvic girdle complex, allowing me to answer a specific question. Is parallel evolution of pelvic complex loss in Scottish populations of three-spined stickleback due to mutation at the *Pitx1* locus or to a mutation in an upstream regulator of *Pitx1?* Due to the complex patterns of inheritance of other body armour traits and financial constraints, the inheritance of dorsal spines, lateral plates and the anal spine are described qualitatively.

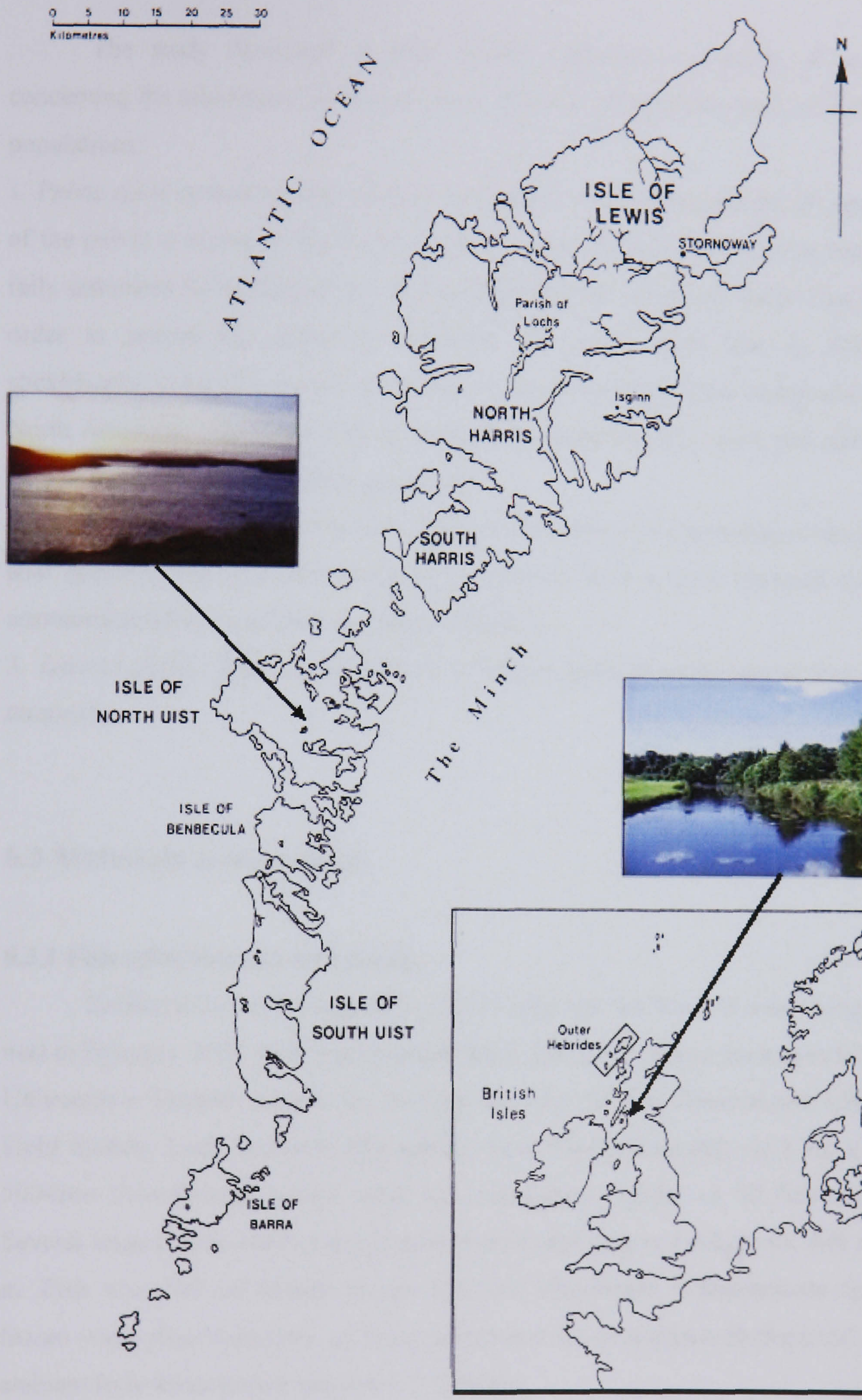

Figure 6.3 Map with the location of the two study sites. Left picture is Loch Fada and left picture is the River Kelvin. Inset is a line drawing of the British Isles with the Outer Hebrides boxed.

6.2 Aims

The study described in this chapter addressed a number of questions concerning the inheritance of armour loss in three-spine sticklebacks from Hebridean populations:

1. *Pelvic spine reduction*: The primary aim was to examine the pattern of expression of the pelvic complex in the F_1 and F_2 generations derived from a cross between a fully armoured River Kelvin fish and a completely un-armoured North Uist fish, in order to answer the following questions. Is pelvic spine loss in Hebridean sticklebacks, controlled by the same major gene controlling spine and girdle loss in North American populations? If so, using QTL mapping does spine and girdle loss map to the distal end of linkage group VII?
2. *Dorsal and anal spine reduction*: What is the pattern of expression of dorsal and anal spines in the F_1 and F_2 generations derived from a cross between the fully armoured and the completely un-armoured fish?
3. *Lateral plates*: What is the pattern of lateral plate development in these same crosses?

6.3 Materials and methods

6.3.1 Fish collection and husbandry

Sticklebacks were caught from Loch Fada and the River Kelvin using hand-nets in February 2003. Fish were transported in aerated 25 gallon buckets to Glasgow University's Scottish Centre for Ecology and the Natural Environment (SCENE) Field Station, Loch Lomond. Populations were held individually in 1.3m x 1.3m, 500-litre flow-through indoor tanks at a maximum density of 40 fish per tank. Several large plastic plants were placed in each tank and as a refuge for fish to hide in. Fish were fed *ad libitum* frozen and live bloodworm (*Chironomous* sp.) and frozen water fleas (*Daphnia* sp.) and maintained on an ambient photoperiod and at ambient loch water temperature (6 ± 2° Celsius).

6.3.2 Breeding programme

Males in breeding condition were moved to sandy-bottomed breeding tanks (45cm × 27cm × 15cm) with nesting material. After nest construction, a gravid female was placed in the tank and the pair left to breed naturally. After spawning, the female was removed and the fertilised eggs placed in an incubator and artificially oxygenated at 16°C. Males rebuilt a nest within one to three days and the same gravid female returned to the tank to breed again. After hatching, fry were fed Liquifry No1 (INTERPET) for one week and thereafter maintained on a mixture of enriched AF high-grade *Artemia* (INVE AQUACULTURE nv) and chopped bloodworm. At 8 weeks fry were moved to small holding tanks (25cm × 20cm × 45cm) and kept in family groups of 10 to 15 fish until maturity, when a number of fish were selected for further breeding.

6.3.3 Phenotype crosses and measurements

An armour-less female stickleback from Loch Fada was crossed with a robustly armoured male stickleback from the River Kelvin (see Figure 6.4). From this cross 40 F_1 progeny were generated. The F_2 generation was generated by four full-sib crosses, from fish that were first to come into breeding condition, one of which had a missing right pelvic spine, producing a total of 177 F_2 progeny (see Table 6.1). F_2 fish were sacrificed at week 24 post-hatching and preserved in 100% alcohol.

Counts of spines and lateral plates were made. Plate counts were made under binocular microscope (X10). First and second dorsal spine and girdle, left and right spine (pelvic complex) lengths were measured from the base of the spine to the tip. Measurements of dorsal spines, pelvic complex and standard body length (tip of snout to end of caudal peduncle) were made with callipers to the nearest 0.1mm.

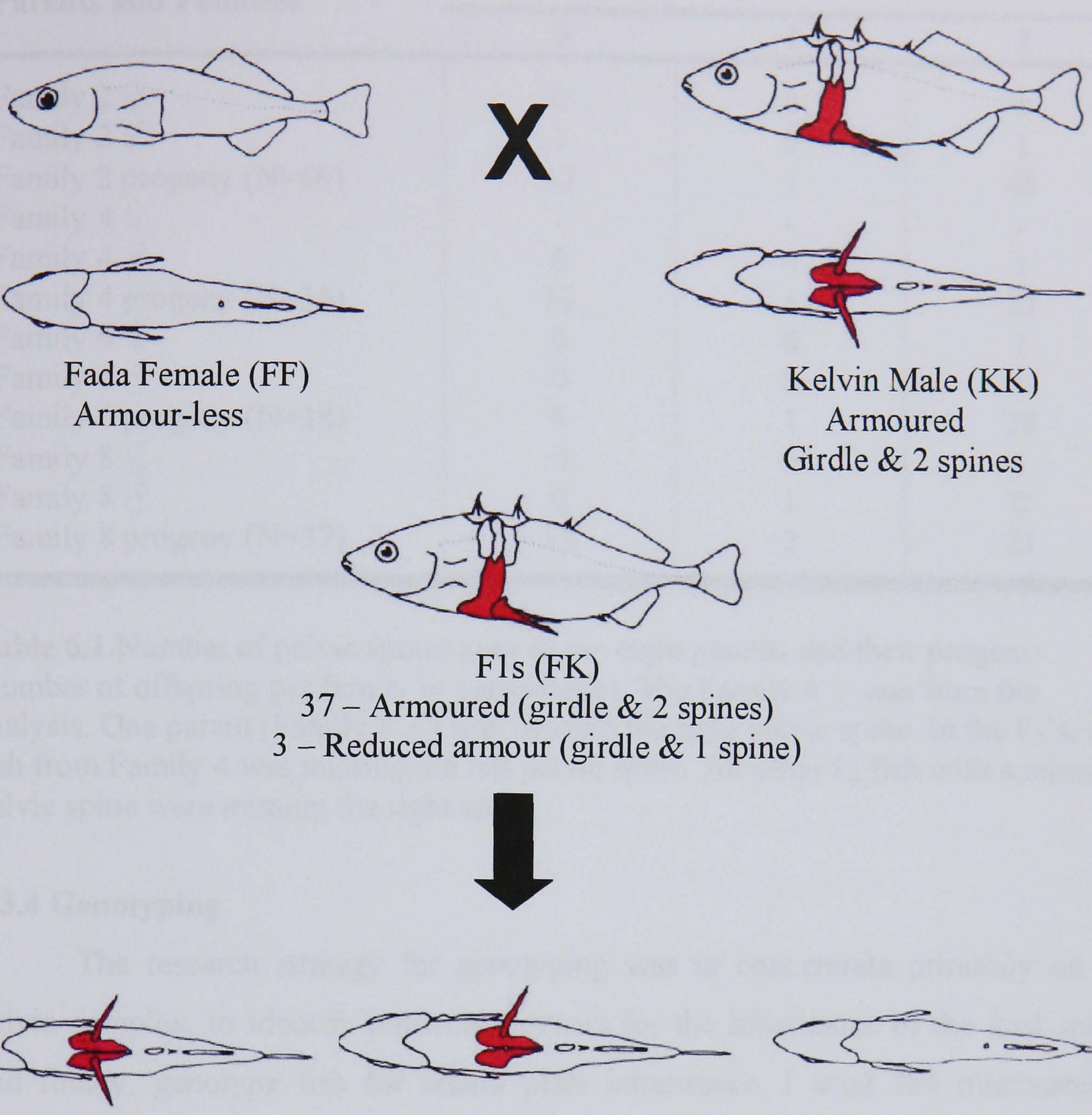

Figure 6.4 Scheme of Fada armour-less fish (alleles for pelvic armour loss = FF) crossed with a Kelvin armoured fish (alleles for pelvic armour = KK) used in the genetic analysis of inheritance of the pelvic complex. Of the 40 progeny in the F_1 generation, 37 have the same phenotype as the P_1 male and 3 F_1's have a pelvic spine missing. The F_2 generation has a 3:1 ratio of armoured to un-armoured fish (pelvic girdle present N = 127; pelvic girdle absent N = 50). Measurement of girdle complex illustrated on left picture of F_2 phenotype.

Parents and Families	Pelvic spine number		
	0	1	2
Family 2 ♀	0	0	1
Family 2 ♂	0	0	1
Family 2 progeny (N=66)	17	1	48
Family 4 ♀	-	-	-
Family 4 ♂	0	0	1
Family 4 progeny (N=36)	12	1	23
Family 6 ♀	0	0	1
Family 6 ♂	0	0	1
Family 6 progeny (N=38)	9	1	28
Family 8 ♀	0	0	1
Family 8 ♂	0	1	0
Family 8 progeny (N=37)	12	2	23

Table 6.1 Number of pelvic spines seen in the eight parents and their progeny (number of offspring per family in parenthesis). The Family 4 ♀ was from the analysis. One parent (Family 8 ♂) was missing the right pelvic spine. In the F_2's, one fish from Family 4 was missing the left pelvic spine. All other F_2 fish with a missing pelvic spine were missing the right spine.

6.3.4 Genotyping

The research strategy for genotyping was to concentrate primarily on the pelvic complex, to identify potential markers for the inheritance of the anal spines and finally, genotype fish for lateral plate inheritance. I used 384 microsatellite markers (previously utilised in the stickleback linkage map) as potential anal spine markers, but none were informative and so no further genotyping was carried out on this trait. Due to both financial and time constraints genotyping with markers for the *Eda* gene was not possible. However, since the Fada/Kelvin crosses displayed a simple pattern of girdle complex inheritance, QTL mapping concentrated on this aspect of armour inheritance.

Genotyping for girdle inheritance was carried out using six previously described and informative microsatellite markers. The markers (*Stn76*, *Stn257*, *Stn80*, *Stn82*, *Stn336/Pitx1*, *Stn342/Pitx1*) from linkage group VII were used to genotype 177 F2 progeny, as well as the grandparent and F_1 parents, using previously described PCR conditions (Peichel *et al.*, 2001). Allele sizes and segregation patterns were determined using ABI GeneMapper 3.7 (Applied Biosystems, Foster City CA). A map of linkage group VII was generated in JoinMap 3.0 (Stam & Van

Ooijen, 1995) using the default settings (LOD threshold of 4.5, significant at P > 0.002) and interval mapping of five pelvic traits (pelvic spine number, left and right pelvic spine length, girdle length and asymmetry) was performed in MapQTL 4.0 (Van Ooijen & Maliepard, 1996) using default settings.

6.4 Data analysis

6.4.1 Quantifying armour development

Eight measures were used to quantify armour status. These were; body size, presence or absence of pelvic spines, pelvic girdle length, length and symmetry of pelvic spines (see below), number and length of dorsal spines, lateral plate number and presence or absence of the anal spine (see Figure 6.5). P_1 grandfather only was used in the genetic analysis, as the grandmother was eaten by a mink. However, the armour phenotype of the P_1 female was known, so data from this fish were used in the analysis of spine, pelvic complex and lateral plate inheritance. Direct measurements of body length were used to look at the differences in standard length (SL) between grandparent, F_1 and F_2 offspring and between the four F_2 families. To account for size differences between individuals, the length of spines and girdle were each regressed onto standard length (tip of snout to end of caudal peduncle) and the residuals used in the analysis of spine and girdle lengths between generations and families.

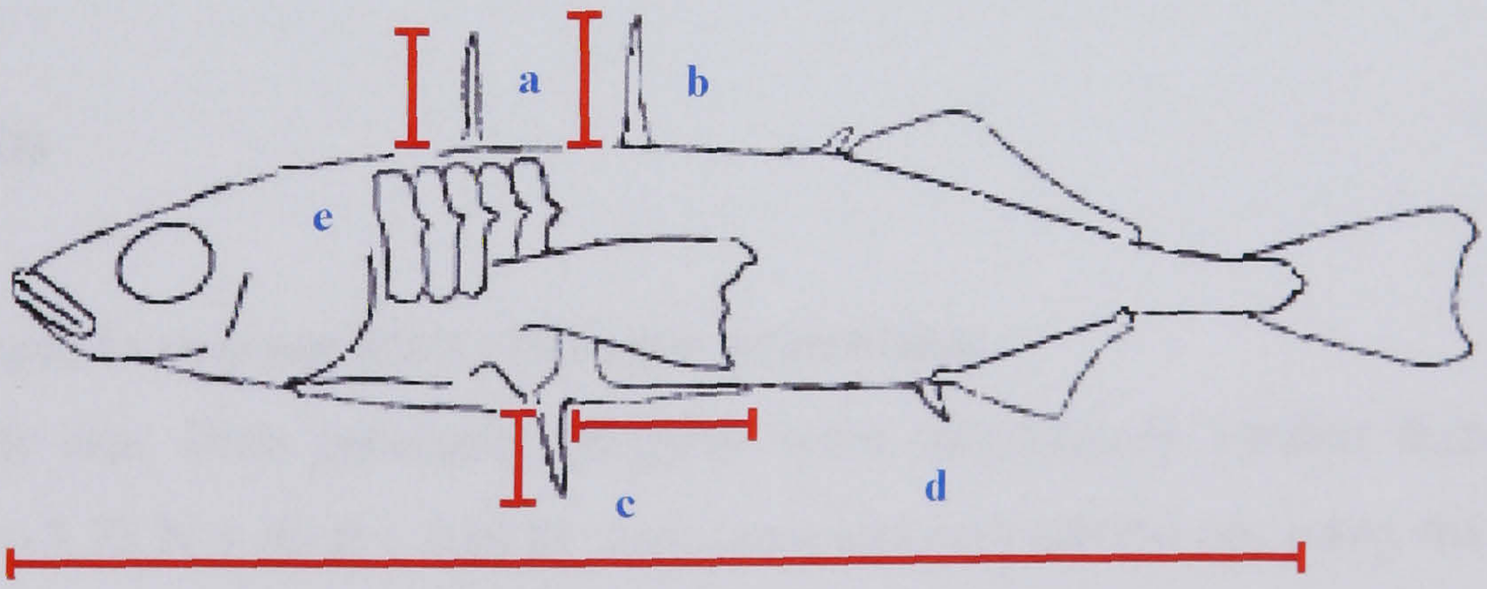

Figure 6.5 Schematic drawing (X3 normal stickleback size) of five quantitative measurements standard length, 1st and 2nd dorsal spine length, pelvic spine length and pelvic girdle length made of the pelvic complex, in red. Direct counts of spine number and plate number are in blue letters.

Absolute length of the right and left spines was used to investigate directional asymmetry between first and second generations and between families. Asymmetry was scored as the ratio of the length of the left spine to the length of right and left spines added together (L/L+R) (Shapiro *et al.*, 2004). All data sets were tested for normality with an Anderson Darling test and non-parametric tests used on non-normally distributed data.

6.4.2 Morphological differences between generations

A one-sample *t*-test was used to compare mean SL, mean girdle length, mean pelvic spine length (both spines), mean dorsal spine length (first and second) (the latter three corrected for body length), mean pelvic spine symmetry and mean lateral plate number in the F_1 and F_2 generations to the P_1 generation. In normally distributed data a two-sample *t*-test was used to compare mean spine lengths and mean plate number between F_1 and F_2 generations. Where data were non-normally distributed a Mann-Whitney U test was used.

6.4.3 Morphological differences between families

The presence or absence of pelvic spines between families was investigated with Chi-square analysis. Standard length was compared between families using Kruskal Wallis ANOVA. Differences in spine length (dorsal and pelvic spines) in the F_2 generation families, was explored using a One-way ANOVA and pelvic spine symmetry with a Kruskal-Wallis (data were not normally distributed).

6.5 Results

6.5.1 Changes in armour status between generations

Body size: First generation progeny were significantly smaller than the P_1 parent ($T = -3.73$ $N = 40$ $P < 0.001$). The same test was carried out using the second generation ($T = -24.32$ $N = 177$ $P < 0.001$) and confirmed the mean size of this generation was also smaller than the SL of the grandfather. As F_2 fish were culled at six months old this result was expected and validates correcting for size differences between generations.

Pelvic complex: Mean size-corrected pelvic girdle lengths (GL) in the first (N = 40) and second (N = 126) generations were compared to the size corrected GL of the P_1 parent and found to differ significantly (T = -7.92 P < 0.001 and T = -21.99 P < 0.001 respectively), decreasing across generations (see Figure 6.6). Between F_1 and F_2 fish, the difference was marginally non-significant (two-sample *t*-test; T = 1.75 DF = 55 P = 0.086). Only fish with a pelvic girdle was used in this analysis.

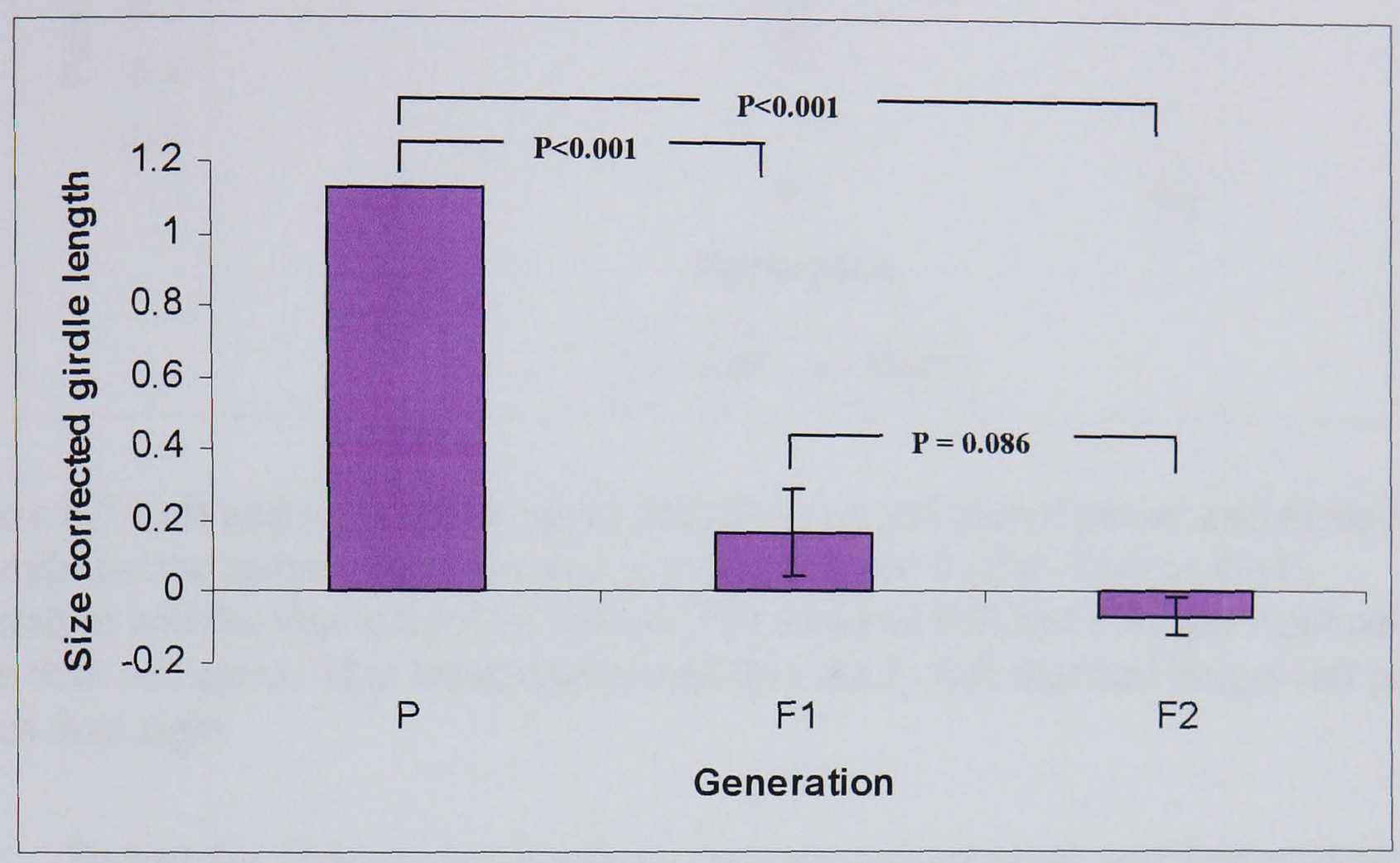

Figure 6.6 Body size corrected pelvic girdle length in the armoured parent and mean (including s. e.) pelvic girdle length in the 40 F_1 and 126 F_2 fish with a pelvic girdle present. Bars show significant differences between generations.

Before analysis of pelvic spine length was carried out, all body size-corrected left and right pelvic spines in the F_1 (N = 40) and the F_2 (N = 177) fish were plotted. The distributions were found to be bi-modal, in that fish grouped into spined or un-spined fish. Removal of the spineless fish normalised the distributions and *t*-tests were carried out on spined fish only. Residual mean spine lengths in each generation were individually compared to the P_1 parent in one-sample tests (see Figure 6.7). In all cases left and right spine lengths were significantly different between the P_1 and each filial generation (P_1 and F_1 left pelvic spine N = 37 T = -9.06 P < 0.001; P_1 and F_1 right pelvic spine N = 37 T = -9.70 P < 0.001; P_1 and F_2 left pelvic spine N = 121 T = -7.25 P < 0.001; P_1 and F_2 right pelvic spine N = 121 T = -10.18 P < 0.001). This was also true for left or right spine lengths between F_1 and F_2 progeny (T value = 2.88 DF = 80 P = 0.005; T value = 3.62 DF = 57 P = 0.001 respectively). The F_1 generation had the shortest pelvic spines and the P_1 parent the longest.

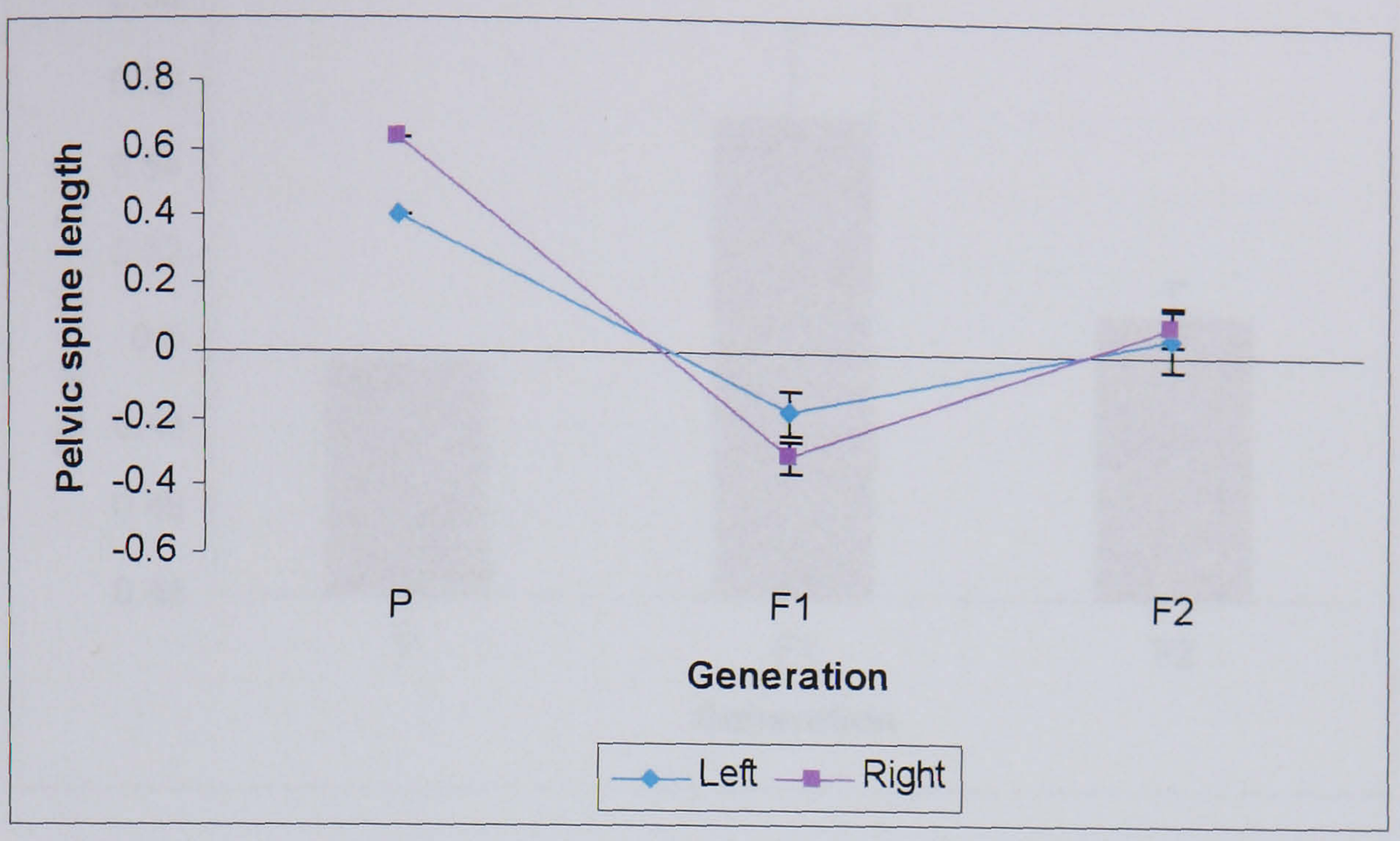

Figure 6.7 Left and right pelvic spine lengths in the armoured parent and mean left and right pelvic spines length (with s. e.) in the F_1 and F_2 fish. Fish in the F_1 generation had the shortest pelvic spines. The parental fish had a longer right pelvic spine than left spine. This trend was reversed in the F_1 fish that had longer left pelvic spines than right

To test for changes in symmetry between grandparent and first and second-generation fish, one-sample *t*-tests were carried out using fish with one or two spines. Spineless fish were not used in the analysis. A difference in asymmetry was detected between the F_1 generation and P_1 ($N = 40$ $T = 2.77$ $P = 0.009$), with the F_1 generation having higher levels of asymmetry than the parental generation. F_1 fish had longer left pelvic spines than right. However, no differences in asymmetry were found between the F_2 generation and P_1 parent. The first generation fish were significantly different in comparison with fish from the second-generation (Mann-Whitney U-test; $W = 0.12608$, $P < 0.001$), the F_2 fish showing lower levels of asymmetry in comparison to the F_1 fish (see Figure 6.8).

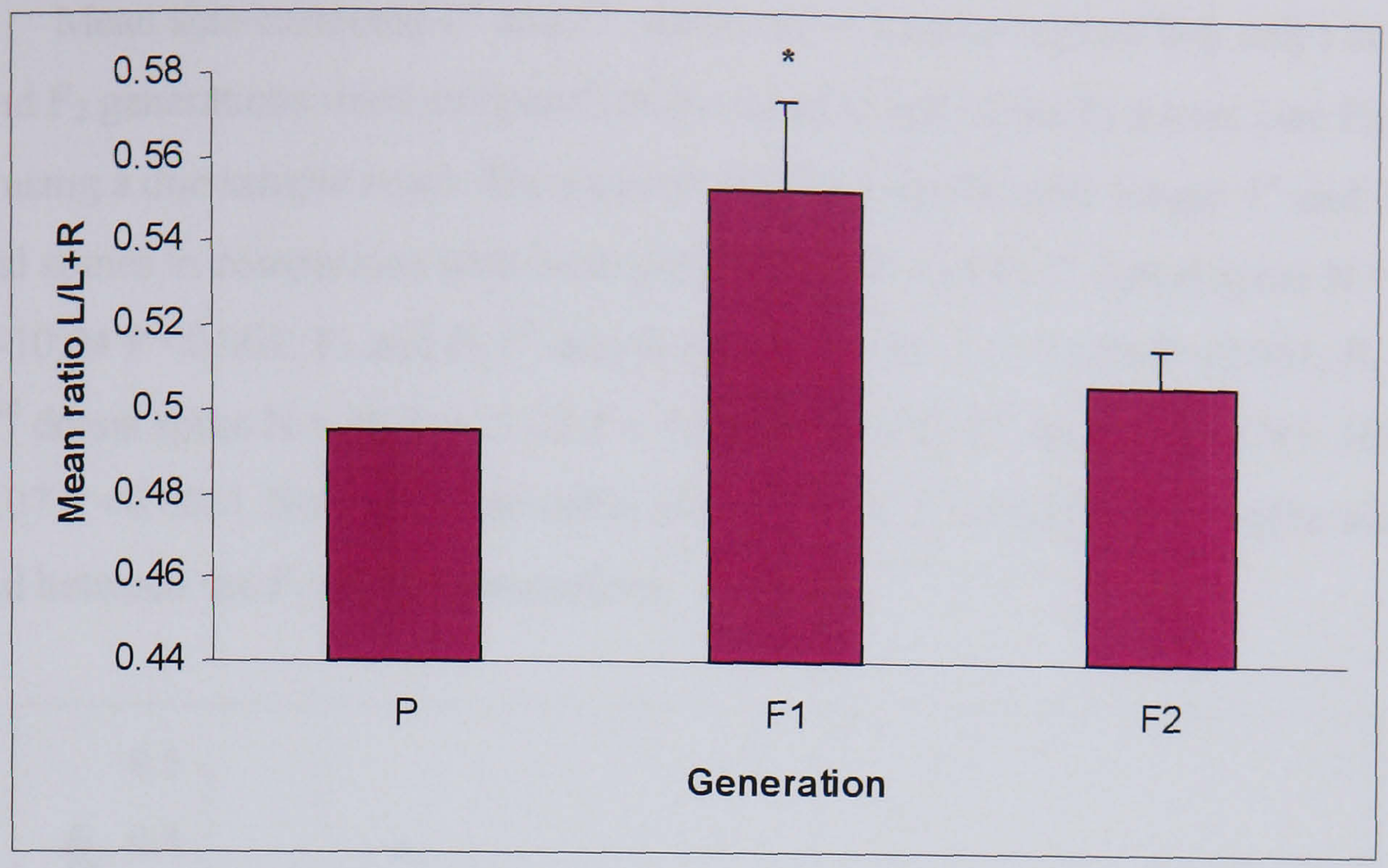

Figure 6.8 Spine asymmetry (L/L+R ratio) in all generations. Fish with a score of 0.5 are perfectly symmetrically, lower than 0.5 signifies that right spine is longer, higher than 0.5 signifies that left spine is longer. Asymmetry is highest in first generation individuals.

Dorsal spines: Between-generation differences in dorsal spine number can be seen in Table 6.2. In the parental generation fish had 0 or 3 spines (female and male respectively). The dominant phenotype in the F_1 was the male parental phenotype of 3 dorsal spines. Only three fish had a reduced number of spines (2). Again in the second generation, the most common phenotype is 3 dorsal spines. Around 10% of F_2 fish had 2 spines and one fish had only 1 spine. None of the fish exhibited the spineless phenotype of the grandmother.

		Dorsal spine number				**Anal spine number**	
	N	0	1	2	3	0	1
P_1 ♀	1	1	0	0	0	?	?
P_1 ♂	1	0	0	0	1	-	1
F_1	40	0	0	3	37	7	33
F_2	177	0	1	18	158	35	142

Table 6.2 Number (N) of fish in each generation. The number of fish with 0, 1, 2 or 3 dorsal spines, in each generation. The number of fish with or without an anal spine. Dorsal spine number in the P_1 ♀ was known but presence or absence of the anal spine was not known (signified by a question mark in the table).

Mean size-corrected 1st and 2nd dorsal spine lengths (spined fish only) in the F_1 and F_2 generations were compared to the spine length of the P_1 parent (see Figure 6.9) using a one sample *t*-test. The parental fish had significantly longer 1st and 2nd dorsal spines in comparison with both generations (P_1 and F_1 1st dorsal spine N = 38 T = -10.04 P <0.001; P_1 and F_2 1st dorsal spine N = 168, T = -12.88 P <0.001; P_1 and F_1 2nd dorsal spine N = 39 T = -3.22 P = 0.003; P_1 and F_2 2nd dorsal spine N = 167 T = -6.07 P <0.001). No significant differences in 1st or 2nd dorsal spine lengths were found between the F_1 and F_2 generations.

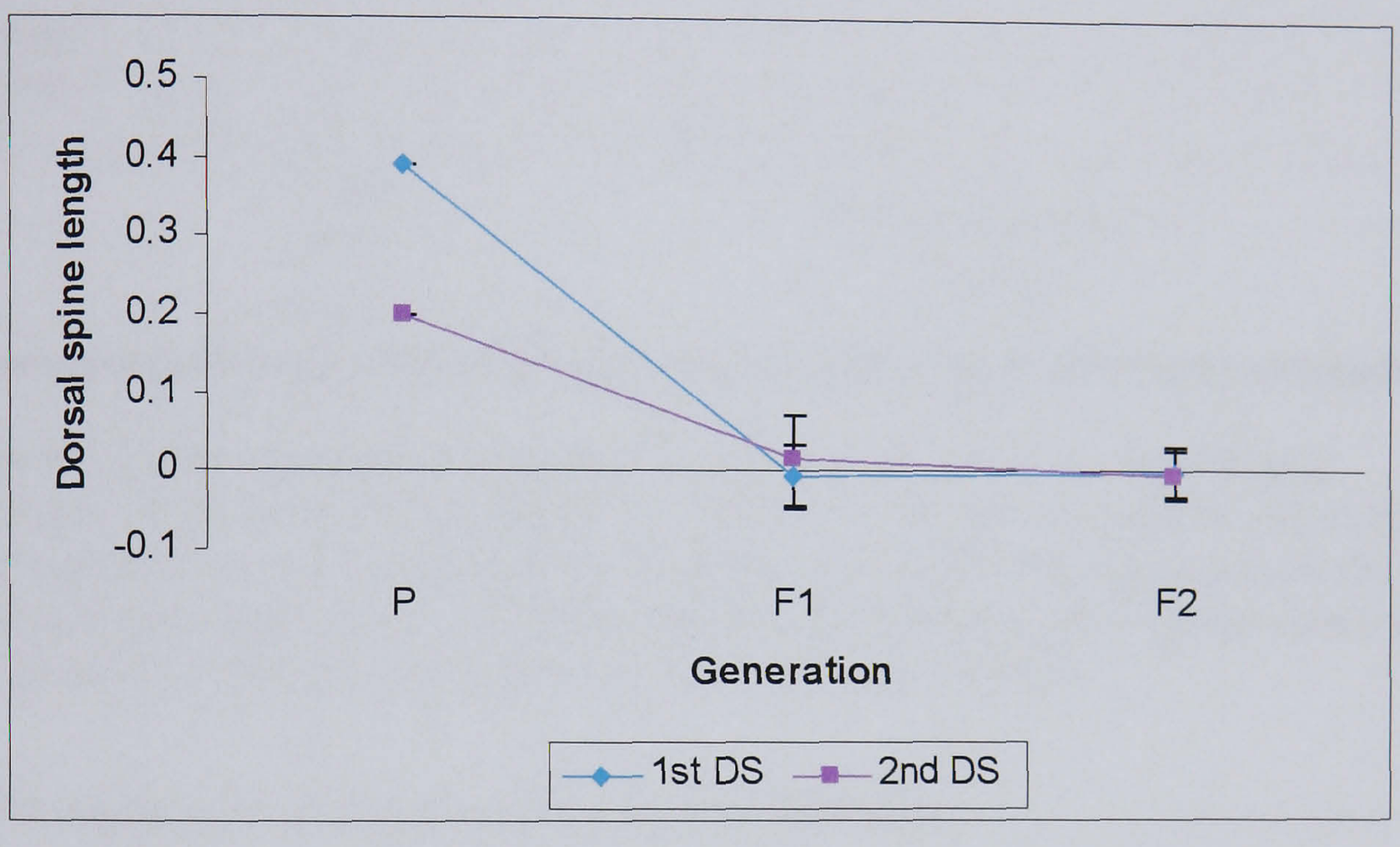

Figure 6.9 1st and 2nd dorsal spine lengths in the armoured parent and mean 1st and 2nd dorsal spine lengths (with s. e.) in the F_1 and F_2 fish.

Anal spines: In each generation approximately one quarter of the fish inherited an anal spine (see Table 6.2). The P_1 female phenotype was unknown, but the P_1 male had an anal spine.

Lateral plates (see Table 6.3): There were no significant differences in mean lateral plate number between generations. Interestingly, some F_2 individuals had three times more lateral plates than the plated P_1 grandfather (left plate N = 3 and right plate N = 4).

Generation	Lateral plate number											
	0	1	2	3	4	5	6	8	9	10	11	12
P_1	*			*	*							
F_1 L plate	0	2	6	15	9	8	0	0	0	0	0	0
F_1 R plate	0	2	4	16	8	9	1	0	0	0	0	0
Total	0	4	10	31	17	17	1	0	0	0	0	0
	Low plate morph Obs =14			Partial plate morph Obs = 66								
	0	1	2	3	4	5	6	8	9	10	11	12
F_2 L plate	16	6	24	36	56	27	8	2	1	0	1	0
F_2 R plate	9	8	18	39	50	26	13	2	0	1	0	1
Total	35	14	42	75	106	53	21	4	1	1	1	1
	Low plate morph Obs=91			Partial plate morph Obs=263								

Table 6.3 Table of lateral plate numbers in three generations. In the parental generation (lateral plates signified by *) the Fada ♀ did not have lateral plates and was classified as a low plated morph. The Kelvin ♂ had 3 left plates and 4 right plates and was classified as a partial plated morph. In the F_1 and F_2 generations there is an almost 3:1 ratio of partial plate morph to low plate morph.

6.5.2 Comparison of armour status between families

Body size (see Table 6.4 and Figure 6.10): A Kruskal-Wallis test of SL between F_2 families (N = 177) revealed significant differences between families (H = 31.55 DF = 3 $P < 0.001$). Longest fish were found in Family 4 (N = 36 Median = 38.85). The female parent of this family was missing but the male was the longest of all the male parents. The second longest was Family 8 (N = 37 Median = 37.70), Family 2 (N = 66 Median = 37.25) was next and the shortest fish were found in Family 6 (N = 38 Median = 33.15). *Post-hoc* tests identified Family 6 as being significantly smaller than all other families. However, the parents of this family were almost identical in length to the parents of Family 8 (see Table 6.4).

Pelvic complex (see Figure 6.4): A chi-square analysis of the four families in the F_2 generation showed that there was no significant difference in the proportion of spined to non-spined fish between families (0 or 2 spines). Additionally, a qualitative comparison of the number of fish with the normal pelvic complex as opposed to complex reduction or loss revealed a near Mendelian ratio of 3:1.

One-way ANOVA of F_2 family length corrected girdle length (N = 124) highlighted significant differences between families (F = 6.66, DF = 3, P <0.001) (see Figure 6.10). *Post-hoc* tests showed that Family 2 had the longest pelvic girdle, significantly so in comparison to Family 6 only (see above). In the parental fish, Family 8 parents had the longest and shortest girdle lengths (see Table 6.4).

A one-way ANOVA showed that there was a significant difference in mean pelvic spine length (corrected for length) between F_2 families. (Left spine, F = 6.14 DF = 3 P <0.001; Right spine, F = 6.75 DF = 3 P <0.001). Family 2 had the longest left and right spines and Family 8 had the shortest left and right spines (see Figure 6.11). Although Family 2 parents did have the longest right pelvic spines, the longest left pelvic spine was observed in Family 6 parents. Although the male parent of Family 8 had the right pelvic spine missing, the shortest right pelvic spine was observed in Family 4 female (see Table 6.4).

Parents	SL	PG	LPS	RPS	PGS	1st DS	2nd DS
Fam2 ♀	47.7	-0.68	0.39	0.53	0.505	0.085	0.128
Fam2 ♂	43.4	-0.49	0.54	0.55	0.511	0.408	0.015
Fam4 ♀	-	-	-	-	-	-	-
Fam4 ♂	46.5	-0.99	0.14	-0.42	0.550	-0.439	-0.663
Fam6 ♀	46.2	-0.14	0.78	0.51	0.526	-0.118	-0.036
Fam6 ♂	44.4	-0.85	0.72	0.32	0.534	0.112	-0.074
Fam8 ♀	46.3	1.03	0.17	0.40	0.500	-0.425	-0.055
Fam8 ♂	44.3	-2.23	-0.26	-3.76	1.000	0.019	0.034

Table 6.4 Table of morphological measurements of F_2 family parents. SL = standard length and all other spine lengths have been corrected for body length. PG = pelvic girdle, LPS = left pelvic spine, RPS = right pelvic spine, PGS = pelvic girdle symmetry, 1st DS = first dorsal spine, 2nd DS = second dorsal spine. Missing fish is identified with by a dash (-).

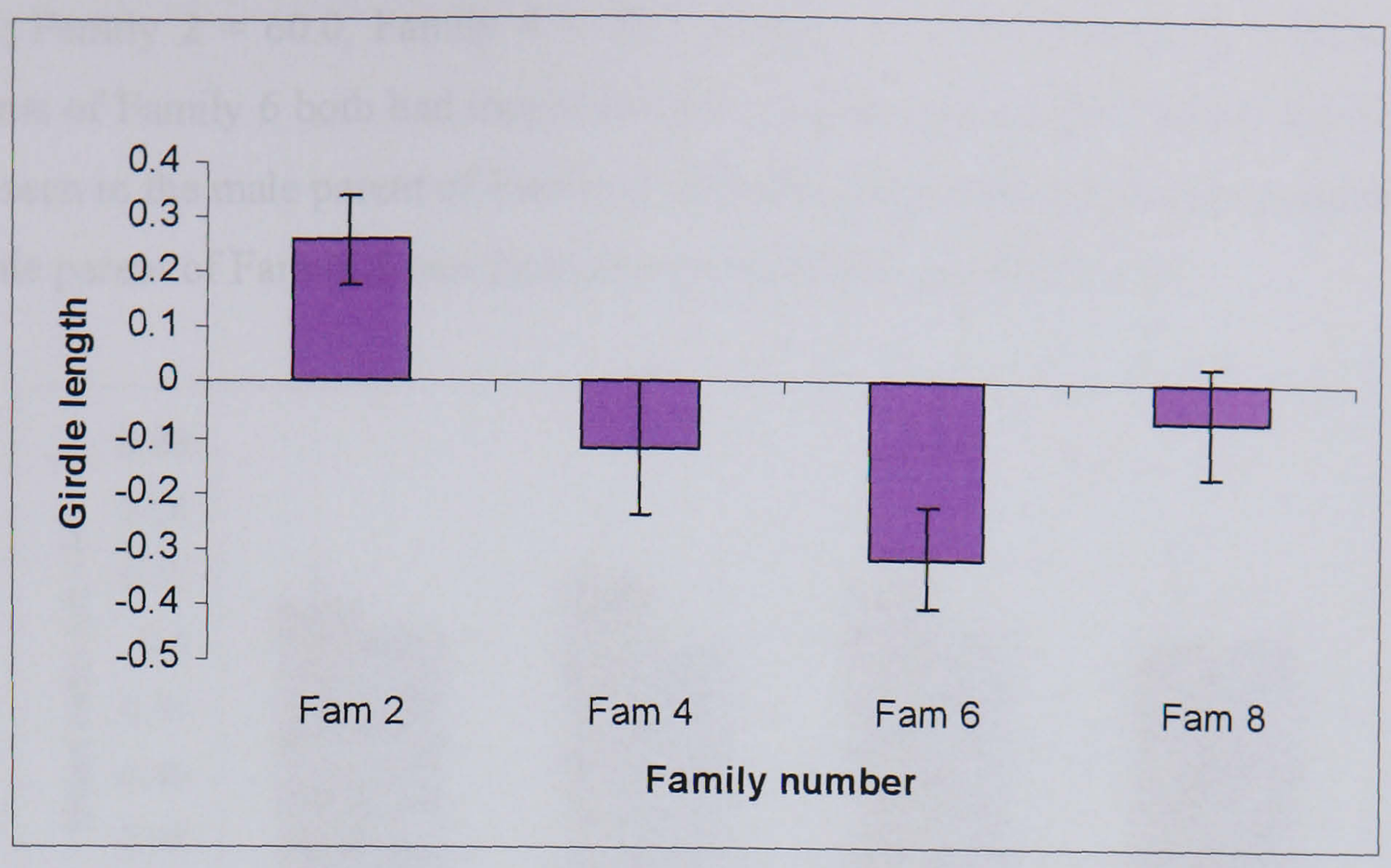

Figure 6.10 Mean size corrected pelvic girdle length (with s. e.) for four F_2 families. Longest girdle length is in Family 2 and the shortest in Family 6.

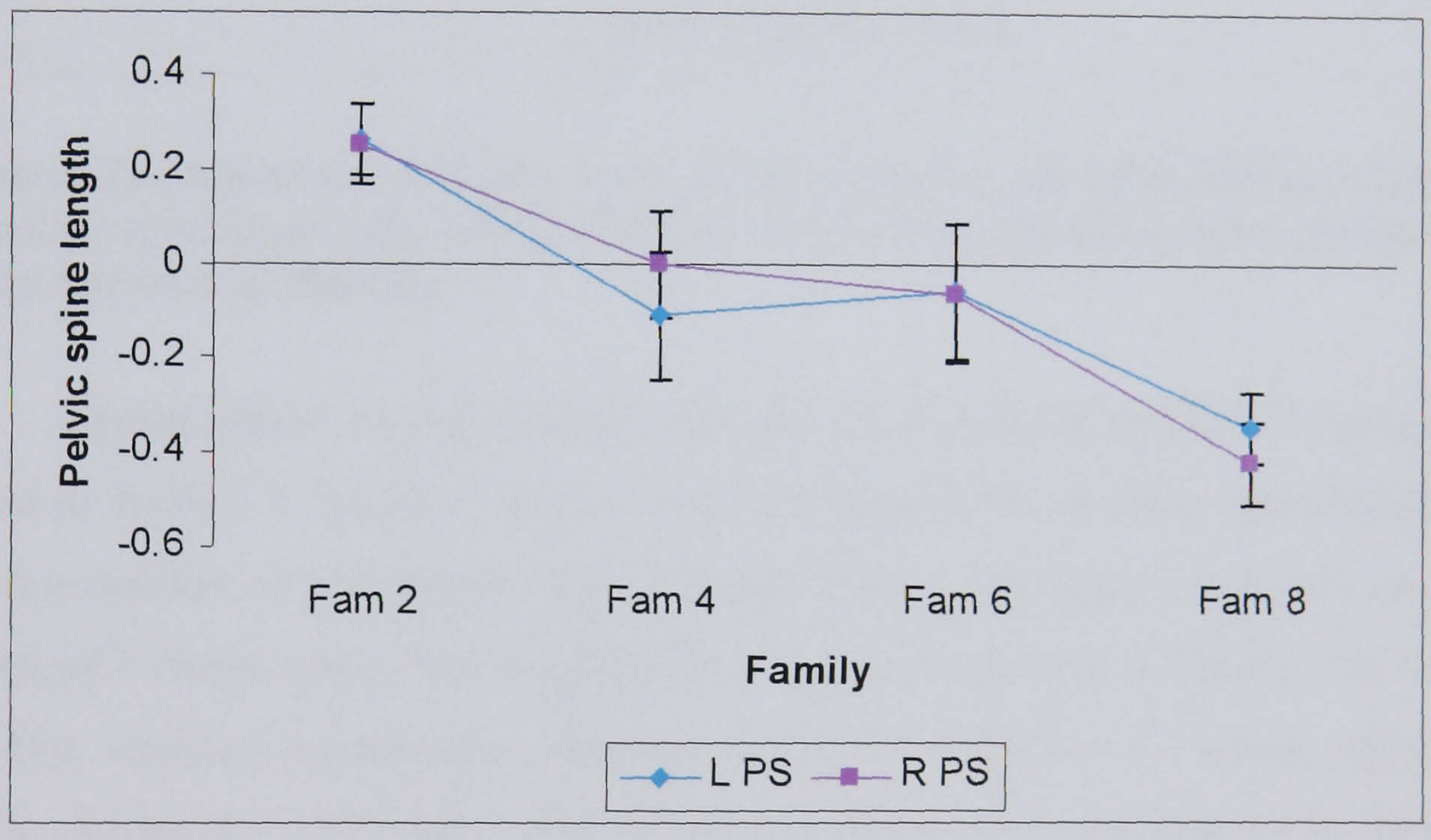

Figure 6.11 Mean size corrected pelvic spine lengths (with s. e.) for four F_2 families. Longest spines are seen in Family 2 and the shortest in Family 8.

Within the F_2 progeny there was a difference in pelvic spine symmetry (Figure 6.12) between families (N = 127) (H = 8.53, DF = 3, P = 0.036) and this was also seen with fish that had only two spines (N = 122) (H = 11.00, DF = 3, P = 0.012). *Post-hoc* testing could not identify which families were significantly different from each other however, the average rank data from the Kruskal Wallis test showed that Family 4 has the lowest level of asymmetry and Family 6 the highest (Average

rank; Family 2 = 60.0, Family 4 = 52.1, Family 6 = 74.6, Family 8 = 71.1). The parents of Family 6 both had longer left pelvic spines than right. Highest asymmetry was seen in the male parent of Family 8, this fish not having a right pelvic spine. The female parent of Family 8 was perfectly symmetrical (see Table 6.4).

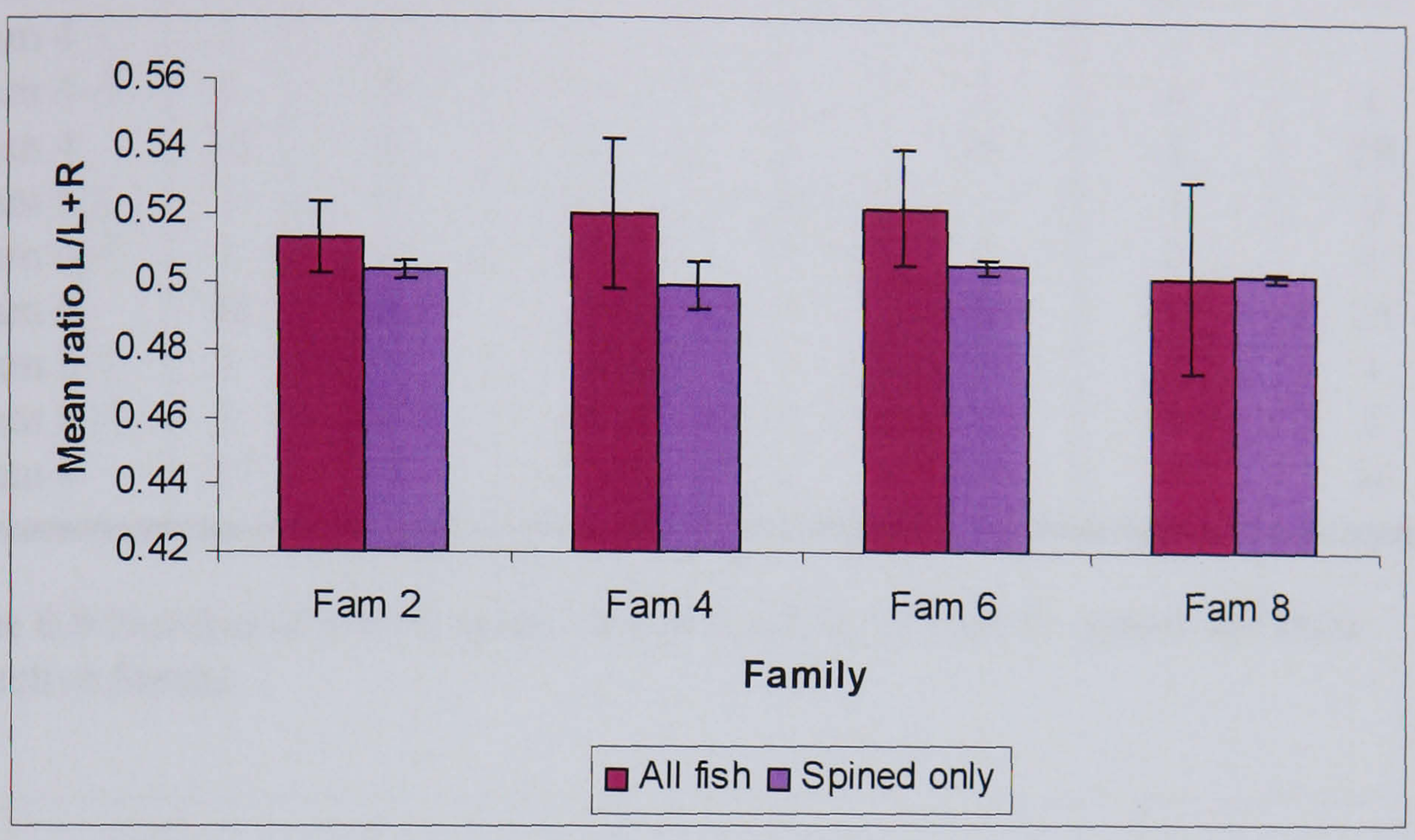

Figure 6.12 Asymmetry is higher in the all fish category, most fish having a longer left pelvic spine than right. Using only fish that have two spines present, symmetry is similar between all families.

Dorsal spines: Dorsal spine development in F_1 parents and their F_2 progeny is shown in Table 6.5. Family 6 was markedly different from the other three families in that the number of individuals with 2 dorsal spines was higher and only one fish expressed 1 dorsal spine. The length of the second dorsal spine (Figure 6.13), but not the first, differed significantly between families (ANOVA 2nd dorsal spine and family number $F = 7.70$ $DF = 3$ $P < 0.001$). Second spine was longest in Family 2 (Family 2 < Family 4 < Family 8 < Family 6) and the parents of this family also had longer second dorsal spines (Female 2nd dorsal spine = 0.128; Male 2nd dorsal spine = 0.015) (see Table 6.4).

		Dorsal spine number				Anal spine number	
	N	0	1	2	3	0	1
F_1 Fam 2 ♀	1	0	0	1	0	0	1
F_1 Fam 2 ♂	1	0	0	0	1	0	1
F_2 Fam 2	66	0	0	3	63	13	53
F_1 Fam 4 ♀	1	-	-	-	-	-	-
F_1 Fam 4 ♂	1	0	0	0	1	0	1
F_2 Fam 4	36	0	0	2	34	8	28
F_1 Fam 6 ♀	1	0	0	0	1	1	0
F_1 Fam 6 ♂	1	0	0	0	1	0	1
F_2 Fam 6	38	0	1	11	23	13	25
F_1 Fam 8 ♀	1	0	0	1	0	0	1
F_1 Fam 8 ♂	1	0	0	0	1	0	1
F_2 Fam 8	37	0	0	2	35	1	36

Table 6.5 Number of dorsal spines and anal spines of each F_1 parent and their respective family.

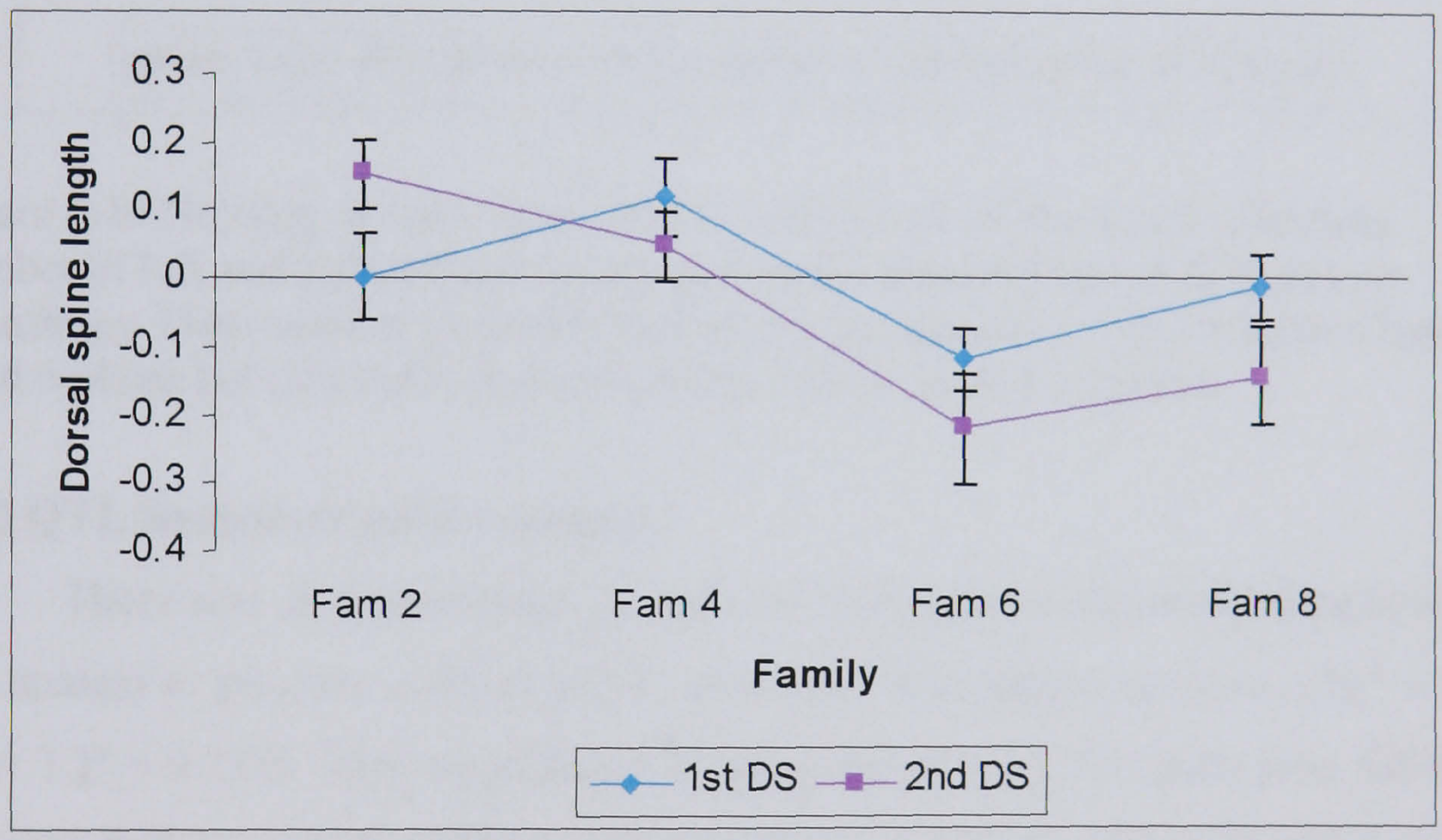

Figure 6.13 Size corrected mean first and second dorsal spine lengths for four F_2 families. Family 2 has the longest second dorsal spine and Family 4 has the longest first dorsal spine.

Anal spines (see Table 6.5): In two families (Family 2 and Family 4), a quarter of the fish did not have an anal spine. This was in common with anal spine inheritance between generations. However, Family 6 and Family 8 showed differing degrees of spine expression. In the former, half of all individuals had an anal spine while in the latter, only 1 of 36 fish had a spine. Both parents of Family 2 and Family

8 had an anal spine but only the male parents in Family 4 and Family 6 had a spine (Family 4 female parent was missing and so anal spine was unknown).

Lateral plates: Figure 6.14 shows the number of lateral plates in each of the F_2 families. Interestingly, all families had higher numbers of plates than their parents with some individuals expressing 8 or more plates.

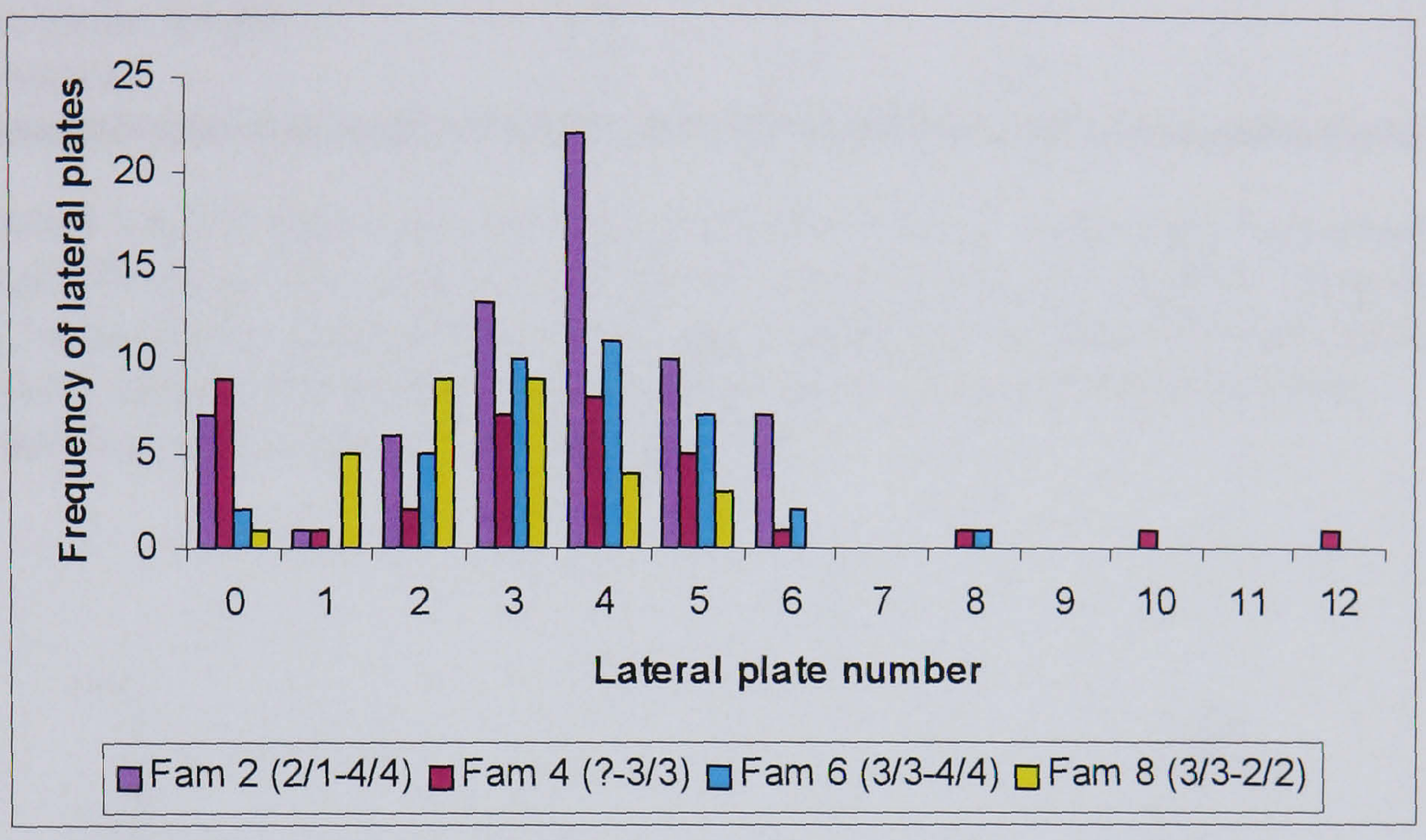

Figure 6.14 Number of right lateral plates within each of the four F_2 families. Number of left and right plate number seen in the parents (female & male), in parenthesis. Plate number in Family 4 mother was unknown. Most fish have between 2 and 6 plates but two individuals in Family 4 have 10 and 12 plates.

6.5.3 QTL analysis of pelvic complex

There was an approximate 3:1 ratio of F_2 progeny with pelvic spines (either 1 or 2 spines) to progeny without pelvic structures (Chi square analysis, $Chi^2 = 0.996$ DF = 1 P = 0.318). This suggested a simple genetic basis for spine loss. Of the F_2 fish that had no pelvic complex expression (N = 50) all fish inherited two Fada alleles (FF) at the *Pitx1* markers (see Figure 6.4). Interval mapping showed that between 85.1 – 96.6% of the variance in pelvic girdle size, pelvic spine size and spine number was explained by the *Pitx1* locus, with LOD scores of between 72.0 and 129.3 (see Table 6.6 and Figure 6.15). The *Pitx1* locus explains 38% of the variance in pelvic spine asymmetry, with a LOD score of 13.2.

Trait	LOD	PVE	Phenotype means		
			KK	FK	FF
Pelvic spine number	129.3	96.6	2.00	1.96	0.04
Left pelvic spine length	73.4	86.0	4.29	3.69	0.08
Right pelvic spine length	72.0	85.1	4.29	3.58	0.00
Pelvic girdle length	73.4	87.0	5.50	5.13	0.10
Asymmetry	13.2	38.0	0.50	0.51	1.00

Table 6.6 Log likelihood ratio of linkage (LOD) score for each of the 5 measured traits (LOD score >3.0 is significant), percentage variance explained (PVE) by the *Ptix1* locus and the mean phenotype values for each trait for fish that inherited alleles two *Pitx1* alleles from the Kelvin grandfather (KK), two alleles from the Fada grandmother (FF) or one allele from each (FK).

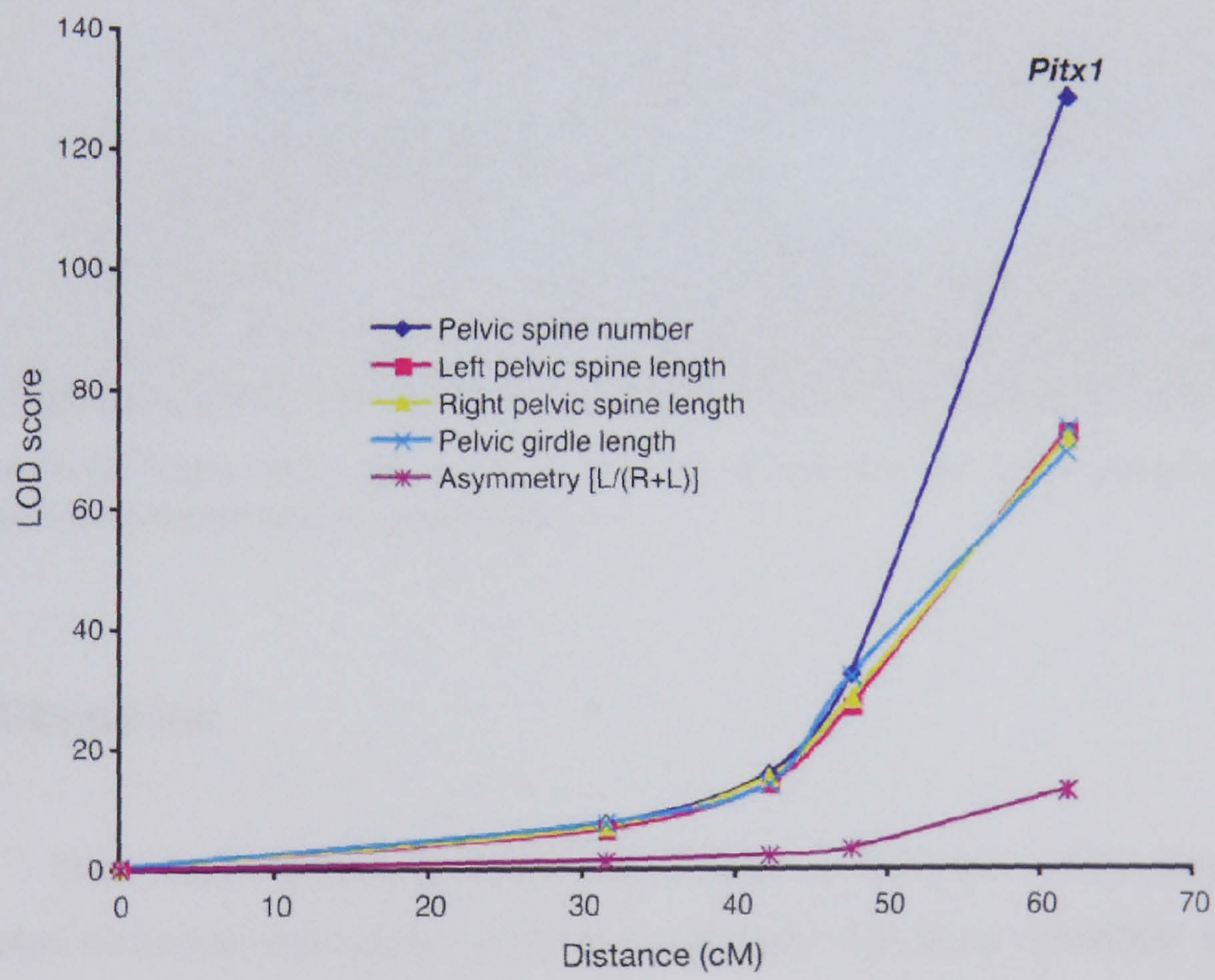

Figure 6.15 LOD scores are plotted as a function of the position in centiMorgan (cM) of markers: *Stn76* (0.0cM), *Stn257* (31.5cM), *Stn80* (42.2cM), *Stn82* (47.6cM), *Stn336/Pitx1* (61.8cM) and *Stn 342/Pitx1* (61.8cM), indicated by points along the curve.

6.5.4 Stumpies

In two of the study families (2 and 8) between 15% and 30% (respectively) of the offspring in each family were categorised as 'Stumpies' (see Figure 6.16). Fish have a highly truncated body and swim in a jerky manner. However, these fish feed well and behave in a normal manner. In the breeding season males exhibited normal nuptial colouration, blue eyes and red throats, and made nests. They also became highly aggressive towards conspecifics, defending their nests vigorously. Females did not develop eggs. Most fish have the normal compliment of three dorsal spines and two pelvic spines but a number of fish are spine reduced (Figure 6.16).

Figure 6.16 Picture of a dead stumpy. The fish is truncated but has 3 dorsal spines, a full pelvic complex and 4 lateral plates.

6.6 Discussion

This study addressed three interconnected questions concerning pelvic complex reduction and loss in the Fada population, plus three subsidiary questions regarding dorsal spine, anal spine and lateral plate inheritance. To achieve this, I chose fish with dissimilar phenotypes and from markedly contrasting environments, oligotrophic low calcium (5mgCa/L) versus mesotrophic, higher calcium (25mgCa/L). Complementation crosses between armour-deficient fish from Fada and armoured fish from Kelvin and a genetic mapping study has allowed me to answer the three main questions. Qualitative measures of spines and plates allowed me to

investigate and make hypotheses on the inheritance of armour, in these Scottish armour-reduced populations. The subsidiary questions will be address first followed by a discussion of the inheritance of pelvic complex reduction.

6.6.1 Pattern of dorsal spine inheritance

The number of dorsal spines in the first and second generation was similar, with approximately 10% of fish having two dorsal spines and 90% expressing the full compliment of three spines. In fish with two spines, there was an almost even distribution of fish having lost the first spine to those that had lost the second (2^{nd} and 3^{rd} spine only N = 8; 1^{st} and 3^{rd} N = 9). Interestingly, although the P_1 female had no dorsal spines, none of the F_1 or F_2 fish showed complete dorsal spine loss and only one fish in the F_2 generation had one spine. The pattern of dorsal spine inheritance suggests a complicated polygenic mode of inheritance influenced by several QTL is responsible for this trait, rather than a simple Mendelian mode of inheritance. This finding is in common with that described by Peichel *et al* (2001) and Summers (pers. comm.).

6.6.2 Pattern of lateral plate inheritance

Parental fish had a minimum of 0 plates (low plate morph) and a maximum of 4 plates (partial plate morph). In their F_1 progeny, plate number range increased from a minimum of 1 plate to a maximum of 6 plates with a 3:1 ratio of partial to low plated morphs, indicating that lateral plate inheritance may be under control of one major locus. In the second generation there was a marked increase in the number of plates. The minimum plate number of 0 mirrored that seen in the P_1 maternal fish, the maximum plate number [(11) seen in Family 4] was almost three times higher that that seen in the paternal fish. It is interesting that in wild caught River Kelvin fish, 4 individuals had up to 15 plates (see Chapter 3). Partial versus low plated morphs in the F_2 generation, showed an almost 3:1 ratio frequency (see Table 6.2). This finding is similar to that of Colosimo *et al.* (2004), who found that lateral plate number was under the control of one major chromosome region on linkage group IV. They also found that these were three further QTL of minor effect that increased plate number in partially plated individuals.

6.6.3 Pattern of anal spine inheritance

The pattern of inheritance is complicated and little information is available in the published literature to aid with its interpretation.

6.6.4 Pattern of pelvic complex inheritance

Lack of the pelvic complex is the principle phenotype in wild caught Fada fish, no fish possessing a girdle or pelvic spines. In contrast, fish from the River Kelvin have a full pelvic complex. My data suggests that these differences have a strong genetic component and persist between same phenotype crosses made and reared in the laboratory. Low levels of asymmetry are common in Kelvin wild caught fish and indeed the Kelvin grandparent had a slightly longer right pelvic spine (+0.1mm) than left. In common with other studies, I found that the inheritance of the pelvic complex had a Mendelian component. In the F_1 generation, heterozygous individuals had a similar phenotype to the fully armoured parent. In the F_2 progeny there was a ratio of 3:1 armoured versus unarmoured fish and this did not differ significantly between the four families.

There is a clear relationship between girdle size and generation. Across successive generations the size of the girdle fell, the effect of Fada alleles inherited from the girdle deficient grandmother. A similar pattern is also seen in other complementation crosses between different populations (Peichel *et al.*, 2001; Shapiro *et al.*, 2004). The pattern is more complicated between F_2 families. There are significant differences in girdle size between two of the families. Family 2 had a significantly larger mean girdle size than Family 6. A tank effect can be ruled out, since all families were kept in smaller family groups of 10 to 15 fish, fed to satiation and held under the same environmental conditions. Differential inheritance of smaller effect modifier genes (minor modifier qualitative trait loci QTL) may be the most likely explanation (Shapiro *et al.*, 2004), although girdle length does not relate in any simple way to parental girdle length, both Family 2 parents having shorter girdles than the female parent of Family 6.

The pattern of spine development across generations is unexpected as spine length is shortest in the F_1 not the F_2 generation and the difference is significant. Again this may be due to the effect of minor QTL inherited from either the spined grandfather or the spine reduced grandmother, but this cannot be resolved without further analysis of these chromosome regions. Again, between-family differences are

apparent, Family 2 having the longest pelvic spines and Family 8 the shortest. This may again be due to differential inheritance of minor modifier QTL but once more the relationship between offspring and parent is complicated. Although the parents of Family 8 did have short spines, the longest pelvic spines were seen in Family 6.

6.6.5 Is pelvic spine loss in Fada fish, controlled by the same major gene controlling spine and girdle loss in North American populations?

Initially identified as a candidate region by Cole *et al.* (2003) using Uist fish and confirmed by Shapiro *et al.* (2004) working primarily on North American and Icelandic fish, the major locus for pelvic reduction and loss seems to be *Pitx1*. However, the gene itself is not modified in phenotypically different population; rather *cis* regulatory components (control regions on the same DNA strand as the *Pitx1* gene) have been modified and this is most likely the underlying cause for pelvic reduction. An indicator of the involvement of the *Pitx1* locus is the asymmetry seen in pelvic reduction. In this study I found that there is indeed directional asymmetry in pelvic spine length, with the right shorter than the left. However the asymmetry is not as strikingly directional as that reported in other populations e.g. mean spine length ratio L/L+R=0.719 (Shapiro *et al.*, 2004). The results in the present study show that the first generation has a higher degree of asymmetry (mean left to both spines ratio of 0.52) than the second (0.502). The results in which this study may be viewed as a further indicator that *Pitx1*, is the prime candidate for pelvic complex reduction.

6.6.6 Using QTL mapping, does spine and girdle loss map to the distal end of linkage group VII?

Girdle loss maps to the distal end of linkage group (LG) VII, the locus responsible for the Mendelian inheritance of pelvic complex reduction. The odds that two gene loci lie close to each other and are therefore liable to be inherited together are 1000 to 1 in favour of linkage at a LOD score of 3. Three markers for pelvic reduction have LOD scores below 3 and lie at the proximal end of LG VII, but moving towards to the distal end the LOD scores increase to over 70 for the two *Pitx1* markers. Coupled with previously published work which demonstrated that *Pitx1* was not expressed in spine-deficient fish (Cole *et al.*, 2003), these mapping data provide compelling evidence that mutations in the *Pitx1* gene or at a tightly

linked gene regulating expression of *Pitx1* are principally the cause of pelvic reduction in the Fada population. It is interesting then that the same locus for pelvic reduction reported in North American and Icelandic populations, is inherited in Scottish populations, even though these populations are geographically distant and under different selection pressure.

Chapter 7. Genetic variation between populations of three-spined stickleback (*Gasterosteus aculeatus*): status of armour-reduced populations

7.1 Introduction

7.1.1 Post-glacial radiation of freshwater fishes

During the last 2 million years, the Earth has been subject to a series of thermal cycles that have had a major impact of the distribution of many species. During periods of warming, plants and animals expanded their geographical ranges and species richness and endemism increased, but in cooling periods, geographical ranges contracted as more habitats at higher latitudes disappeared in the face of advancing glaciers (Hewitt, 2000). During major glaciations, species went extinct, retreated to new locations or survived in refugia. Those that remained in refugia stayed there until the ice and permafrost retreated after which they were able to expand and repopulate inhospitable northern areas once again (Hewitt, 1999, 2000).

In Northern Europe during the last glacial period (24,000 to 10,000bp) the ice sheet divided North and South into two distinct areas. In the northern part, regions were covered with permafrost or ice. In contrast southern regions, particularly around the Mediterranean Sea and the Balkans (hereafter referred to as the Mediterranean region) and as far east, as the Caspian Sea, were largely unaffected (Hewitt, 2000). Consequently, many species found refuge in and around the Mediterranean region and post-glacial dispersals after the ice sheet retreated can be traced from here (Hewitt, 1999). However, although numerous plants, animals and birds migrated from the Mediterranean region, freshwater fishes faced a particular problem in that they were confined to inland drainage systems, being unable to disperse through the sea. Consequently, postglacial colonisation of freshwaters in more northerly regions such as the British Isles or Iceland (and also in North America), were undertaken by fish that could tolerate both fresh and saline waters, for example, salmonids and gasterosteids (Gíslason *et al.*, 1999; Taylor & McPhail, 1999; Brunner *et al.*, 2001; Reusch *et al.*, 2001; Kristjánsson *et al.*, 2002; Wilson *et al.*, 2004).

Having successfully invaded these newly-created freshwater habitats, pioneers encountered little competition for resources and the radiation of morphology (Gíslason *et al.*, 1999; Kristjánsson, 2005), colouration (Adams *et al.*, 1998) and behavioural traits (Foster, 1995) has in geological terms been rapid. The profuse diversification among populations of the same species has important implications in both theoretical and practical terms. Theoretically, these populations offer an unparalleled opportunity to examine incipient speciation (Skúlason & Smith, 1995); practically from a conservation point of view, they allow enquiry into what the unit of conservation should be. With the advent of genetic molecular tools such as neutral microsatellite markers (Kingsley *et al.*, 2004), it is now possible to investigate the genetic variation between such geographically distinct populations (Storz, 2005).

In the three-spined stickleback, the colonisation of freshwater habitats is thought to have occurred by invasion of migratory marine sticklebacks (Hagen & McPhail, 1970). In the breeding season, marine sticklebacks move into brackish water to mate and produce offspring, after which they return to the sea (Wootton, 1976). However, a number of individuals are thought to have remained and moved from brackish to freshwater, ultimately establishing new populations. Many of these populations became isolated in lochs as a result of isostatic rebound and gene flow with either the ancestral population or geographically close neighbouring populations ceased. Consequently adaptations to local environmental conditions lead to population differentiation (Kristjánsson, 2002).

Several studies on the genetic diversification in three-spined stickleback have recently been published. For example, Reusch *et al.*, (2001) used 7 microsatellite loci among 16 populations to explore common ancestry, geography and genetic divergence in German river, lake and estuarine postglacial populations. They used F_{ST} ratios to summarise population structure. The F-statistic is a measure of population substructure that does not make assumptions about population size, sample number or frequency of heterozygotes (Weir & Cockerham, 1984). To quote Hartle & Clark (1997) "the fixation index F_{ST} compares the least inclusive to the most inclusive levels of the population hierarchy and measures all effects of population sub-structures combined". It is derived from the equation $F_{ST} = (H_T - H_S)/ H_T$. Were H_T is the average heterozygosity among organisms (in Hardy-Weinberg Equilibrium) within the total area and H_S is the average heterozygosity among

organisms (in Hardy-Weinberg Equilibrium) within randomly mating subpopulations. Reusch *et al.*, (2001) found that the genetic divergence of the Germany populations was correlated with habitat type (lake, river or estuary) and weakly with geographical distance (Mantels test; correlation analysis of distance matrices of pair-wise comparisons e.g. lake vs. river $F_{ST} = 0.18$). A similar but larger study carried out by Mäkinen *et al.* (2006) at 74 marine and freshwater locations and using 18 microsatellites found the reverse, with genetic variation correlating weakly with geographical regions and habitat type having a negligible effect ($F_{ST} = 0.11$-0.30).

Due to the immense phenotypic variation seen in the three-spined stickleback, it was originally classified as 40 different species. For example, behaviourally the species exhibits great variation in anti-predator responses (McLeod & Huntingford, 1994), aggression (Bakker, 1994) risk-taking (Huntingford, 1976; Bell, 2005) and physiologically, sticklebacks differ in their immune response to parasitic infection (Kalbe & Kurtz, 2006). However, the most striking feature of three-spined stickleback is the diversity in body armour. In marine sticklebacks fish are heavily armoured, with three dorsal spines, a robust pelvic girdle with two pelvic spines and a continuous of row of bony lateral plates extending from the head to the tail. Anadromous sticklebacks are less heavily armoured, having a reduced number of lateral plates (Campbell, 1985). However, the greatest variation in armour is seen in freshwater populations where sticklebacks may have 0 to 3 dorsal spines, reduced or absent pelvic girdle complex and 0 to 20 plates (Bell & Foster, 1994). The reduction of skeletal armour has evolved in several locations around the Northern hemisphere including North America (Moodie & Reimchen, 1976; Cresko *et al.*, 2004), Iceland (Kristjánsson *et al.*, 2002) and Scotland (Campbell, 1984) and in a small number of populations completely armour-less fish have been recorded (Giles, 1983).

The Outer Hebridean islands off the West coast of Scotland (UK) are home to a number of spine-reduced populations of three-spined stickleback. One location in particular, Loch Fada, has attracted the attention of several researchers (Giles, 1983; Cole *et al.*, 2003; Shapiro *et al.*, 2004; Coyle *et al.*, 2007) as being one of the few locations where completely armour-less stickleback are found. It is in our interest to know whether fish from this loch are genetically distinct from fish in geographically close proximity, where armour reduction is also prevalent. And if so, should Loch Fada be afforded a higher conservation status than other nearby loch? Before these

questions can be addressed, the genetic structure of the Uist populations must first be classified. I compared Uist populations, in relation to genetic distance, geographical distance, morphological distance and an ecological parameter, in this case calcium concentration, to a population on the mainland and populations on other islands.

7.2 Aims

The main aim of this study was to examine genetic differentiation in eight populations of three-spined stickleback from geographically differing locations, using 16 microsatellite markers. Six populations were located on the Outer Hebrides, one on the Orkney island of Stronsay and one on the Scottish mainland. A subsidiary aim was to explain any interpopulation genetic differences in terms of biological or environmental distances.

7.3 Material and methods

7.3.1 Study sites

Eight populations were chosen from locations ranging in geographical distance from each other (see Figure 7.1). All sites except one were located along the Western coast of Scotland. Beginning at the most southerly site, the River Kelvin was considered as kilometre 0 (see Table 7.1). Fish from this site were normally armoured, some possessed a keel and water calcium level was regarded as high. At kilometre 250 on the Outer Hebridean island of South Uist, fish from Loch Druidibeg were almost all fully armoured although one fish did not have a pelvic complex and another had 4 dorsal spines. Calcium level was low at this site. On the Isle of North Uist there were four study sites (see figure 1.1, Chapter 1), Lochs a'Bharpa, Scadavay, Fada and Grogary (kilometre 270, 275, 280 and 280 respectively) and fish from the first three sites were armour-reduced. Almost none of the fish sampled possessed lateral plates or pelvic complex, and at Loch Fada, approximately one third of the sampled fish were completely armour-less. All three sites were categorised as low calcium. Conversely, Loch Grogary was a high calcium

site and fish here were normally armoured. At Kilometre 345, Loch Charrasan on the Isle of Lewis, fish were armour reduced.

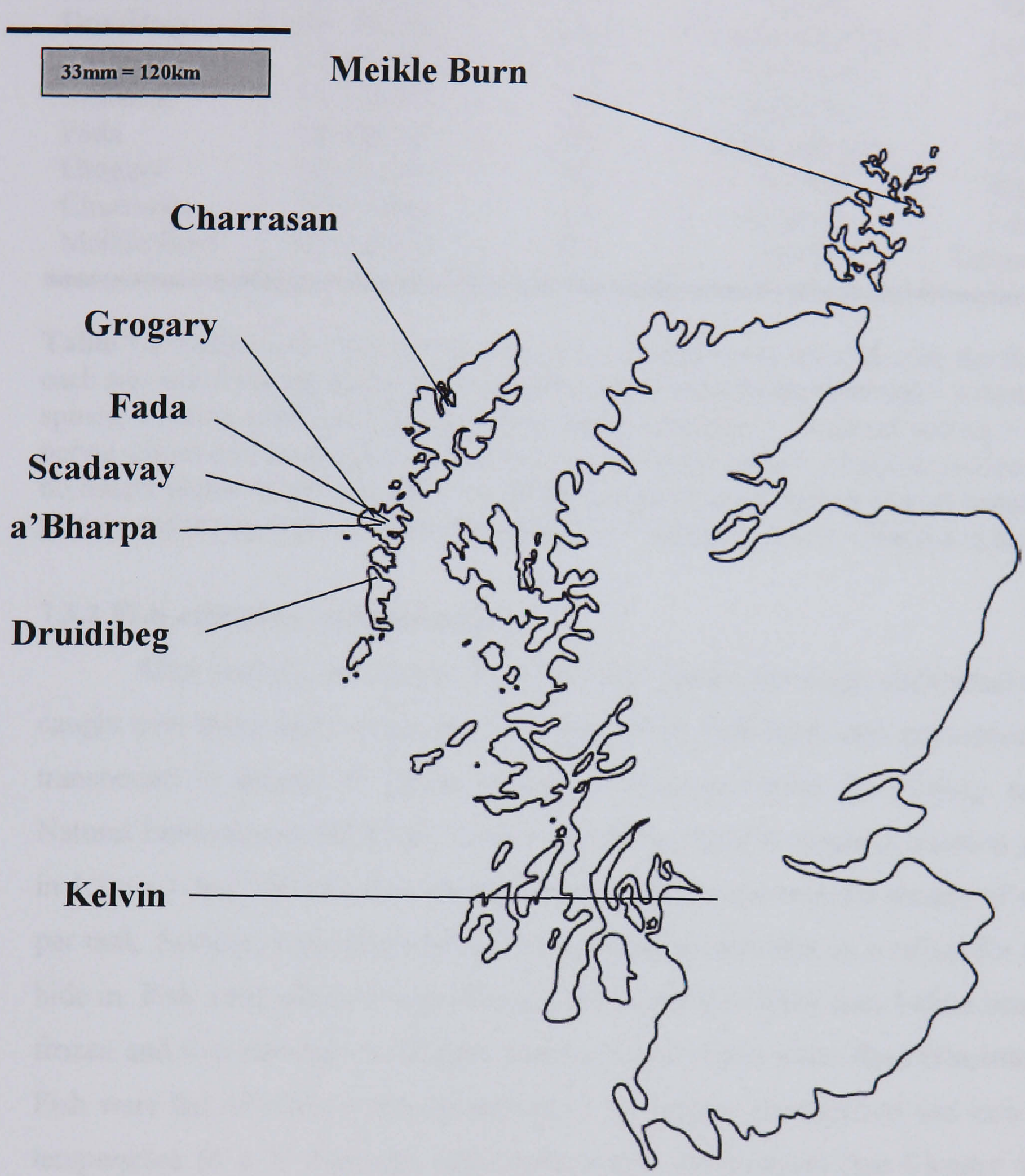

Figure 7.1 Outline drawing of Scotland showing the location of the study sites.

None had lateral plates, but 80% had a pelvic complex and more than 90% had three dorsal spines. The final sampling site, Kilometre 375 from the River Kelvin, was Meikel Burn, on the Orkney Isle of Stronsay. Fish here were normally armoured with respect to dorsal spine number and pelvic complex expression but 30% of fish did not have lateral plates. Of those fish with plates, plate number ranged from 1 to 24 and three fish had a keel. Calcium concentration at this site was unknown (see below).

Population	Location	Kilometres S to N	Morphology	Calcium
Kelvin	NS 570674	0	Normal	High
Druidibeg	NF 788384	250	Some reduction	Low
a'Bharpa	NF 833656	270	Reduction	Low
Scadavay	NF 871672	275	Reduction	Low
Fada	NF 891706	280	High reduction	Low
Grogary	NF 717709	280	Normal	High
Charrasan	NF 19886-	345	Reduction	Low
Meikle Burn	HY 659254	375	Normal	Unknown

Table 7.1 Table of the eight study sites, the grid references for each site, the distance each site was from the River Kelvin, body armour expression (Normal = 3 dorsal spines, 2 pelvic spines and lateral plates; Some reduction = >3 dorsal spines, > 2 pelvic spines and lateral plates; Reduction = >3 dorsal spines, >2 pelvic spines and no lateral plates; High reduction = 0 dorsal spines, 0 pelvic spines and no lateral plates) and the calcium concentration (High = >25mg/L; Low = <5mg/L) at each site.

7.3.2 Fish collection and husbandry

After seeking permission from Scottish Natural Heritage, sticklebacks were caught over three days in January 2004 (N = 342). Fish from each population were transported in aerated 25 gallon buckets to Scottish Centre for Ecology and the Natural Environment (SCENE), Loch Lomond and held in single population groups in 1.3m x 1.3m, 500-litre flow-through indoor tanks at a maximum density of 40 fish per tank. Several large plastic plants were placed in each tank as a refuge for fish to hide in. Fish were allowed to acclimatise for a period of three days before being fed frozen and live bloodworm (Chironomus sp.) and frozen water fleas (Daphnia sp.). Fish were fed *ad libitum* and maintained on an ambient photoperiod and loch water temperature (6 ± 2° Celsius). After behavioural observations (see Chapter 4) fish were killed by over-anaesthesia of Benzocaine and preserved in 100% ethanol. Fish not used for behavioural observations were killed immediately by an over dose of Benzocaine and preserved in 100% ethanol. Dr. Colin Bean, Scottish Natural Heritage (SNH), supplied fish from Meikle Burn, preserved in 100% ethanol.

7.3.3 Genetic analysis: tissue preparation

A sub-sample of 24 fish from each population was used for population differentiation (N = 192) with 16 microsatellite markers. Number of fish samples and marker number were chosen for several reasons. Firstly, sample sizes ranging in size from N = 17 to N = 50 have been used to successfully genetically differentiate populations using 6 or 7 microsatellite markers (Reusch *et al.*, 2001; Wilson *et al.*, 2004; Hasselquist Langefors, 2005). Secondly, 8 populations with 24 individuals from each resulted in all samples being analysed on two PCR plates (96 x 2 wells), removing the necessity of using more than two thermo-cyclers.

Fin clips were taken by removing a portion of the left pectoral fin with sterile scissors. Tissue was placed into a 1ml labelled eppendorf tube. After each sample was taken scissors were cleaned by washing first with sterile water and then with 60% Hydrochloric acid and then again with sterile water. Samples were held on dry ice until fin clipping was complete. Tissue was then shipped on dry ice to the Peichel Laboratory, Fred Hutchinson Cancer Research Center (FHCRC), Seattle WA 98109, for analysis.

0.1mg of pectoral fin tissue was placed in a 1ml eppendorf tube containing 600µl lysis buffer 0.5% and 10µl Proteinase-k (20mg/ml) was added. The samples were briefly vortexed before being placed in a 55°C water bath and incubated overnight. An equal volume (600µl) of a 1:1 mixture of phenol: chloroform was added to each eppendorf and pipette tip was changed each time. All samples were vortexed until completely mixed and spun at room temperature, at 12,100 revolutions per minute (rmp) for 10 minutes. The upper aqueous (top) layer (~500µl) was removed into a fresh, labelled 1.5ml eppendorf tube containing 1000µl of 100% ethanol. Samples were inverted several times to mix the solution and spun again at room temperature, at 12,100 rmp for 10 minutes. The ethanol was decanted without disturbing the DNA pellet before 70% ethanol was added to the eppendorf. The samples were spun at room temperature, at 12,100 revolutions per minute (rmp) for 5 minutes. The ethanol was then removed with a pipette and the samples spun. Any remaining residue ethanol was removed. After this washing procedure the DNA pellet was dried at room temperature for 10 to 20 minutes. The DNA was then re-suspended in 50µl buffer and stored at 4°C (Peichel *et al.*, 2001). To standardise the amount of DNA in each sample, concentration was determined using a BioPhotometer 6131 and appropriate dilutions made.

7.3.4 Genetic analysis: Polymerase Chain Reaction (PCR)

Microsatellite loci were amplified by polymerase chain reaction (PCR) using fluorescent-labelled primers (blue, yellow and green) in a PTC-200 DNA Engine thermocycler (MJ Research) using 10μl reactions (Master Mix 1; 7μl water + 1μl forward primer + 1μl reverse primer + 1μl DNA) (Master Mix 2; 6.45μl water + 2μl x10 buffer + 1.2μl $MgCl_2$ + 0.25 dNTP's (Pharmacia) + 0.1μl Taq polymerase (PE Applied Biosystems)). All samples were vortexed before being placed in the thermocycler. Conditions for all reactions were: 1 cycle of 95°C for 1 min 45s, 56°C for 45s and 72 °C for 45s; 5 cycles of 94 °C for 45s, 56°C for 45s and 72°C for 45s; 30 cycles of 90°C for 45s, 56°C for 45s and 72°C for 45s, plus a final cycle of 72°C for 5 min.

All samples were sexed using isocitrate dehydrogenase (Idh) (Peichel *et al.*, 2004) and 12 females and 12 males from each population were chosen for analysis with 16 microsatellite markers. The exception was Loch Druidibeg where the number of females to males was unbalanced (17 females and 7 males). In fact, out of all the Druidibeg samples that were genotyped, only 7 males were present. Markers used for population differentiation were C9yellow3, F1yellow3, G6yellow3, A1green1, A5green1, B1green1, C1green1, C7green1, D6green1, E8green1, G6green1, D2green1, H4green1, F2blue5, F8blue5, and G10blue5. All markers were designed and provided by Dr. Peichel (FHCRC). PCR products (Ihd - 9μl DNA + 2μl loading dye; Microsatellite marker 5μl DNA + 5μl loading dye) were run out on a 2% agarose gel (200ml TE Buffer + 4g agarose powder + 20μl ethidiumbromide) to verify that the reactions were successful before further analysis was carried out.

7.3.5 Analysis of microsatellite markers

Diluted PCR products (1μl DNA:50μl H_2O) from each primer pair were individually analysed on a 96-lane gel and allele sizes determined using Genemapper 3.7 (Applied Biosystems, Foster City CA). Scoring of individual markers was carried out six times (by myself) to ensure markers were correctly identified. After this rigorous process, the markers were then scored blind (by Dr. Peichel) and crossed referenced with the original calls. Three calls out of 3072 were amended. Markers that were uninformative i.e. monomorphic were discarded, as were markers that did not amplify well or were difficult to score (F1yellow3, G6yellow3, A1green1, A5green1, C1green1, C7green1, D2green1, H4green1). After this rigorous process a

total of 8 informative markers (G10blue5, D6green1, G6green1, F8blue5, F2blue5, E8green1, B1green1 and C9yellow3) were used for population differentiation (see Table 7.2). All markers were previously utilised by Peichel *et al.* (2001) in the construction of the stickleback linkage map.

Locus	No. alleles	Size range in bp	$U_{samples}$
G10b5	29	161 – 199 (6)	28 (15.6%)
D6g1	33	121 – 181 (4)	47 (24.5%)
G6g1	61	151 – 293 (9)	47 (24.5%)
F8b5	81	188 – 371 (44)	45 (23.4%)
F2b5	58	169 – 267 (6)	45 (23.4%)
E8g1	8	135 – 164 (16)	119 (62.1%)
B1g1	10	179 – 189 (2)	79 (41.1%)
C9yel3	33	175 – 265 (14)	36 (18.7%)
Mean	39.12	81.25	55.75 (29.1%)

Table 7.2 Table of three-spined stickleback microsatellite loci, with loci name, number of alleles identified, size-range of alleles and the maximum mutational steps from nearest allele neighbour in parenthesis, plus the number of unsuccessful samples and the percentage of unsuccessful samples in parenthesis.

Before a comparison of populations was carried out, a number of statistical analyses were first performed. As sample size was small, an exact test (probability test) was used to test deviation from Hardy-Weinberg. Deviations from Hardy-Weinberg equilibrium and genetic linkage disequilibrium were analysed using free software on the web, GENEPOP (Raymond & Rousset, 1995). Estimation of P-values (at the highly conservative value of $P<0.0006$ to correct for multiple comparisons) was carried out using the Markov chain method with 1000 permutations. To estimate genetic distance for all loci, F_{ST} values were calculated using Fstat 2.9.3.2 (Goudet, 2001) at the highly conservative value of $P<0.0004$. F_{ST} was used in place of R_{ST} (an alternative method for calculating genetic distance) as F_{ST} is more conservative when sample sizes are small (Gaggiotti *et al.*, 1999). The difference for each population pair in terms of allelic (F_{ST} values), geographic ("as the crow flies"), morphologic (spines and plates) and ecologic (calcium concentration) distances was found by subtracting one measurement from the other. A Partial Mantel test (Fstat 2.9.3) was then used to investigate the correlation between allelic, geographical morphological and ecological distances for all

population pairs. Morphology was used in two contexts. Firstly, corrected spine length, the lengths of first and second dorsal spines added together plus dorsal spine lengths and divided by fish standard length, for all fish caught in each population. Secondly plate number, the mean number of plates for all sampled fish. A direct measurement of calcium concentration (mg/L) was used as a parameter of ecological distance.

Inferences on the population structure of Loch Charrasan were made with the free software package STRUCTURE 2.1 (described in Pritchard *et al.*, 2000). The model-based analysis clusters individuals into populations on the basis of multilocus genotypes data using a Bayesian approach. The model accounts for linkage disequilibrium, genes that are not in random association, by structuring populations into groups not in disequilibrium. Simulations were first carried out with 1 population (K = number of populations), K = 1, 2, . . . 6, to identify which clustering model was the most suitable for interpreting the observed data (Pritchard *et al.*, 2000). The burn-in period was 1000 with a Marvoc chain Monte Carlo (MCMC) of 1000. The value of K was given by the log probability and a peak in this value (seen by graphing the values) indicates the true value of K (Evanno *et al.*, 2005).

7.4 Results and discussion

7.4.1 Microsatellite amplification

Microsatellite alleles normally follow a stepwise mutation model, gaining or loosing a single repeat unit (Valdes *et al.*, 1993). However, in approximately 20% of mutation events, mutation does not follow this stepwise model (Weber & Wong, 1993). Of the eight-microsatellite markers chosen for population differentiation all loci contained 1bp mutational steps and across all samples, a total of 313 alleles were detected. The largest mutational step was seen at loci F8b5, where allele 327 and allele 371 differed by 44 bp steps (see Table 7.2). At one locus (E8g1) no samples from Loch Fada and the River Kelvin amplified. This locus also had the lowest number of alleles present, (8 alleles). The low number of alleles may have been related to the high failure rate of this marker, almost two thirds of the samples failed to amplify (see Table 7.2). The most allelic-rich loci were G6g1 (61 alleles), F8b5

(81 alleles) and F2b5 (58 alleles). These loci also had the widest size ranges for all markers, with 142 base pairs (bp), 183bp and 98bp respectively. The largest mutational step between one allele and its neighbour (44bp) was recorded at locus F8b5.

7.4.2 Test of equilibrium

Of the eight populations tested for Hardy-Weinberg equilibrium, seven conformed to the test, indicating that the chosen markers were indeed neutral and not under selection (see Table 6.3). In Loch Charrasan, the test was rejected at three loci (G10b5 P<0.0001; F8b5 P<0.0001 and F2b5 P<0.0002), a result that may be an indicator of the Wahlund effect i.e. the population at Charrasan has subdivided into two distinct breeding units (Hoarau *et al.*, 2002).

Population	**Locus name & P-value**							
	G10b5	D6g1	G6g1	F8b5	F2b5	E8g1	B1g1	C9yel3
a'Bharpa	0.3057	0.9879	0.0036	1	0.0008	0.1728	0.7075	0.7444
Charrasan	**0.0000**	0.0497	0.0293	**0.0000**	**0.0002**	0.0386	0.1210	0.0308
Druidibeg	0.0051	0.3714	0.4461	0.0029	0.760	0.5365	0.0658	0.0062
Fada	0.1724	0.0508	0.3033	0.6205	1	-	0.4896	0.6971
Grogary	0.3199	0.0251	0.0158	0.4233	0.0314	1	0.2260	0.1996
Kelvin	0.2397	0.5737	0.5237	0.2285	0.7117	-	0.1434	0.3198
M. Burn	0.4329	0.0736	0.3354	0.2318	0.7125	0.2268	0.1346	0.7089
Scadavay	0.1289	0.2838	0.0008	0.8571	0.2022	0.0610	0.0439	1

Table 7.3 Results using GENEPOP analysis of Hardy-Weinberg test of equilibrium using eight micro-satellites in eight populations. Significant P-values are in bold.

7.4.3 Test of heterozygote deficiency

Four instances of heterozygote deficiency were identified in two populations and at four different loci (see Table 7.4), Loch a'Bharpa (G6g1 P<0.0001; F2b5 P<0.0001) and Loch Charrasan (F8b5 P<0.0001; C9y3 P<0.003). This may be indicative of inbreeding due to small population size or to the presence of null alleles that could have arisen from poor amplification of one of the alleles.

Population	Locus name & P-value							
	G10b5	D6g1	G6g1	F8b5	F2b5	E8g1	B1g1	C9yel3
a'Bharpa	0.0709	0.3931	**0.0000**	1	**0.0000**	0.2038	0.7535	0.9784
Charrasan	0.0020	0.2409	0.0248	**0.0001**	0.2372	0.0399	0.9418	**0.0003**
Druidibeg	0.0272	0.5392	0.2536	0.0185	0.5740	0.5441	0.0832	0.0102
Fada	0.0792	0.0446	0.2010	0.4378	1	-	0.9000	0.6793
Grogary	0.1408	0.0079	0.037	0.7368	0.7318	1	0.4587	0.1504
Kelvin	0.5481	0.3357	0.3075	0.4713	0.2865	-	0.5108	0.3197
Orkney	0.0452	0.5154	1	0.1297	0.0718	0.0898	0.3040	0.2993
Scadavay	0.2487	0.1402	0.2511	0.4220	0.2918	0.2447	0.2494	0.7821

Table 7.4 Results using GENEPOP analysis of Hardy-Weinberg test of heterozygote deficiency using eight micro-satellites in eight populations.

7.4.4 Population differentiation

F_{ST} ratios for all alleles showed that all populations were genetically different from each other (see Table 7.5). Although the overall mean value for all population pairs was moderate ($F_{ST} = 0.09$), the significance of the result was high ($P<0.0004$). Population differentiation was highest between fish from Loch Fada and the River Kelvin ($F_{ST} = 0.20$) and Loch Fada and Loch Charrasan ($F_{ST} = 0.19$).

Population	Charrasan	Druidibeg	Fada	Grogary	Kelvin	M.Burn	Scadavay
a'Bharpa	0.14	0.04	**0.15**	0.07	0.05	0.04	0.07
Charrasan		0.10	**0.19**	0.11	**0.16**	0.10	0.09
Druidibeg			0.14	0.04	0.05	0.05	0.06
Fada				0.11	**0.20**	0.06	0.12
Grogary					0.11	0.04	0.05
Kelvin						0.10	0.10
M.Burn							0.04
Mean F_{ST} value for all pairs = 0.09							

Table 7.5 Table of F_{ST} ratios between population pairs. All population differ significantly at $P>0.0004$. Loch Fada was marked different from Lochs a'Bharpa and Charrasan and the River Kelvin (bold text). Loch Charrasan was also markedly different from River Kelvin.

A Partial Mantel test of genetic variation, geographical distance, morphology (spines and plates) and calcium concentration was non-significant for all tests (see Table 7.6 and Figure 7.2). Analysis with STRUCTURE 2.1 showed that the 8

populations clustered into 4 genotypically similar groups (Figure 7.3). Cluster 1 was made-up entirely of Loch Charrasan. Cluster 2 was formed by Loch Fada and Meikle Burn. The River Kelvin and Loch Druidibeg made-up cluster 3 and the remaining three lochs, Lochs a'Bharpa, Grogary and Scadavay formed cluster 4. Loch Charrasan was made up of more than one population (Figure 7.4).

Variable	r^2	P-value
Geography	-0.0918	0.614
Spine morphology	0.0252	0.560
Plate morphology	-0.1634	0.595
Calcium concentration	-0.0661	0.737

Table 7.6 Table of Partial Mantel test results of genetic distance in relation to geographical distance (in kilometres and as the 'crow flies'), morphological distance (spines and plates) and calcium concentration (mg/L).

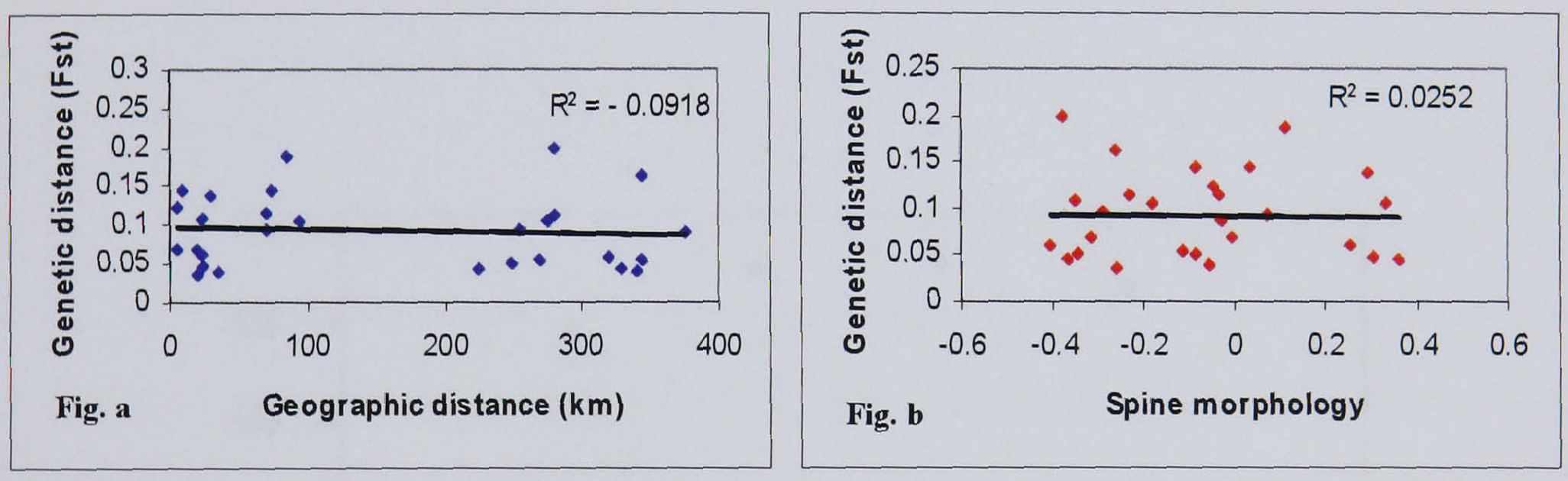

Figure 7.2 Figures of four variables used to explain genetic variation between population pairs (N = 28). There was no correlation between genetic distance, geographical, morphological or ecological distance. Fig. a shows genetic distance (F_{ST} value) plotted against geographic distance for all populations pairs. Fig. b shows genetic distance plotted against spine morphology (2 dorsal spines + 2 pelvic spines/fish length). Fig. c shows genetic distance plotted against plate morphology (mean plate number). Fig. d shows genetic distance plotted against Ca^{2+} (mg/L).

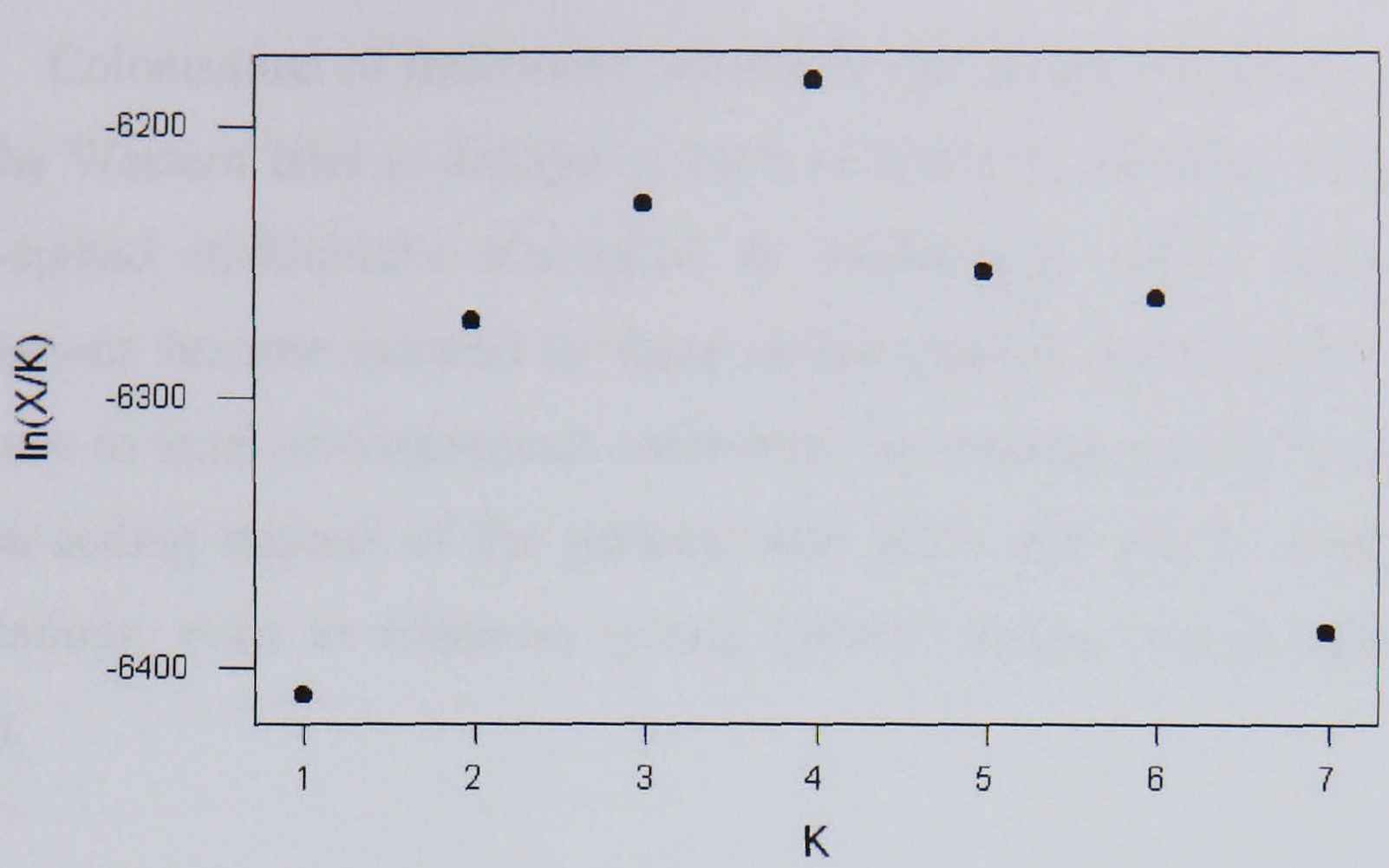

Figure 7.3 Graph of genotypic structure for all study populations (Structure 2.1). *K* (number of inferred genotypic clusters) on the x-axis. Log probability of the data fitting into each group on the y-axis. Peak in the graphed *K* value was evident suggesting that populations could be grouped into 4 genetic clusters.

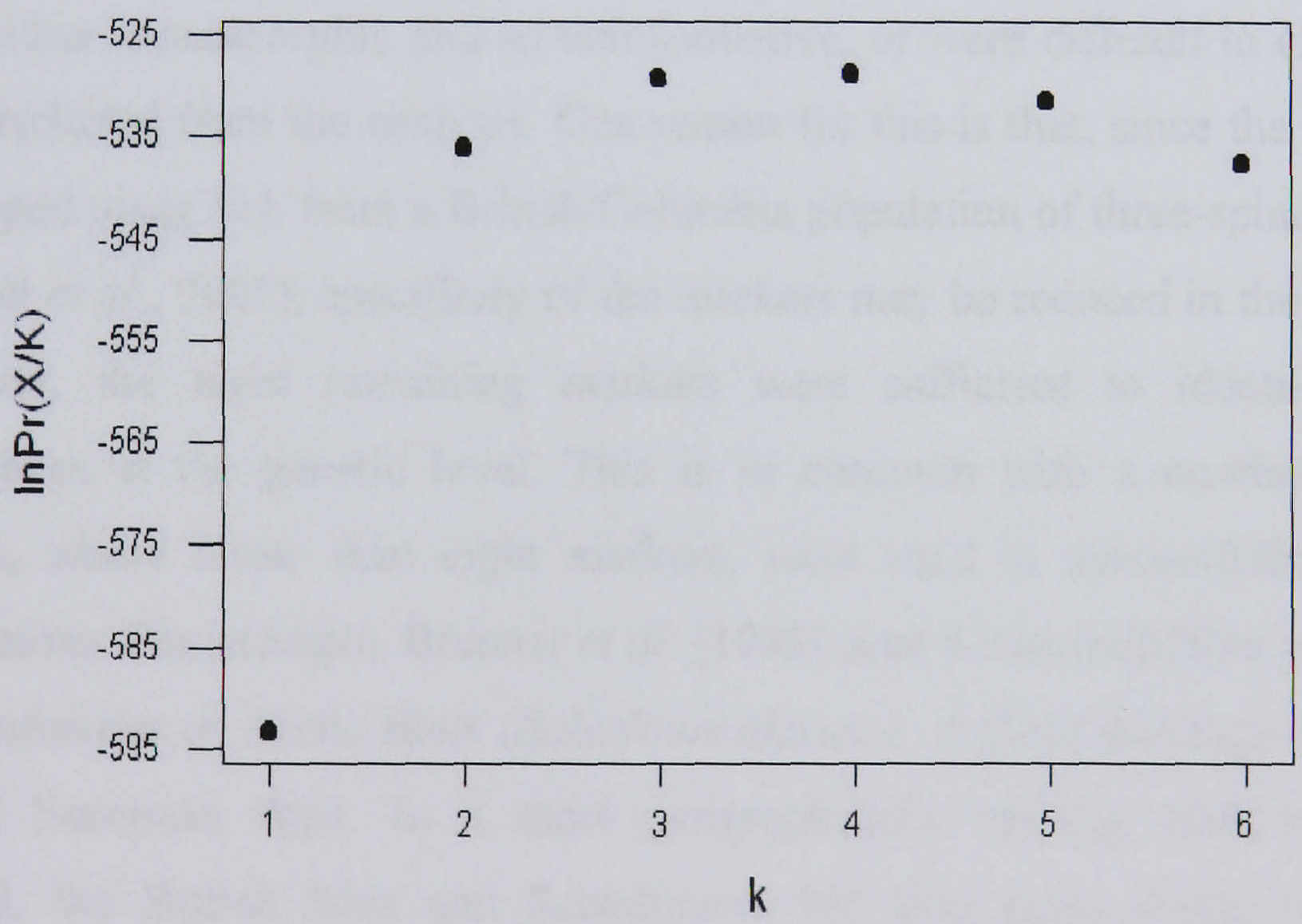

Figure 7.4 Graph of the population structure in Loch Charrasan (Structure 2.1). *K* (number of inferred populations) on the x-axis. Log probability of the data fitting into each group on the y-axis. No peak in the graphed *K* value was evident and so the true number of populations at Loch Charrasan was not found.

7.5 Discussion

Colonisation of freshwater habitats in and around the West coast of Scotland and the Western Isles is thought to have occurred by invasion of migratory marine three-spined sticklebacks (Campbell & Williamson, 1979). Subsequently, many populations became isolated in these newly-created environments and evolved in response to local environmental conditions. In tandem, genetic mutations occurring in non-coding regions of the genome also arose and can be used to differentiate populations, even in relatively young (10,000 years) systems (Zhang & Hewitt, 2003).

7.5.1 Population differentiation

The main aim of this study was to determine whether eight populations of three-spined stickleback from geographically differing locations could be differentiated on the basis of genetic variation. This aim was successfully accomplished, in that discrimination between populations was achieved, although only half of the microsatellite markers were utilised. 8 of the 16 selected markers were either monomorphic and so uninformative, or were difficult to classify and so were excluded from the analysis. One reason for this is that, since the markers were developed using fish from a British Columbia population of three-spined stickleback (Peichel *et al.*, 2001), specificity of the markers may be reduced in the Scottish fish. However, the eight remaining markers were sufficient to identify population differences at the genetic level. This is in common with a number of previous studies, where fewer than eight markers, were used to successfully differentiate populations. For example, Brunner *et al.* (1998) used 6 microsatellites to differentiate 10 populations of Arctic charr (*Salvelinus alpinus*), in three drainage systems in the central European Alps. In a more geographically ranging study encompassing Iceland, the British Isles and Scandinavia but also using Arctic charr and six microsatellite markers, Wilson *et al.* (2004) identified genetic diversity both within and between populations. Genetic differentiation and the colonization history of Atlantic salmon (*Salmo salar*) in the north-eastern Atlantic and Baltic Sea was elucidated utilising 6 microsatellite markers coupled with the major histocompatibility complex (Hasselquist Langefors, 2005). Additionally in

sticklebacks Reusch *et al.* (2001) utilised 7 microsatellite markers to genetically differentiate 16 populations (see above).

A peak in the graphed *K* value is indicative of the true number of populations in an analysis of how genotypically similar fish are. Across all study populations, individuals grouped mainly into 4 genotypically similar clusters. However, the clusters are difficult to explain, as individuals from populations that are geographically quite distant are clustering with each other. For example, most Loch Fada fish clustering with Meikle Burn and Loch Druidibeg fish clustering with River Kelvin individuals. Analysis of Loch Charrasan fish failed to identify the true number of populations found at this site. However, as there was no peak in the graphed *K* value, the true value of K remained a mystery but is greater than one. This result may be due to the small sample number (N = 24), or where fish were collected from. Approximately half of the fish were sampled from the loch and half were sampled from a small pond (<1m in diameter) by the side of the loch. Potential differences in male and female genotype were not taken into account and this may have masked the true number of populations found at Loch Charrasan, although no gender effects were found at other study sites.

7.5.2 Explaining genetic variation

A secondary aim of this study was to explain any observed genetic variation in terms of biological and environmental distances between the population pairs. However, genetic differences were not related to geographical distance. For example, at the geographical extremes of the eight study populations the River Kelvin and Meikle Burn were 375km apart, but in terms of genetic distance (F_{ST} = 0.10) not the most dissimilar. In fact, the River Kelvin and Loch Fada were the most genetically different (F_{ST} = 0.20) and were 280km apart. Loch Grogary and the River Kelvin were also 289km apart, but the genetic difference between this population pair was not so particularly great (F_{ST} = 0.11). The second most genetically differentiated populations were Fada and Charrasan (F_{ST} = 0.19) but were 85km apart. Genetic differences were also not related to armour expression even though study populations did differ in amour expression (both plates and spines). This finding was also true of the environmental parameter, calcium concentration, which was not correlated to genetic differences seen between population pairs.

The discovery that all the study populations were genetically different from each other was a surprising one. Populations that are geographically distant would be expected to differ; for example, it seems unlikely that fish from the River Kelvin could exchange genes with fish from Meikel Burn, simply because they are so far apart. However the Uist populations, in particular Lochs a'Bharpa, Scadavay and Fada, are in such close proximity (see Figure 1.1, Chapter 1), less than 10km in a water-rich landscape, that they might well be expected to represent a single population. Yet these populations are genetically distinct. Indeed Fada and a'Bharpa have an F_{ST} value of 0.15. The island itself is on average no more than 17km in length and width (Beverage, 2001) and has around 180 lochs and lochans (Giles, 1983), many interlinked. It seems surprising then that there has been no gene flow between these three closely situated lochs.

7.5.3 Implications for conservation

The result of this research has implications for the conservation of the study populations. All populations are genetically distinct and as such, may be viewed as unique evolutionary events and thus evolutionary significant units (Manel *et al.*, 2003).

Chapter 8: General discussion

8.1 Thesis overview

In this thesis I addressed five questions in order to understand and describe biodiversity within and between 12 Scottish populations of three-spined stickleback. The work integrates three distinct fields of research: morphology, behaviour and genetics. The results from all studies have allowed a comparison of geographically distinct populations to be made. I have been able to look at the interactions between water chemistry and body armour, and between risk-taking and body armour. By establishing two breeding programmes, for the inheritance of both risk-taking behaviour and protective body armour, I have also been able to make population comparisons at the genetic level.

I carried out chemical analysis of water samples at 10 of the study sites, to measure calcium, phosphorous and pH level. A qualitative survey of invertebrates and plants was made at 3 representative sites, two low calcium oligotrophic lochs (Fada and a'Bharpa) and one high calcium mesotrophic loch (Grogary). Morphology of wild caught fish was investigated at all sites and individuals from 3 of these sites were crossed and the inheritance of protective body armour studied in the resulting first and second generations. Using molecular tools and the genetic linkage map, a genetic study of the inheritance of the pelvic complex was carried out using one of these lines. To explore risk-taking in wild caught fish, individuals were screened using a novel environment test. Intrapopulation and interpopulation comparisons were made and several individuals (risk-takers or risk-averse) were chosen for a breeding programme to investigate the inheritance of this behaviour. Two generations of fish were produced and risk-taking in each generation and in each family studied. Finally, genetic differentiation of 8 populations was studied using microsatellites. The genetic distance between populations was compared to the actual distance (in kilometres), the distance in body armour expression and ecological distance in the form of calcium concentration.

8.2 Answers to 5 questions

The aims of this thesis are contained in five questions:

1. Does the calcium hypothesis hold up when one looks at a wider geographical range of sites?
2. Do armoured and unarmoured fish differ in one aspect of their protective behaviour, namely readiness to explore a novel environment?
3. Are such differences in behaviour inherited and if so:
    a. What is the genetic mechanism?
    b. What are the major loci responsible and can QTL mapping be used to find the loci?
4. What is the pattern of inheritance of variable body armour?
5. Are the various sites with reduced armour genetically distinct populations, and how distinct are they from nearby armoured sites?

8.2.1 Does the calcium hypothesis hold up when one looks at a wider geographical range of sites? (Chapter 3 and 7)

The hypothesis that reduction in protective body armour is associated with and possibly a response to low levels of calcium was investigated. I categorised each study site, into high or low calcium sites depending on the concentration of calcium found at each location. The exceptions to this were Loch Quien and Meikle Burn, as water samples were not taken at these sites. The chosen sites were spread over a wide geographical distribution, but some sites, in particular those on North Uist, were in very close proximity to each other. The maximum distance between study sites was 354km (River Kelvin and Loch Charrasan) and the minimum 5km (Loch Scadavay and Loch a'Bharpa; Loch Scadavay and Loch Fada). At several sites, measurements were taken over a three-year period. Overall calcium level remained stable. Calcium concentration was reflective of variation in armour expression across the study sites, in that reduction in armour only occurs at low calcium sites. However, the relationship was not perfect in that (as found by Giles, 1987) armoured fish were also found at low calcium sites. A relevant point from chapter 7 is that there is no relationship between genetic distinctness of each pair of populations and the difference in calcium level between them.

My data therefore support the hypothesis that in populations of stickleback from a wide range of geographical sites in Scotland, low calcium levels are part of the reason for reduction in armour.

8.2.2 Do armoured and unarmoured fish differ in one aspect of their protective behaviour, namely readiness to explore a novel environment? (Chapter 4)

Within most of the study populations there was a spread of behavioural phenotypes, ranging from very risk-prone to very risk-averse. This axis of behaviour (also termed the bold-shy continuum) has been observed in other populations of stickleback (Giles & Huntingford, 1984; Bell & Stamps, 2004) and other species of animals (Gosling, 2001; Dingemanse *et al.*, 2003; Sih *et al.*, 2004). Levels of risk-taking did not differ between study populations. Bell (2005) suggests that predation regime may be an important factor in selecting for risk-taking and for its association with aggression. As all populations in this study are under similar predation risk from birds, fish and invertebrates, lack of difference in predation risk may explain why there were no interpopulation differences. Given the striking difference in body armour among the populations this seems unlikely. It is also possible that populations may indeed differ in risk-taking behaviour, but this was not reflected in the chosen test, exploration of a novel environment. Indeed, casual observation shows that fish from unarmoured populations are very agile and hard for humans to catch. At the population level there was no correlation between risk-taking and the degree of morphological protection from body armour. At the individual level, there is a weak relationship between risk-taking and protection, but only in two populations.

My data shows that armoured and unarmoured fish do not differ in their level of risk-taking.

8.2.3 Are such differences in behaviour inherited and if so: What is the genetic mechanism? What are the major loci responsible and can QTL mapping be used to find the loci? (Chapter 5)

In the two lineages of within and between population crosses, there were trends in risk-taking across the generations, but as these were in opposite directions, the biological significance (if any) is not clear. There were also some significant family differences in both the F_1 and F_2 generations in both lineages, but offspring

phenotype showed little relationship with the parental phenotype. Overall, therefore, although some significant differences in risk-taking between families in both generations and in both lineages were found, risk-taking is not inherited in any simple way. This finding is in contrast to other studies where risk-taking was found to be a heritable trait (Dingemanse *et al.*, 2002; Bell 2005). A possible explanation is that risk-taking is influenced by social interactions within rearing groups, as found by Magnhagen & Staffan (2005) and that holding fish in isolation (as I did to standardise social experience) removes this influence. Such effects would also explain the inconsistent levels of repeatability that I found (Chapter 2).

My data show that risk-taking is not inherited in any simple sense. For this reason, QTL analysis was not carried out.

8.2.4 What is the pattern of inheritance of body armour? (Chapter 6)

In common with other studies (Peichel *et al.*, 2001; Summers B, pers. com.) my data showed that the pattern of dorsal spine inheritance was complicated. Crosses between a fully spined fish and a spine deficient fish resulted in two generations of fish, all with 2 or more dorsal spines and none that were spine deficient. Where spines were lost there was no simple pattern, some fish expressing the first and third dorsal spines and some the second and third spines. The results from this breeding programme suggest that inheritance of the dorsal spine has a polygenetic mode of inheritance. The inheritance of anal spines was also complicated and no clear pattern of inheritance was apparent.

The pattern of lateral plate inheritance was clearer. Parental fish used in this cross were a low plated morph (0 plates) and a partially plated morph (4 plates). In the first filial generation of fish, the pattern of plate inheritance followed a near Mendelian ratio of 3:1 partially plated to low plated morphs, with a minimum plate number of 1 and a maximum of 6. Interestingly, in the second filial generation, a similar 3:1 ratio of plate inheritance was observed, but the minimum and maximum number of plates was 0 and 11 respectively. This indicated that, inheritance of plates maybe under the control of one major locus. QTL of minor effect may also be present increasing plate number in partially plated individuals. This result is similar to Colosimo *et al.* (2004), who found that plate number is under the control of one

major chromosome region on linkage group VI, with 3 QTL of minor effect (found on other chromosomes) increasing plate number in partially plated fish.

The inheritance of the pelvic complex seems to be under the control of one major locus, namely *Pitx1*. The gene mapped to linkage group VII, the locus responsible for the Mendelian inheritance of pelvic reduction defined by Shapiro *et al.* (2004) in North American populations. The result adds to the mounting evidence that pelvic girdle reduction in the armour-less population of Loch Fada fish is most likely due to a null mutation in the same *Pitx1* gene, even though selection pressure is different. The result suggests that pelvic complex loss in Scottish populations of three-spined stickleback is a case of parallel evolution with North American populations of stickleback.

My data show that the pattern of dorsal and anal spine inheritance is complicated but that lateral plates and the pelvic complex have a relatively simple Mendelian mode of inheritance.

8.2.5 Are the various sites with reduced armour genetically distinct populations, and how distinct are they from nearby armoured sites? (Chapter 7)

Using 8 microsatellites developed by Dr. Peichel (a further 8 microsatellites were rejected due to poor amplification or difficultly in identification), I found that 7 populations were in Hardy-Weinberg equilibrium. Using F_{ST} ratios, all populations were significantly distinct from each other. Furthermore, I found that the one population for which two alleles were not in Hardy-Weinberg equilibrium (Loch Charrasan) was composed of at least two genetically distinct populations. I used geographical, morphological and ecological distance in an attempt to explain genetic distances between population pairs, but failed to find any significant relationships. This is in contrast to a number of studies that have also used this method to discriminate stickleback populations on the basis of genetic and geographical distance. For example, Reusch *et al.*, (2001) used 7 microsatellite loci among 16 populations of stickleback to explore genetic divergence, common ancestry and geography in German river, lake and estuarine postglacial populations. They find that genetic divergence was correlated with habitat type, and weakly, with geographical distance. Mäkinen *et al.* (2006) used 18 microsatellites at 74 marine and freshwater

locations and found that genetic variation correlated weakly with geographical regions, habitat type had a negligible effect.

My data confirm that the study populations are genetically distinct and the difference is significant regardless of the geographical distance between populations.

8.3 Broader implications

8.3.1 Adaptation and evolutionary change

In North American populations of sticklebacks, the main driving force for armour reduction seems to be the presence of piscine predators (Reimchen, 1980). This cannot be the case for the Scottish populations of stickleback used in this study as a variety of predators were present at all sites. The results of my thesis support and amplify the hypothesis proposed by Giles (1983), that armour loss in Scottish populations of three-spined stickleback is driven by reduced levels of calcium. Although Giles (1983) concentrated on armour reduced populations on North Uist, my study included a number of sites on other Hebridean islands and on mainland Scotland, and found that calcium levels remained a good predictor of armour reduction.

As the selective force for armour reduction in Scottish populations seems to be low calcium level, one would not necessarily predict that the same genetic mechanism would be involved as found for North American populations. For example, the gene for girdle reduction might have been a gene controlling calcium regulation and the distribution of calcium among body compartments. The discovery then, that a mutation in the *Pitx1* gene, rather than a mutation in an upstream regulator, is the most likely underlying cause for pelvic girdle loss in my study sites as well as that found in North American fish, will add to the mounting evidence of parallel evolution in the loss of protective body armour.

8.3.2 Biodiversity and conservation

Although some of the populations used in this study are in close geographic proximity to each other, they are indeed just as distinct as those populations found on

other islands or on the mainland. By using 8 microsatellite markers, I have demonstrated genetic diversity and, as this is one of the three components of biodiversity (Redford & Richter, 1999), has raised issues for conservation. Many lochs on the Outer Hebrides already have a high protection status under UK and European law, only at one (Loch Druidibeg) is there a specific mention of spine-reduced stickleback on the sites citation. This means that genetically distinct populations of armour-reduced stickleback are not specifically protected, even though the habitat within which they live is. In my opinion, the populations of armour-reduced stickleback on the Outer Hebrides are not interchangeable and so to protect biodiversity, must be taken into consideration in any future plans for these sites.

8.3.3 Behavioural syndromes

In the 1990's and the early part of this century, the concept of behavioural syndromes and coping strategies has had a high profile. The thrust of much of the literature has been founded on the idea that risk-taking is a highly repeatable trait, whether in the context of encounters with a potential predator or aggressive interactions with a conspecific. However, recent studies have highlighted some complexities. Though many studies have shown significant repeatability's of risk-taking in the face of novelty (Dingemanse *et al.*, 2002; Drent *et al.*, 2003), this is not always a repeatable trait. Thus relative risk-taking may vary with context (Coleman & Wilson, 1998) and over longer periods of time (Bell & Stamps, 2004). Variable external influences are also important in determining individual levels of risk-taking, even once obvious variables such as hunger, temperature and recent predatory encounters are allowed for. Another such influence is interactions within social groups, Magnhagen & Staffan (2005) finding that boldness is modulated by social interactions and Frost *et al.* (2006) finding that social relationships affect risk-taking in fish.

My findings are in line with these more recent studies, since my measures of consistency over two tests ranged from 0.1 to > 0.3. Over five tests with fish kept in isolation, consistency is completely absent and repeatability measure is >0.01. My data therefore suggest that when fish are kept in isolation this has a strong effect on the consistency of individual level of risk-taking. Clearly such effects need further study.

8.4 Questions arising from this thesis, implications for other studies and future work

A number of questions have arisen from the work carried out in this thesis. I will try and address each as they arise in each chapter.

8.4.1 Questions from Chapter 2: Problems with screening risk-taking and Chapter 5: A study of the inheritance of risk-taking.

A recurring problem throughout the behavioural screening process has been the lack of consistency in risk-taking from one test to the next. The possible reasons for this have been discussed at length in chapters 2 and 5. However, it is worth noting that, although consistency seems to increase from generation to generation, the highest levels of consistency were seen in F_2 families tested in 2006. This cohort was treated in a different manner to all other fish screened for behaviour. All other fish were screened at the university field station (SCENE), but fish tested in 2006, although born and raised at SCENE, were moved at the age of 6 months, to the University's fish laboratory in Glasgow. Fish were screened in the same way as before and held under the same conditions, being in isolation before and after testing. However one thing was different. There was an air-conditioning unit in the fish laboratory in which fish were housed and screened, which made a constant loud noise. It is possible that this 'white noise' had an effect on the fish, possibly blocking out all other noise and ultimately having a calming effect on the fish. This is similar to the use of white noise to block out intermittent sounds aiding sleep in humans (Stanchina *et al.*, 2005). The question then is, does white noise increase consistency in risk-taking behaviour in sticklebacks?

In chapter 5, although some significant family effects in risk-taking were found, the picture was somewhat complicated and no clear pattern of inheritance of risk-taking was identified. A possible explanation may be that risk-taking is influenced by social interactions within rearing groups. Is risk-taking influenced by social interactions?

Implications for other studies: The assumption that the novel environment test provides results that are always consistent and repeatable should be treated with caution. I have demonstrated consistency between tests in first and particularly in

second generation fish, but not in wild caught fish. Also, without removing the effect of social interactions a true picture of the inheritance of risk-taking will not seen and any attempt at finding QTL for this behaviour will be doomed.

Future work: A possible experiment to test whether white noise aids consistency in the novel environment test would be to split a group of fish from one population or one family into two treatment groups, one exposed to white noise and the other held in quiet conditions and examining consistency of behaviour in the novel environment test. To test the hypothesis that risk-taking is influenced by social interactions a replicated study should be carried out. Fish could be held in mixed behavioural groups and screened for risk-taking while a second group of fish may be held in isolation before and after screening.

8.4.2 Questions from Chapter 3: Reduction in body armour and the role of calcium.

In order to fully understand the relationship between three-spined stickleback and their habitat, it was first necessary to characterise study sites primarily in terms of water chemistry but also in terms of ecology. Although a limited qualitative survey of invertebrates was carried out at 3 study sites and a literature review of known fish and bird predators recorded at all sites, no attempt was made to quantify the number and location of stickleback predators, nor how often stickleback came into contact with predators. Since predators have been demonstrated to influence risk-taking in stickleback (Giles & Huntingford, 1984; Bell, 2005) it may be important then to quantify this effect by surveying the number and location of potential stickleback predators. If predation pressure is an important influence on risk-taking behaviour, ecological surveys should be carried out into the location number and location of potential predators and the position of sticklebacks in relation to their predators.

Implications for other studies: Meaningful assumptions concerning predation pressure on wild fish cannot be made without a true measure of the number and location of predators and the number and location of their prey.

Future work: Counts of predatory bird and possible observations of birds feeding on stickleback could be carried out. Likewise, and perhaps easier it may be possible to identify which predatory fish species are present at each site, the location of these fish and the number and size of these fish. Coupled with this approach, stomach

sampling in a sub-set of predatory fish may also be carried out to quantify the number and size of stickleback being eaten. Also, since nothing is known of the exact number of armour-reduced fish at each study site or precisely where these fish are located, this too should be studied. From a conservation point of view this information is imperative if proper protection of these distinct populations is to be carried out. Therefore I suggest that in tandem with a survey of piscivorous fish, a survey of armour-reduced stickleback could also be carried out.

8.4.3 Questions from Chapter 4: Reduction of body armour and implications for risk-taking.

The novel environment was chosen to quantifying risk-taking because it is a well-established technique, broadly predictive of some aspects of the sticklebacks' response to a predator (Huntingford, 1976; Bell and Stamps, 2004) and is easier to standardise. In retrospect however, this test alone may not have been suitable in eliciting the full response of anti-predator behaviour differently armoured fish. Differences in behaviour between armoured and unarmoured fish might well be revealed in response to a model predator or a live predator behind glass.

The number of spines was used as a measure of how well a fish is armoured but the 'quality' of the spine was not taken into account. When dorsal and ventral spines are locked into position they form a triangle of protection protecting the fish from gape limited predators. However, thin and flimsy spines offer less protection than robust and rigid spines. Therefore spine quality is an important measure of how well a fish is protected that was not taken into account in this study. Is spine quality an important component of protection?

Implications for other studies: Use of the novel environment test alone may not be sufficient in identifying risk-taking in an animal. Using spine counts and spine lengths may give a false impression of how well armoured an armour-reduced fish really is and so caution should be taken when using these measurement alone.

Future work: To screen for a broader spectrum of anti-predator behaviour fish should be screen using the novel environment test in conjunction with a predator test. A test of spine quality, similar to that carried out by Reimchen (1983) would be of use in quantifying body armour protection so that a meaningful comparison of armour protection can carried out between armoured and armour reduced stickleback.

8.4.4 Questions from Chapter 6

Due to time and financial constraints, the inheritance of lateral plates, dorsal spines and the anal spine was not investigated using molecular tools. Although preliminary work was carried out to identify microsatellite markers suitable for finding the genes important in anal spine inheritance, these did not work and so the question was not pursued any further. Further work could usefully be carried out as all fish samples are still available. Summers *et al.* (Stanford University) are working to identify useful markers for dorsal spine inheritance, once those markers have been found they could be used on my samples. What is the genetic basis of dorsal spine and anal spine inheritance?

Future work: As all the fish used in my study are available for further research, once the gene or genes underlying the reduction of dorsal spines are identified, work currently being carried out in a number of labs, markers for this trait can be utilised in my study fish. This may provide a further example of parallel evolution in Scottish and North American fish. A similar study could be carried out using markers, once identified, for the anal spines.

8.4.5 Questions from Chapter 7

All the populations used in my investigation into population differentiation were found to be genetically distinct. This was a surprising result, as some of the water-bodies were in close geographical proximity and previously thought to be connected. Although the populations are also morphologically distinct, interestingly morphology was not a useful predictor of the extent of genetic differentiation between populations. This was also true of the ecological variable I considered, calcium concentration, which also differed significantly between sites. One outstanding question then is how the differences at the genetic level can be explained by other morphological or ecological variables? A second surprising result was that the fish from Loch Charrasan was in fact two or more distinct populations. On reflection, while most of the fish from this site were caught in the loch itself, a significant number were caught in a small pond less that a metre from the loch. Could this pond represent a novel habitat, isolated from the loch and where fish are genetically distinct? Do these fish differ morphologically? Also it has been demonstrated that the populations are genetically different but how differentiated are they in comparison to the ancestral population?

Implications for other studies: Fish that are caught in one location, even a small loch, should not be assumed to be genetically the same.

Future work: Further analysis using F_{ST} ratios generated from data obtained in the molecular study could be used in a Mantel test with different variables such as a measure of predation pressure at each site to elucidate why the populations are genetically distinct. These data could also be used in conjunction with data from marine stickleback, captured close to the each study site, to compare genetic difference if freshwater populations to that in the nearby ancestral population. To find out how many populations there are at Charrasan further sampling should be carried out at this site and the exact location of where the fish were caught noted.

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Appendix 1

Repeatability *r* is derived from the following equation (Lessells & Boag, 1986);

$$r = s^2_A/(s^2+s^2_A)$$

s^2_A is the variance among groups component and s^2 is the variance within groups component, both are calculated using the mean squares in an analysis of variance.

Source of variation	df	Sum of squares	Mean squares	F ratio
Among groups	df_1	SS_A	MS_A	F
Within groups	df_2	SS_W	MS_W	

$$s^2 = MS_W$$

$$s^2_A = (MS_A - MS_W)/n_o$$

In the analysis of variance n_o is the coefficient related to sample size in each group. If samples sizes are the same n_o is the same as group size n. When group sizes are different, n_o is less than group size mean $\bar{u}$.

$$n_o = [1/(a-1)].\left[\sum n_i - \left(\sum n_i^2/\sum n_i\right)\right]$$

a = group number and n_i = size of the sample in the ith group.

Appendix 2

How consistent are individual differences in risk-taking in three-spined stickleback?

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Summary

Variability in risk-taking is thought to have a heritable component and has been demonstrated in several studies. We screened 25 fish for risk-taking in a novel environment. Factor scores from a Principle Component Analysis were used in categorising fish into three groups; risk-takers, risk-intermediate or risk-averse. Individual fish were tested 5 times over a period of 6 months and were held in social isolation before and between testing. Contrary to expectations we found no individual consistency in risk-taking even though screening protocols were carefully standardised and the effects of extraneous uncontrolled variables allowed for. The key difference between studies that find repeatability and those that do not, is that fish were held in groups in the former and in isolation in the latter. We suggest then, that differences in risk-taking are produced and maintained by social forces but what those forces are, remain obscure.

Keywords: risk-taking, repeatability, three-spined stickleback

Introduction

Consistent individual differences in risk-taking (also termed 'boldness') in response to a novel environment have been reported in studies using several different species of fish, birds and mammals (see Table 1). Where such differences exist, it is important to know whether these are consistent individual

traits or temporary responses to current or recent circumstances. Many studies show significant repeatability, in the sense that an individual's relative position between tests does not significantly change. For example, Dingemanse et al. (2002) found significant repeatability estimates (r) of between 0.27 and 0.66 in exploratory behaviour in individuals from two populations of wild caught great tits (*Parus major*). The majority of birds were tested within 24hs of capture and after screening, released in the location from where they were taken. Around a fifth of birds were recaptured several months later and re-tested.

One fish species in which individual differences in response to a novel environment have been tested is the three-spined stickleback (*Gasterosteus aculeatus* L.). Thus Huntingford (1973) screened sticklebacks in groups of three to four in an unfamiliar environment with weed cover and repeated the test without protective weed, several weeks later. The behaviour of the fish was summarised in a single 'boldness' score, reflecting the rate at which fish emerged from cover and explored the unfamiliar tank. The fish remained in their groups between tests. Individual scores in the second test were significantly predicted by those in the first test (Regression analysis; $r^2 = 26\%$ $p = 0.001$). In a later study, sticklebacks were screened singly for boldness in an unfamiliar tank on two occasions separated by several weeks, being housed in groups in between tests. Here again, individual scores in the second test were significantly related to those in the first test, though the association was weaker ($r^2 = 13.3\%$, $p = 0.005$; Huntingford, unpublished data). The response to a novel environment of the fish used in the earlier published study was predictive of how fish behaved towards a predator several weeks later and of response to an intruder, several months after the initial tests (Huntingford, 1976). The implication of these results is that individual responses to a novel environment reflect a consistent feature of the 'personality' of these fish. Such consistency within and correlations across contexts has been described as a behavioural syndrome (Sih et al., 2004)

Comparison of sticklebacks from high and low predation regimes, revealed population differences in boldness in response to a model predator that persisted in fish reared in the lab without direct experience of a predator (Huntingford et al., 1994). This suggested a possible heritable component to the original behavioural difference, which presupposes that boldness is at least a partially fixed trait. However, the finding was only true in respect to those fish

reared by their father (Tulley & Huntingford, 1987). Bell (2005) found weak heritablities for activity in a novel environment in two populations of three-spined stickleback (Population A, heritability = 0.048; Population B, heritability = 0.156).

With this background, as part of an investigation of the inheritance of variable risk-taking in sticklebacks (in particular, we wished to look for Quantitative Trait Loci underlying this behaviour) we undertook a study designed to confirm that this is a consistent and repeatable trait. The same fish were screened for risk-taking in a novel environment on 5 separate occasions, with possible confounding factors either controlled for or allowed for statistically. We report here on our failure to find significant repeatability and discuss possible reasons for this finding.

Materials and methods

Study fish

In order to investigate repeatability of risk-taking at the level of the individual a sub-set of first filial (F1) generation fish from a larger related study into the inheritance of risk-taking was used. The parents of these F1 fish were categorised as risk-taking (female) and risk-averse (male). This cross was chosen as it provided the widest distribution of risk-taking scores. Fish were five months old at the beginning of behavioural screening.

Fish husbandry

Fish were housed and screened for risk-taking at the Scottish Centre for Ecology and the Natural Environment (SCENE), Loch Lomond and fed *ad libitum*, on a diet of frozen and live bloodworm (Chironomus sp.) and frozen water fleas (Daphnia sp.). Fish were not marked and, to keep track of individual identity, after behavioural observations, were held individually in chambers. The chambers (120mm x 100mm, with mesh windows and a mesh bottom) were held in an outdoor flow-through tank (1m x 1m, depth 50cm) under natural light conditions and ambient loch water temperature (9 ± 4°C). To provide a hiding place a $2cm^2$ piece of black plastic was placed in each cell. Uneaten food and faeces were removed daily.

Behavioural screening

Risk-taking was assessed using a version of the standard test of exploration/activity in a novel environment (Verbeek, et al., 1994). The method is a well-established technique for quantifying risk-taking that is broadly predictive of some aspects of the sticklebacks' response to a predator (Bell & Stamps, 2004) and is repeatable (Dingemanse et al., 2002). A further advantage of this approach is that it is easier to standardise than using a live predator or even a model predator.

A number of potentially confounding variables were identified and, where possible, controlled for, to ensure that individual risk-taking was measured accurately. Fish were deprived of food for a minimum of 20 hours prior to testing to minimise differences in hunger level, which are known to influence risk-taking (Lima & Dill, 1990). To standardise stress levels, fish were allowed to settle for a minimum of one hour in aerated holding tanks and under low light conditions before testing. Each fish was tested in turn in one observation tank, to remove any possible confounding effect of tank, over a two-day period. Fish were tested 5 times, Test 1: 19-20 October, Test 2: 17-18 November, Test 3: 15-16 December, Test 4: 13-14 January and Test 5: 6-7 March, in sequence and at the same time of day to maximise the chances of obtaining repeatability.

One observer (SC) carried out all observations to reduce any possible observer effects. Each flow-through observation tank (100cm x 40cm x 40cm) was surrounded on all sides with cardboard to conceal external movements, with a horizontal slit along the front wall for observation (see Figure 1). A 40 (watt) light 30cm above the tank was the sole source of illumination. Sand covered the bottom of the test arena. A 30cm high plastic plant for refuge was located in the area where the fish was released (home-base). A large stone was positioned under the in-flow pipe, 75cm from the home-base, to prevent sand being moved around while water flowed into the tank. A grid was drawn on the back of the tank to divide the tank into twelve equal lettered areas (25cm x 10cm) for use in the full behaviour test. Each fish was allowed to settle in a lowered holding chamber (10cm in diameter by 50cm high) for 15 minutes prior to the start of each test. Tank temperature was noted and in-flowing water was turned off 20 seconds before the start of the test. Fish were observed for up to 15 minutes from the time

at which the chamber was raised. The test was terminated when a fish crossed the vertical time line AB. Time (in seconds) at which the fish crossed line AB (latency to move from home-base) was recorded, as well as the number of squares entered once (tank use) and the total number of squares used (activity). Fish that failed to cross the vertical line were given an arbitrary score of 1000. At the end of each test water was turned on to refresh the tank while the next test fish was settling. After testing, fish were returned to their individual holding chamber until the next behavioural screening was carried out.

Data analysis

Combining behavioural variables

All data were checked for normality with an Anderson Darling Test. Data not normally distributed were analysed with non-parametric tests. Principle Component Analysis (PCA) was used to condense the behavioural data, summarising the measured variables into a single factor score for each fish (Huntingford, 1976). Variables used were latency to cross line AB, activity and tank use. The latter two variables were divided by time to cross AB to account for the shorter test experienced by fish that explored quickly. The truncated test is predictive of risk-taking scores obtained from a longer version of the test using 15 minutes for all fish, four time lines and movement/activity in twelve squares (Coyle, unpublished data). The first principle (PC1), which accounted for 77.7% of the total variance, opposed latency to cross AB (negative loading) against activity and tank use (positive loadings). This was interpreted as an index of risk-taking and is comparable to the index of boldness identified by Huntingford (1976) and Bell (2005).

Allowing for potentially confounding variables

Before making comparisons of risk-taking between tests it was necessary to determine whether confounding factors influenced behaviour and where this occurred, apply corrections. A Spearman rank order correlation was used to identify variables effecting behaviour. There was a significant but weak association between temperature and behaviour of the fish ($r_s = 0.298$ $N = 125$ $p = 0.001$). To correct for variables effecting behaviour, scores were expressed as residuals from the linear regression of risk-taking (boldness) against temperature

(Boylan, 2005). Although fish were tested on different dates, there was no effect of date on behaviour and no corrections were made. Clean risk-taking scores were then used to investigate repeatability between tests.

Comparison between tests

Consistency of individual levels of risk-taking, were investigated in two ways. Firstly, scores in successive tests were compared, using Spearman rank order correlation followed by regression analysis. Secondly, Kruskal-Wallis was used to look for significant individual differences across all five tests. To obtain a measure of repeatability we used a parametric test, One-way Analysis of Variance (ANOVA) (Lessells & Boag, 1987).

Results

Table 1 and Figure 2 show the relationship between individual scores in successive tests. Although there were marginally-significant positive relationships between tests 1 & 2 and tests1 & 4, overall there was very little relationship between the scores of the same fish in successive tests. There was no significant difference in risk-taking behaviour across the 5 tests (Kruskal-Wallis; $H = 2.52$ $df = 4$ $p = 0.641$) nor were there any obvious changes in variability (see Figure 3). Repeatability (r) was non-significant ($r = 0.013$).

Discussion

It is quite clear from this study that there was no overall consistency in risk taking across the 5 tests. This is in contrast to the published studies summarised in Table 1 were tests were carried out on two occasions only. It is also in contrast to Huntingford's earlier studies of sticklebacks (1973, 1976). However, it does agree with Bell & Stamps (2004), who did not find a significant correlation between individual levels of activity in a novel environment in the same sticklebacks tested as juveniles and (c. 9 weeks later) as sub-adults (held in groups between trials).

There are a number of possible reasons for our failure to find significant repeatability. One arises from the fact that the 5 tests were carried out over a period of declining water temperature. If all our fish became increasingly reluctant to leave cover at low temperatures, loss of variability in later tests might explain

the lack of relationship between successive tests. Another possibility is that over the 5 tests, the fish might well have habituated to the initially novel environment (Brown, 2001). Such an effect might cause all fish to emerge from cover quickly, gaining high risk-taking scores. Here again, loss of variability in later tests might explain the lack of relationship between successive tests. Unfortunately, since temperature and test number were strongly associated (Spearman rank order correlation; $r_s = 0.247$, $N = 5$, $p = 0.005$) we could not separate their effects statistically. However, as Figure 3 shows, the range of variability did not decline over the study period, so we believe that we can rule out these two possible explanations for the lack of individual consistency in our tests.

A third possibility is that our failure to find individual consistency in risk taking where other studies have done so, arises from the fact that our subjects were held in isolation between successive tests (partly to allow fish to be identified, but also to rule out possible influences of social interactions within groups). Perhaps individual differences in risk-taking are somehow reinforced by experience within groups and change when fish are separated from the group. That interactions within groups can influence risk taking is demonstrated by Magnhagen & Staffan (2005), who found that in young of the year perch (*Perca fluviatilis*) time to emerge from cover (reflecting a shy-bold continuum) was affected by group composition. When a shy individual was placed in a group consisting of bold fish only it became bolder, spending more time in open water, and when a bold fish was placed in a shy group, it became shyer, conversely spending more time hiding.

Wilson et al. (1993) found that differences between sunfish (*Lepomis gibbosus*) classified as shy or bold in a field test were stable so long as fish remained in the field, but broke down after a period of social separation in the laboratory. It is possible then that differences in risk-taking in the three-spined stickleback are produced and maintained by social forces. Removal of these forces, for example holding fish in social isolation, allows risk-averse individuals to become risk-takers and visa versa. Quite what such social forces might be, requires further research.

Acknowledgements

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Table 1. Selection of papers on personality traits, syndromes or coping strategies, species used, conditions animals were held under (before, during and after testing), the type and number of times the test was used and the paper reference.

Personality trait	**Species**	**Conditions**	**Tests**	**Number of times tested**	**Reference**
Boldness Aggression	Lion-headed cichlid *Steatocranus casuarius*	Fish held in groups. Individually tested.	Novel environment. Inter-specific inspection. Mirror test.	Twice for each test.	Budaev et al., (1999)
Exploration	Great tit *Parus major*	Caught from wild and tested individually within 24hrs of catching.	Novel environment.	Fifth of birds re-caught and retested.	Dingemanse et al., (2002).
Neophobia Boldness	Sardinian & garden warbler *Sylvia melanocephala momus & S. borin*	Caught from wild, held in groups one week before testing.	Novel object. Novel environment.	Twice for each test. Repeated 10 months later.	Mettke-Hofmann et al., (2005)
Boldness Aggression Feeding competition	Domestic pig *Sus scrofa domesticus*	Kept in groups. Tested in groups and individually.	Back test. Novel environment. Feeding competition test.	Twice for each test.	Ruis et al., (2000)

Table 2. Results of a regression analysis of tests 1 & 2, 2 & 3, 3 & 4 and 4 & 5, with r and p values.

	Dependent variable			
Independent variable	Test 2	Test 3	Test 4	Test 5
Test 1	$r^2 = 8.9, p =$ 0.08	$r^2 = 0.0, p =$ 0.50	$r^2 = 9.1, p =$ 0.08	$r^2 = 0.0, p =$ 0.41
Test 2		$r^2 = 7.7, p =$ 0.10	$r^2 = 1.3, p =$ 0.26	$r^2 = 2.1, p =$ 0.23
Test 3			$r^2 = 0.0, p =$ 0.92	$r^2 = 0.0, p =$ 0.77
Test 4				$r^2 = 0.0, p =$ 0.85

FIGURE LEGENDS

Figure 1. Diagram of behavioural observation tank.

Figure 2. Test pairs 1 & 2, 2 & 3, 3 & 4 and 4 & 5, plotted against each other. Stars signify the position of the two fish with repeatable risk-taking behaviour.

Figure 3. Representation the median and range of rank risk-taking scores of 25 individual fish tested 5 times over a period of 6 months.

Figure 1.

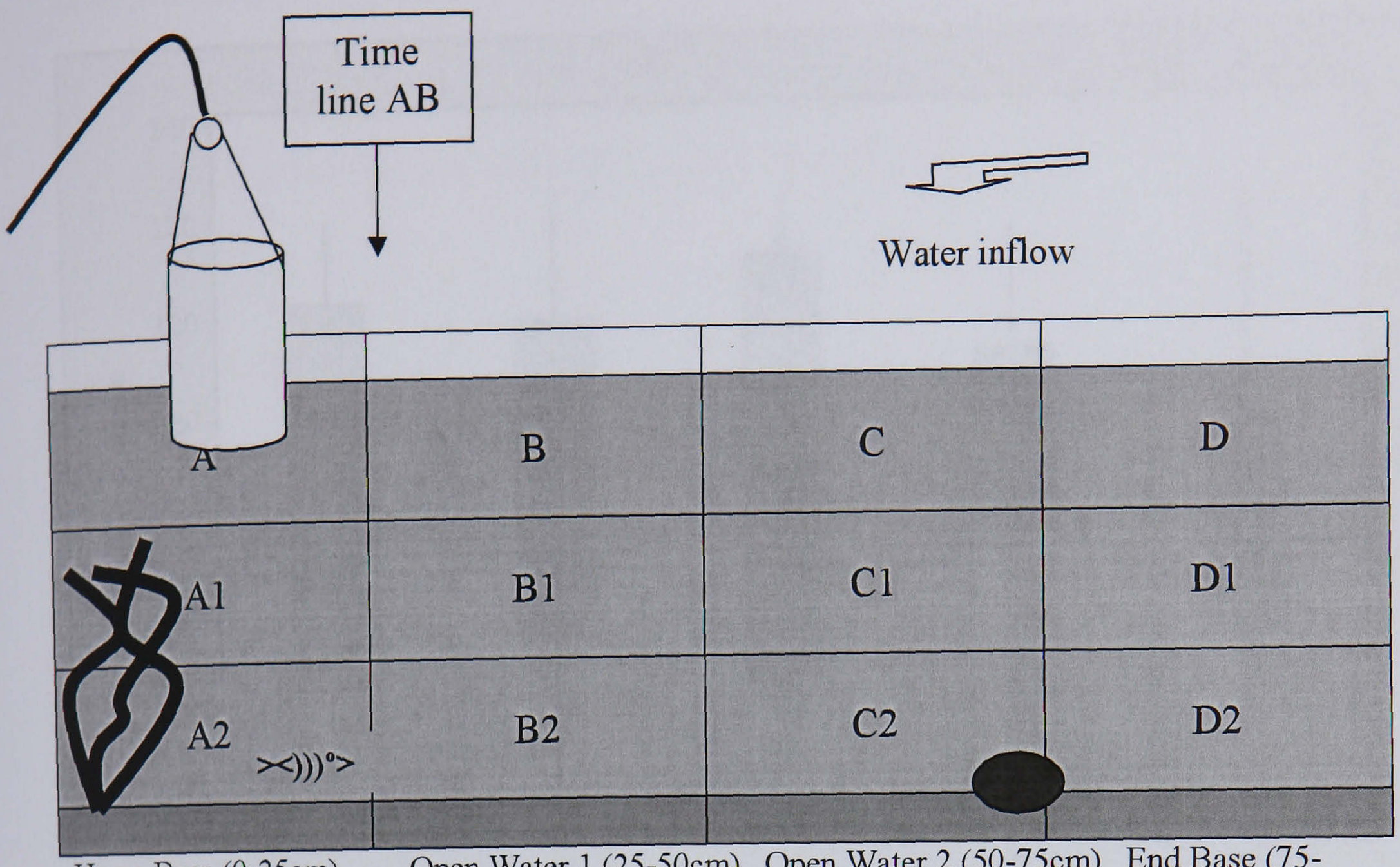


Figure 2.

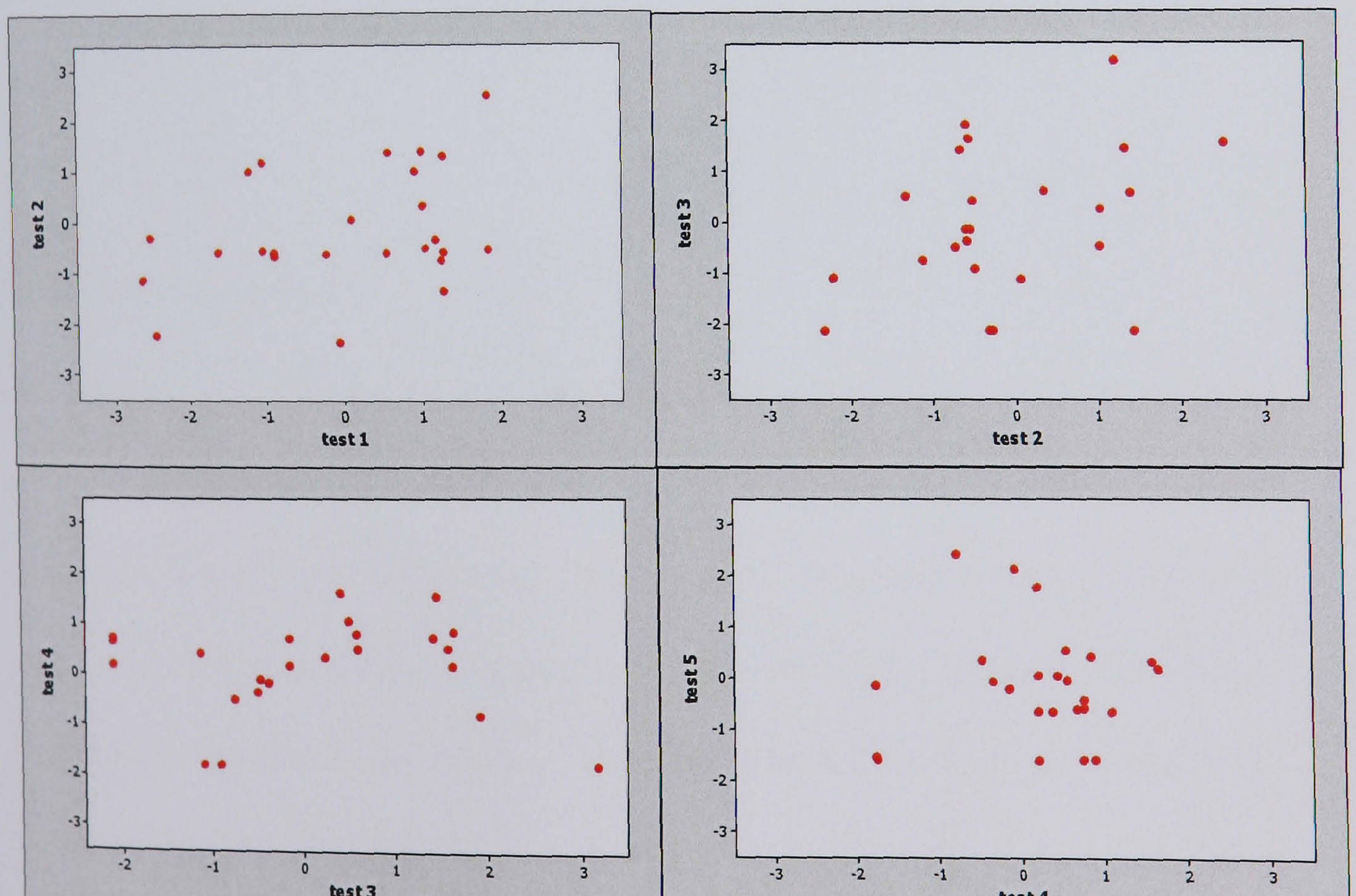
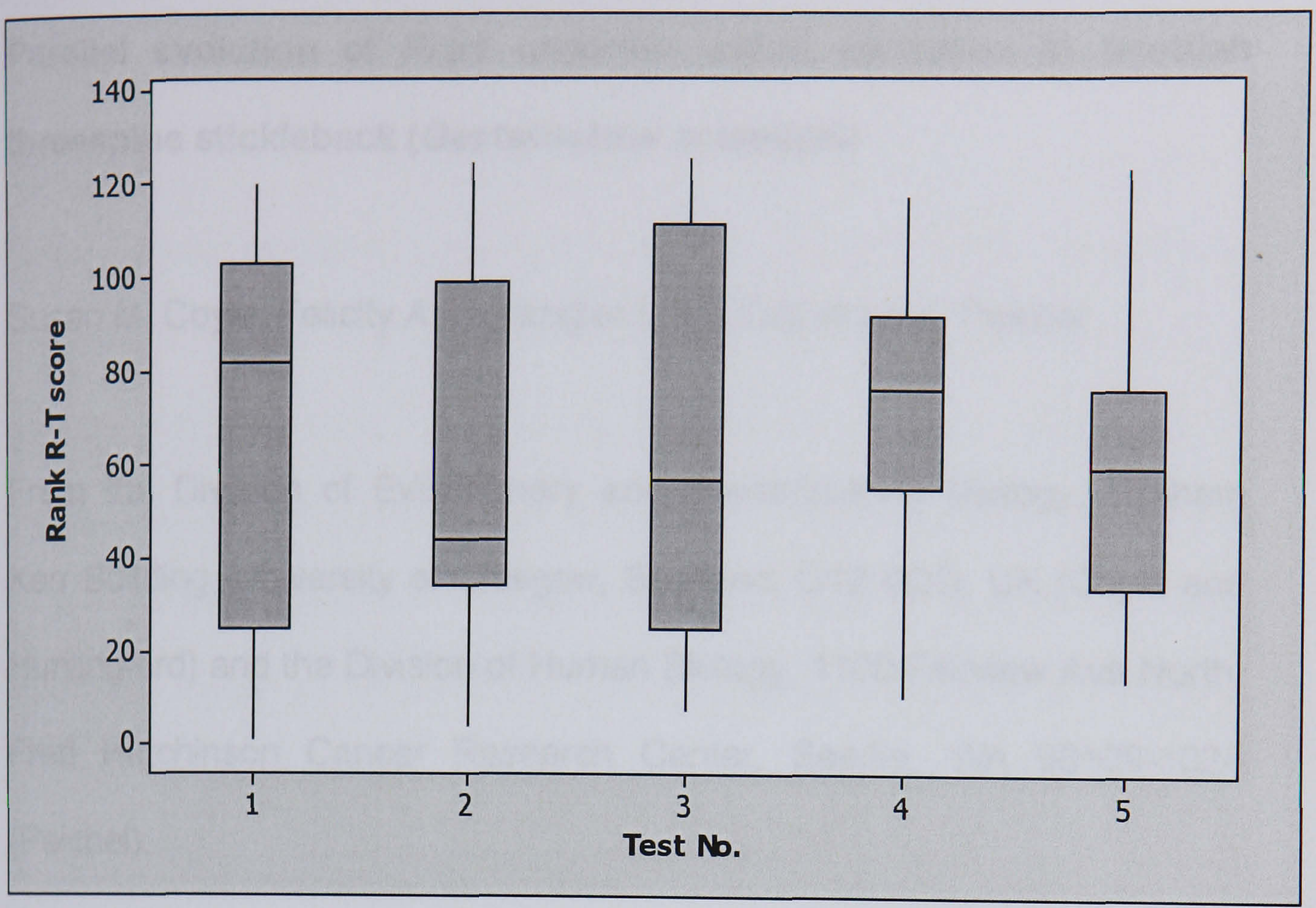


Figure 3.



Appendix 3

Parallel evolution of *Pitx1* underlies pelvic reduction in Scottish threespine stickleback (*Gasterosteus aculeatus*)

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Running title: *Pitx1* and pelvic reduction in Scottish threespine stickleback

Abstract

Little is known about the genetic and molecular mechanisms that underlie adaptive phenotypic variation in natural populations, or whether similar genetic and molecular mechanisms are utilized when similar adaptive phenotypes arise in independent populations. The threespine stickleback (*Gasterosteus aculeatus*) is a good model system to investigate these questions because these fish display a large amount of adaptive phenotypic variation, and similar adaptive phenotypes have arisen in multiple, independent stickleback populations. A particularly striking pattern of parallel evolution in sticklebacks is reduction of skeletal armor, which has occurred in numerous freshwater locations around the world. New genetic and genomic tools for the threespine stickleback have made it possible to identify genes that underlie loss of different elements of the skeletal armor. Previous work has shown that regulatory mutations at the *Pitx1* locus are likely responsible for loss of the pelvic structures in independent stickleback populations from North America and Iceland. Here we show that the *Pitx1* locus is also likely to underlie pelvic reduction in a Scottish population of threespine stickleback, which has apparently evolved pelvic reduction under a different selection regime than the North American populations.

Introduction

Although the selective forces that contribute to adaptive phenotypic variation are beginning to be understood, very little is known about the genetic changes upon which selection acts during phenotypic evolution (Orr 2005). The threespine stickleback (*Gasterosteus aculeatus*) is an excellent model system in which to integrate ecological and genetic studies to gain a greater understanding of the process of phenotypic evolution (Foster and Baker 2004; Gibson 2005; Kingsley et al. 2004; Kingsley and Peichel 2007). This small teleost fish displays such great phenotypic variation that different forms were originally classified as over 40 different species, with some of the most obvious phenotypic changes in stickleback populations occurring in skeletal armor. In most locations, these fish are encased in bony armor that consists of lateral plates, dorsal spines, and two pelvic spines supported by a pelvic girdle. However, armor reduction has occurred in freshwater locations that have only existed since the retreat of the glaciers less than 20,000 years ago. In particular, pelvic reduction has occurred in a small number of independent locations ranging from the northwest coast of Scotland to Iceland to several sites along the Pacific coast of North America (Bell 1974, 1987; Bell et al. 1993; Campbell 1979; Moodie and Reimchen 1976; Shapiro et al. 2004). The evolution of armor reduction is associated with specific predation regimes and low calcium levels in North American populations (Bell et al. 1993; Reimchen 1980) but is solely associated with low calcium levels in Scottish populations (Giles 1983).

The development of genetic and genomic tools for threespine stickleback has made it possible to identify the genetic and molecular basis of phenotypic variation in natural populations of this species (Peichel 2005; Kingsley and Peichel 2007). Genome-wide linkage mapping carried out using Canadian pelvic-reduced populations identified the *Pitx1* gene as a candidate locus of large effect for pelvic size (Shapiro et al. 2004). Genetic crosses suggest that the same locus also underlies pelvic reduction in Icelandic and Alaskan threespine stickleback populations (Cresko et al. 2004; Shapiro et al. 2004) as well as pelvic reduction in a closely related species, the ninespine stickleback (Shapiro et al. 2006). In Canadian and Scottish pelvic-reduced populations, gene expression studies have demonstrated that *Pitx1* is not expressed in the developing pelvic region (Cole et al. 2003; Shapiro et al. 2004). However, mapping studies had not been carried out in the Scottish population, so it was unknown whether the loss of *Pitx1* expression in this population is genetically linked to the *Pitx1* locus or results from a mutation in an unlinked gene that regulates *Pitx1* expression. Here we show that the pelvic reduction phenotype is tightly linked to the *Pitx1* locus in a Scottish population. Although our data do not rule out the possibility that the loss of *Pitx1* expression in this Scottish population results from changes in a closely linked gene that regulates *Pitx1* expression, this result provides evidence that changes at or near the *Pitx1* locus have occurred in multiple, independent stickleback populations with pelvic reduction.

Materials and Methods

Fish

Sticklebacks from the River Kelvin (Glasgow, Scotland) and Loch Fada (North Uist, Scotland) were caught in February 2003 and transported in aerated 25 gallon buckets to the Glasgow University Field Station (SCENE), Loch Lomondside. Fish from a single population were kept together in 1.3 m x 1.3 m, 500-liter flow-through indoor tanks at a maximum density of 40 fish per tank. Several large plastic plants were placed in each tank as a refuge for fish to hide in. Fish were fed frozen and live bloodworm (*Chironomous* spp.) and frozen water fleas (*Daphnia* spp.) *ad libitum* and maintained on an ambient photoperiod at ambient loch water temperature (6 ± 2°C).

Crosses

For genetic mapping, one Loch Fada female that had no pelvic spines or pelvic girdle structures (pelvic score = 0; Bell et al. 1993) was crossed with one River Kelvin male that had two pelvic spines and complete pelvic girdle structures (pelvic score = 8) to produce 40 F1 offspring. Eight of these F1 fish were intercrossed, resulting in 177 F2 progeny from four F2 families (Table 1). The F1 parents of all families had complete pelvic girdles and two pelvic spines (pelvic score = 8), except for the Family 4 F1 father, which had a complete pelvic girdle but was missing the right pelvic spine (pelvic score = 7).

Males in breeding condition were moved to sandy bottomed breeding tanks (45 cm × 27 cm × 15 cm) with nesting material. After nest

construction, a gravid female was placed in the tank and the pair was left to breed naturally. After spawning, the female was removed and the fertilized eggs placed in an incubator and artificially oxygenated at 16°C. Males rebuilt a nest within one to three days and a gravid female was returned to the tank to breed again. Fry were fed Liquifry No1 (INTERPET) for one week post-hatching and then maintained on a mixture of enriched AF high-grade *Artemia* (INVE AQUACULTURE nv) and chopped bloodworm. At 8 weeks, fry were moved to small holding tanks (25 cm × 20 cm × 45 cm) and kept in family groups of 10 to 15 fish.

Phenotypic analysis

Parental and F1 adults, and F2 fish at 24 weeks post-hatching were killed with an overdose of anaesthetic (benzocaine). Fins were clipped for DNA extraction, and the bodies were preserved in 100% ethanol. The number of pelvic spines was counted and measurements of standard length (tip of snout to end of caudal peduncle), pelvic spine lengths (tip to anterior edge of spine) and ventral pelvic girdle length (between spines to posterior tip of girdle) were made with calipers to the nearest 0.1mm. Asymmetry was calculated as the ratio of the length of the left pelvic spine to the combined length of the left and right pelvic spines, such that a value of 0.5 indicates perfect symmetry, a value of 0.0 indicates the loss of the left pelvic spine, and a value of 1.0 indicates loss of the right pelvic spine.

Genetic analysis

Six microsatellite markers (*Stn76*, *Stn257*, *Stn80*, *Stn82*, *Stn336*, *Stn342*) previously mapped to linkage group (LG) 7 were used for genotyping (Shapiro et al. 2004). These markers are found in the *G. aculeatus* sequence assembly BROAD S1 (http://www.ensembl.org/Gasterosteus_aculeatus/index.html) at approximately 5.22 Mb (*Stn76*), 25.10 Mb (*Stn257*), 26.40 Mb (*Stn80*), and 26.66 Mb (*Stn82*) on LG7, and on scaffold 76 (*Stn336*, *Stn342*), which has not been assigned to a linkage group in the sequence assembly. These markers were used to genotype 177 F2 progeny as well as the grandparents and F1 parents, using previously described PCR conditions (Peichel et al. 2001). However, the DNAs of two fish were not included in the genetic analysis (Loch Fada grandmother and Family 2 F1 mother). PCR reactions were analyzed on an ABI 3100 (Applied Biosystems, Foster City CA). Two people independently determined genotypes by manually calling the allele sizes and segregation patterns, which were visualized with ABI GeneMapper 3.7 (Applied Biosystems, Foster City CA). A map of LG7 was generated in JoinMap 3.0 (Van Ooijen and Voorips 2001) using the default settings, and interval mapping of five pelvic traits was performed in MapQTL 4.0 (Van Ooijen et al. 2002) using default settings. Likelihood of odds (LOD) significance thresholds were determined for each trait by permutation tests in MapQTL 4.0 using a chromosome wide significance threshold of α = 0.01 for n = 1000 permutations. Because only one chromosome was genotyped and tested, the chromosome-wide and genome-wide LOD significance thresholds are equivalent.

Results and Discussion

We crossed a female completely lacking pelvic spines and pelvic girdle structures from an armor-reduced Scottish population (Loch Fada) known to lack pelvic *Pitx1* expression (Cole et al. 2003) to a male with a complete pelvic complex from a robustly armored Scottish population (River Kelvin). From this cross we generated 40 F1 progeny, which all had complete pelvic structures, except for three fish that had complete pelvic girdles, but showed loss of the right pelvic spine. Four pairs of full-sib F1 hybrid fish were intercrossed to generate four F2 families, producing a total of 177 F2 progeny (Figure 1; Table 1). In the F2 progeny, there was an approximate 3:1 ratio of progeny with complete pelvic girdles and either 2 or 1 pelvic spines to progeny with no pelvic structures (Figure 1; X^2 = 0.996; P = 0.318; 1 d.f.). These data suggested that there might be a major locus responsible for loss of the pelvic complex in this Scottish population, as seen in Canadian and Alaskan populations (Cresko et al. 2004; Shapiro et al. 2004).

To determine whether *Pitx1* is genetically linked to the major pelvic reduction locus in the Loch Fada population, we genotyped F2 fish with 6 informative microsatellite markers from stickleback LG7, including two markers in the *Pitx1* gene, to which the major pelvic locus has been mapped in other populations (Cresko et al. 2004; Shapiro et al. 2004). Of the 50 F2 fish that had complete loss of pelvic structures, all inherited two Fada alleles at the *Pitx1* markers (Figure 1). Interval mapping showed that the *Pitx1* locus explains between 85.1 and 96.6% of the variance in

Interestingly, three of the F1 fish and four of the F2 fish had complete pelvic structures, except for loss of the right pelvic spine, while a single F2 fish had complete pelvic structures except for loss of the left pelvic spine (Figure 1; Table 1). This left-biased asymmetry is consistent with previous results in both natural populations (Bell 1974, 1987; Bell et al. 1985, 2007; Moodie and Reimchen 1976) and lab crosses (Cresko et al. 2004; Shapiro et al. 2004). This observation is also consistent with the fact that the loss of *Pitx1* can be partially compensated by *Pitx2*, which is preferentially expressed on the left side of the body (Marcil et al. 2003). Of the five F2 fish with a single pelvic spine, two were homozygous for the Fada alleles at the *Pitx1* markers, while three (including the fish that had lost the left pelvic spine) were heterozygous at the *Pitx1* markers (Figure 1). Interval mapping revealed that the *Pitx1* locus explains 38% of the asymmetry in pelvic spine length, with a significant LOD score of 13.2 (Figure 2; Table 2), consistent with previous results (Shapiro et al. 2004).

We thus conclude that pelvic reduction in the Scottish Loch Fada population, which has likely evolved as a result of selection under low calcium levels rather than lack of piscivorous fish predators (Giles 1983), represents a case of parallel evolution at or near the *Pitx1* locus. Recent genetic studies in a number of taxa have uncovered other examples of parallel evolution involving the same locus. For example, the *yellow* gene controls changes in wing pigmentation in different *Drosophila* species (Gompel and Carroll 2003; Gompel et al. 2005; Prud'homme et al. 2006), the *shavenbaby/ovo* gene is responsible for changes in hair patterns in *Drosophila* species (Sucena et al. 2003), coding mutations in the *Mc1r*

gene result in melanism in many vertebrate lineages (Eizirik et al. 2003; Hoekstra et al. 2006; Mundy et al. 2004; Nachman et al. 2003; Römpler et al. 2006; Rosenblum et al. 2004; Theron et al. 2001), and the *Oca2* gene is responsible for albinism in fish and mammals (Protas et al. 2006). The recurrent use of a small set of genes during morphological evolution suggests that genetic and/or developmental bias may play an important role in adaptation. Additional studies to identify the molecular changes that give rise to loss of pelvic structures in Canadian, Alaskan, Icelandic, and Scottish populations will allow us to determine whether parallel evolution of pelvic reduction results from standing genetic variation in the ancestral marine population as found for loss of bony lateral plates (Colosimo et al. 2005), or from independent mutations, implicating genetic and/or developmental bias as a key factor in the evolution of pelvic reduction in stickleback.

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Figure legends

Figure 1. Intercross for genetic mapping of pelvic reduction. Line drawings of representative fish, with pelvic structures highlighted in red are shown for the parental, F1 and F2 generation. Both lateral and dorsal views of the parental fish are shown to highlight the complete absence of pelvic structures (girdle and spines) in the Loch Fada mother. The ascending branch of the pelvic girdle can be seen in the lateral view of the River Kelvin father, and the ventral pelvic girdle and two pelvic spines can be seen in the ventral view of the River Kelvin male. Only ventral views of representative F2 fish are shown to emphasize the different pelvic spine classes. Genotypes at the *Pitx1* microsatellites (*Stn336*/*Stn342*) are indicated in parenthesis.

Figure 2. Interval mapping of pelvic traits on LG7. LOD scores are plotted as a function of the position in cM of markers: *Stn76* (0.0 cM), *Stn257* (31.5 cM), *Stn80* (42.2 cM), *Stn82* (47.6 cM), *Stn336/Pitx1* (61.8 cM), and *Stn342/Pitx1* (61.8 cM), which are indicated by points along the curve.

Figure 1.

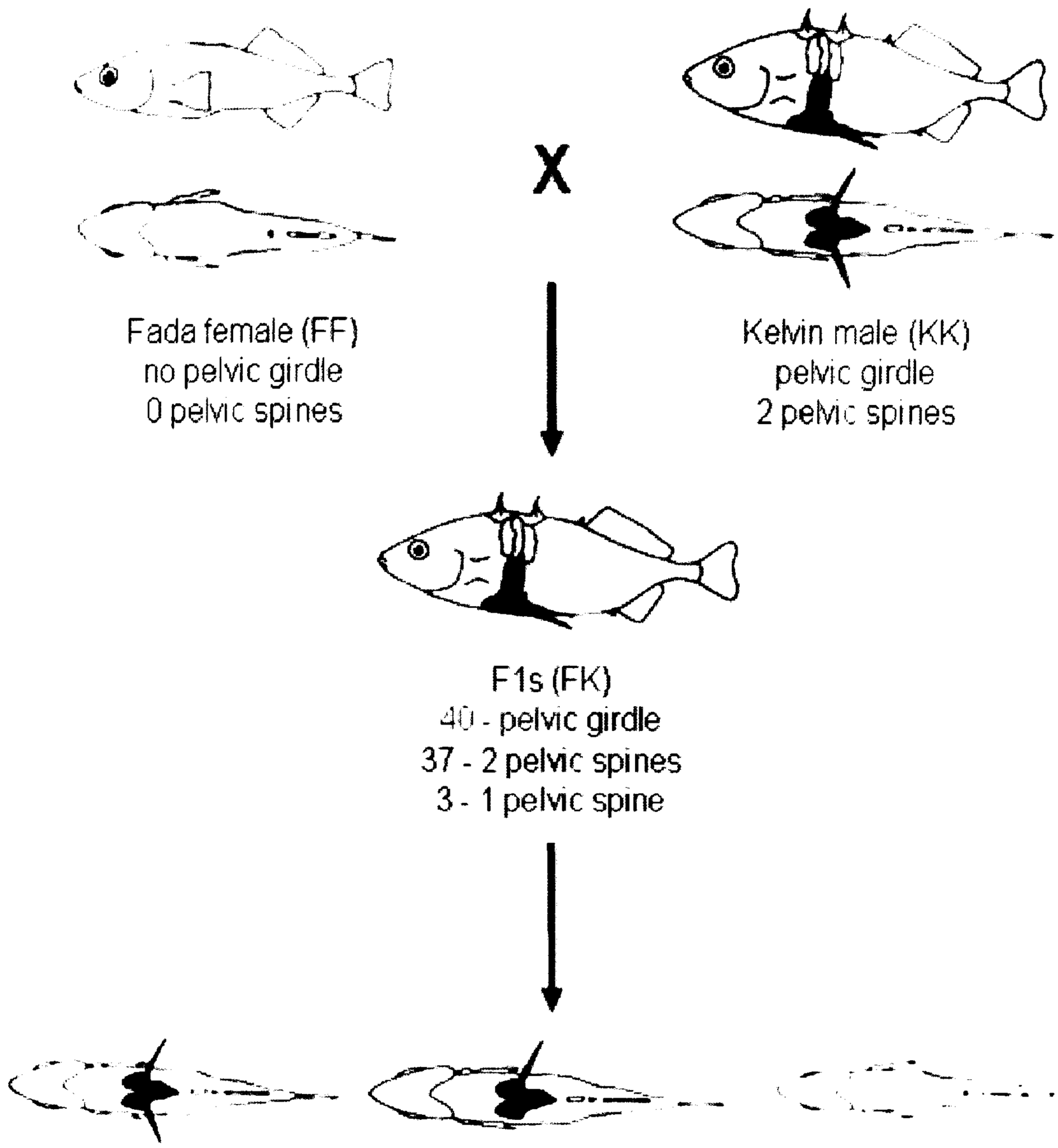

X
Fada female (FF)
no pelvic girdle
0 pelvic spines
Kelvin male (KK)
pelvic girdle
2 pelvic spines
F1s (FK)
40 - pelvic girdle
37 - 2 pelvic spines
3 - 1 pelvic spine
122 F2s
(50 KK, 72 FK)
pelvic girdle
2 pelvic spines
5 F2s
(3 FK, 2 FF)
pelvic girdle
1 pelvic spine
50 F2s
(50 FF)
no pelvic girdle
0 pelvic spines

Figure 2.

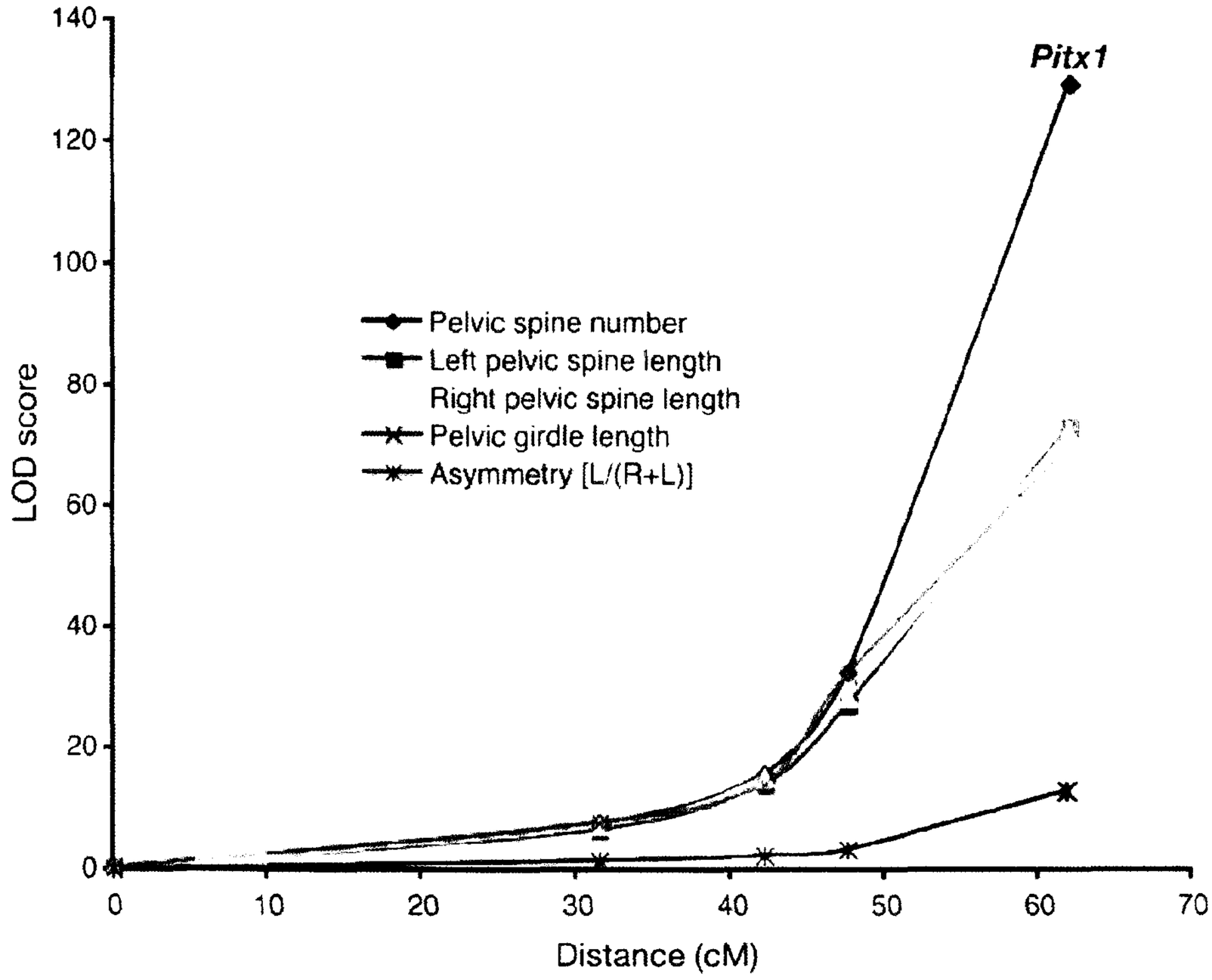

140
120
100
80
60
40
20
0
LOD score
Pitx1
Pelvic spine number
Left pelvic spine length
Right pelvic spine length
Pelvic girdle length
Asymmetry [L/(R+L)]
0
10
20
30
40
50
60
70
Distance (cM)

Table 1. Pelvic spine number in F2 cross fish

	Pelvic Spine Number		
F2 family	0	1[a]	2
Family 1 F2s (N=66)	17	1	48
Family 2 F2s (N=36)	12	1	23
Family 3 F2s (N=38)	9	1	28
Family 4 F2s (N=37)	12	2	23

The number of F2 fish in a family with 0, 1 or 2 pelvic spines is indicated.

[a]One of the F2s from Family 4 was missing its left pelvic spine; all additional fish with 1 pelvic spine were missing the right pelvic spine.

Table 2. Effect of *Pitx1* on pelvic phenotypes

Trait	LOD	PVE	Phenotype means		
			KK	FK	FF
Pelvic spine number	129.3	96.6	2.00	1.96	0.04
Left pelvic spine length	73.4	86.0	4.29	3.69	0.08
Right pelvic spine length	72.0	85.1	4.29	3.58	0.00
Pelvic girdle length	73.4	87.0	5.50	5.13	0.10
Asymmetry	13.2	38.0	0.50	0.51	1.00

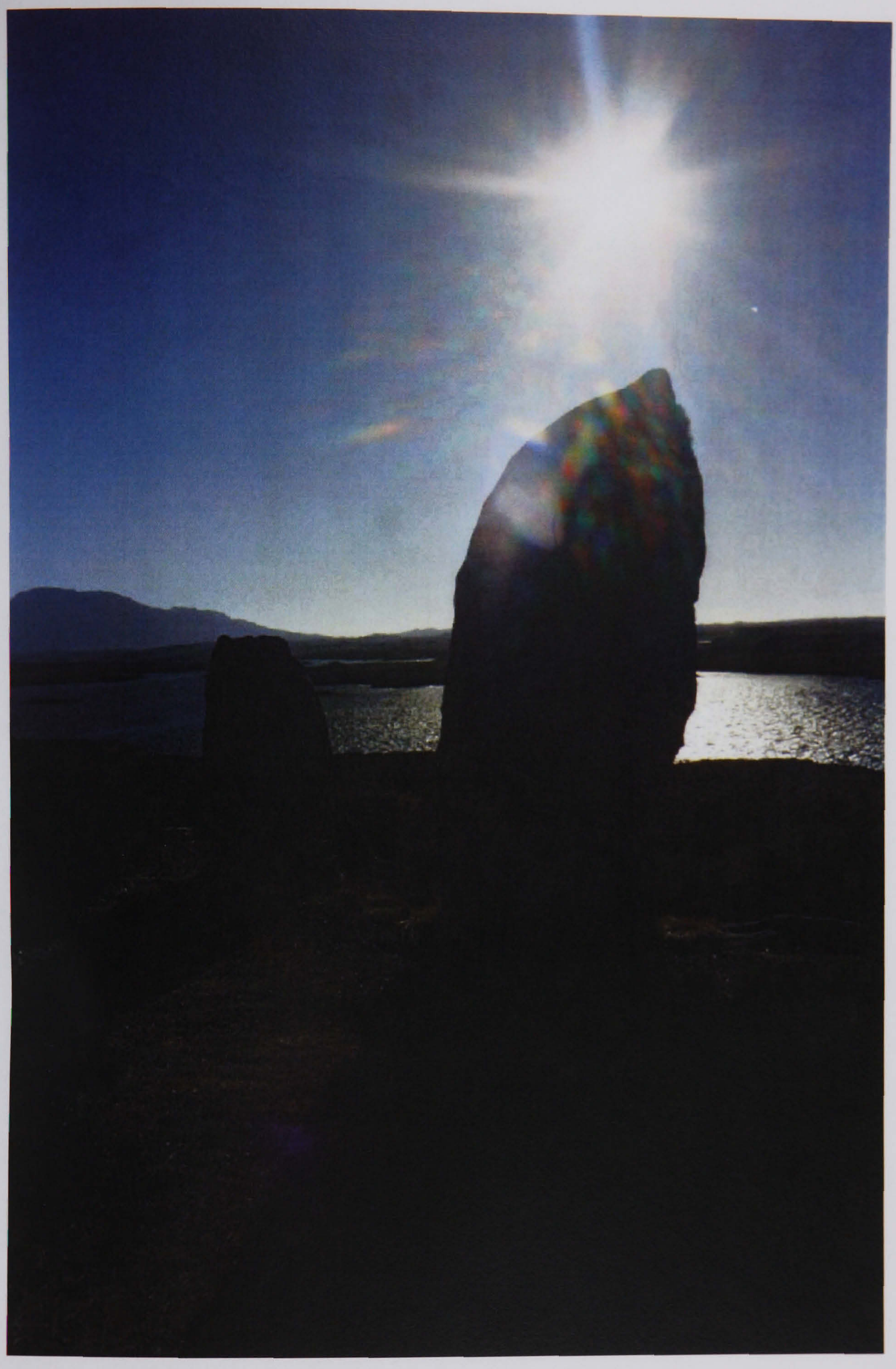
The likelihood of odds (LOD) score and percent variance explained (PVE) at the *Pitx1* locus (*Stn 336/Stn342*) is shown for each of five pelvic traits measured. For each trait, a LOD score of greater than 3.0 is considered significant by permutation testing using a chromosome wide significance threshold of $\alpha = 0.01$. Mean phenotypic values of each trait were calculated for progeny that inherited two *Pitx1* alleles from the Kelvin grandparent (KK), two *Pitx1* alleles from the Fada grandparent (FF), or one *Pitx1* allele from each (FK).

Supplementary Table 1. Effect of *Pitx1* on pelvic phenotypes within each F2 family

Trait	LOD	PVE	Phenotype means		
			KK	FK	FF
Pelvic spine number					
Family 1 (N = 66)	56.4	98.1	2.00	2.00	0.06
Family 2 (N = 36)	27.2	97.1	2.00	2.00	0.08
Family 3 (N = 38)	27.8	96.6	2.00	1.94	0.00
Family 4 (N = 37)	23.0	94.3	2.00	1.91	0.00
Left pelvic spine length					
Family 1 (N = 66)	33.6	90.8	4.35	4.21	0.17
Family 2 (N = 36)	16.4	88.5	4.82	3.70	0.21
Family 3 (N = 38)	13.2	79.9	3.78	3.37	0.00
Family 4 (N = 37)	15.1	84.7	4.00	3.50	0.00
Right pelvic spine length					
Family 1 (N = 66)	38.2	93.3	4.36	4.05	0.00
Family 2 (N = 36)	18.3	91.0	4.81	3.80	0.00
Family 3 (N = 38)	10.1	70.4	3.74	3.15	0.00
Family 4 (N = 37)	14.5	83.5	4.00	3.41	0.00
Pelvic girdle length					
Family 1 (N = 66)	28.9	87.2	5.57	5.75	0.33
Family 2 (N = 36)	22.4	94.7	6.00	5.12	0.00
Family 3 (N = 38)	13.8	81.3	4.71	4.15	0.00
Family 4 (N = 37)	14.6	83.7	5.83	5.37	0.47

The likelihood of odds (LOD) score and percent variance explained (PVE) at the *Pitx1* locus (*Stn 336/Stn342*) is shown for each of five pelvic traits measured. For each trait, a LOD score of greater than 2.2 is considered

significant by permutation testing using a chromosome wide significance threshold of α = 0.05. Mean phenotypic values of each trait were calculated for progeny that inherited two *Pitx1* alleles from the Kelvin grandparent (KK), two *Pitx1* alleles from the Fada grandparent (FF), or one *Pitx1* allele from each (FK).



"When we try to pick out anything by itself, we find it hitched to everything else in the universe."

John Muir