

Natural Selection in Three-Spined Stickleback

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Abstract

The evolution of a monophyletic group of species is known as a radiation, although it is often difficult to determine whether they are adaptive or non-adaptive (Gittenberger, 1991). Large scale radiations are often assumed to be due to adaptive radiation without further study, however there are many environmental or genetic factors which may produce the same effect.

In this thesis, three-spined stickleback (*Gasterosteus aculeatus*) from North Uist, Outer Hebrides, Scotland are used as a model organism to explore the role of predation within a suspected adaptive radiation. Firstly, an exploratory study was carried out to establish the viability of using model replica stickleback in order to infer predation rates of stickleback by trout (*Salmo trutta*). Relative predation rates could be established using this method, on the condition that it was an enclosed system without a seasonal influx of piscivorous fish.

Within the stickleback species, armour characteristics (such as dorsal spines and plates) are under selection within different populations. On North Uist, stickleback armour traits differ widely between each loch, however the exact causes are unknown. Selection on armour traits was investigated within five different populations with differing predation regimes, predatory fish or insects. It was hypothesised that lochs containing piscivorous predators would produce heavily armoured stickleback, compared to less armoured stickleback in insect predated lochs. Stickleback from the same cohort were sampled before and after winter, and morphological traits were analysed against length. There was evidence of selection in three lochs containing insect predators, as several armour traits had significantly reduced relative to stickleback length. However, there was no evidence of selection against armour traits in the lochs containing piscivorous fish as the main predator. The reduction of armour traits such as plates and spines in lochs with trout present suggests that there may

be an alternative cause, such as an environmental factor, resulting in adaptive radiations within these lochs.

Lastly, nucleotide and amino acid mutations that have occurred within stickleback populations since the end of the last ice age were investigated. Three major lineages are currently present: trans-Atlantic, Black Sea and European. With both the European and Black Sea lineages diverging from the ancestral trans-Atlantic lineage, the European sequences, which includes North Uist populations, appear to be accumulating mutations much more quickly than the Black Sea individuals. The high frequency of amino acid changes within European populations suggests that there is adaptive radiation occurring to produce some minor genetic radiations within Europe. On the other hand, the low number of haplotypes seen within the Black Sea lineage suggests that it may be the result of a non-adaptive radiation.

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Chapter 1: Introduction

There have been many definitions of an adaptive radiation, such as “the differentiation of a single ancestor into an array of species that inhabit a variety of environments and that differ in traits used to exploit those environments” (Schluter, 2000). Non-adaptive radiation can be described as speciation without significant ecological correlation and evolution of corresponding traits, often associated with allopatric speciation (Givnish and Sytsma, 1997). Non-adaptive radiations occur due to genetic drift or founder effects creating new phenotypes by chance, however adaptive radiations imply that natural selection is at work.

Whether adaptive or non-adaptive, radiations occur due to random mutations which occur in the nucleotide sequences of individuals over time: either synonymous (which do not alter the corresponding amino acids once translated) or nonsynonymous (which do alter the amino acids after translation) (Wildman et al., 2003). Deleterious amino acid mutations which reduce the survival or fecundity of an individual are usually removed from the population and silent variants, which alter the amino acid but do not cause any phenotypic changes to the individual, persisting at low levels (Loewe et al., 2006). On the other hand, nonsynonymous mutations which assist in survival or reproduction are often passed onto the next generation. In this way, mutations which contribute to the overall fitness of a population often become prevalent: the process of natural selection.

There are varying hypotheses as to how selection may work over long periods of time. It has been suggested that species are continuously under some form of directional selection, although field studies have suggested that stabilising selection and/or oscillating directional selection occurs more commonly. Stabilising selection is present when a population reaches an intermediate phenotype between two extremes, whereas oscillating selection varies in

direction and intensity over time (Ollerton and Diaz, 1999; Barnes, 1968; Gibbs and Grant, 1987). Alternatively, it has been suggested that there are many peaks across the adaptive landscape, across which populations may move between over time (Schluter, 1996). Populations of a species, which have become separated, may carry out this process of natural selection independently of each other, producing an adaptive radiation.

Adaptive radiations are known to have played a fundamental role in the evolution of biological diversity, however the underlying causes of these radiations can be difficult to understand (Rainey and Travisano, 1998). Studies of radiations that have appeared within tens to hundreds of thousands of years have suggested that divergent natural selection between differing environments plays a large part in their production (Rundell and Price, 2009). Although adaptive radiations and the role of the environment in their creation are well-understood, it can be difficult to study such radiations in the wild due to the slow evolutionary process. However, there are some species in which adaptive radiations are thought to be occurring more rapidly.

A well-known example of a species which has undergone adaptive radiation is the Hawaiian honeycreepers (subfamily Drepanidinae), which have radiated into over fifty different species over approximately 5.7 million years, both extant and extinct, occupying a wide range of niches (Lerner et al., 2011). This variety of niches has allowed for a corresponding abundance of bill morphologies, for example nectarivores with long curved bills (Smith et al., 1995). Across the islands, parallel evolution of similar traits in species that inhabit similar niches (i.e. all seed eaters possessing short thick bills) strongly suggests natural selection is occurring, as it is highly unlikely that random genetic drift would produce change correlated with the environment in different lineages.

Many other lineages have failed to undergo adaptive radiations on similar island chains such as the Hawaiian thrushes, suggesting that there are also non-

environmental factors which could affect particular lineages. For example, the timing of different colonisations, species plasticity, and founder effects can all play a role in the ability of a species to adapt and undergo a radiation (Lovette et al., 2002).

The three-spined stickleback (*Gasterosteus aculeatus*) is another well-studied species that provides an example of adaptive radiations. Stickleback are originally marine fish that have undergone adaptive radiation wherever they have invaded freshwater habitats. Changes in bony armour are an especially obvious outcome of these radiations (Taylor and McPhail, 1999). Marine fish are encased in a suit of bony spines and lateral plates (unlike many other fish species, stickleback lack scales). Marine armour is composed of a full set of 30-35 plates along their entire trunk from posterior to the head to the tip of the caudal fin, three dorsal spines (Reimchen, 1992), a bilateral pelvis which consists of a spine on each side and a pelvic girdle (Bell et al., 1993). The dorsal spines can be erected and locked in to position in order to enlarge the effective body diameter of the stickleback during a predation event (Reimchen, 1983). They may also pierce the mouthparts of a predator whilst the stickleback is being manipulated, increasing the possibility for the stickleback to escape (Reimchen, 1982). The four plates in the centre of the trunk are used to buttress the dorsal and pelvic spines and all plates protect the trunk of the stickleback from injuries from predatory fish (Reimchen, 1992). This completely plated morph is considered the ancestral state of three-spined stickleback, largely monomorphic for the marine stickleback which inhabit the Atlantic and Pacific oceans (Reimchen, 2000).

During adaptive radiation, stickleback in many populations lose elements of their armour, leading to the emergence of 'complete', 'low' and 'plateless' morphs through reduction in the size and number of parallel plates (Figure 1). The partial morph has lateral plates towards both the head and caudal fin, separated by a region without any plates. A low morph only has a single row towards the head (Bell, 1981). Alongside this reduction in plates, the spines and

pelvis are often found greatly reduced or absent in many radiations (Bell and Ortí, 1994; Chan et al., 2010). This difference in armour can be so drastic that the different morphs were originally classified as different species (Cuvier, 1829).

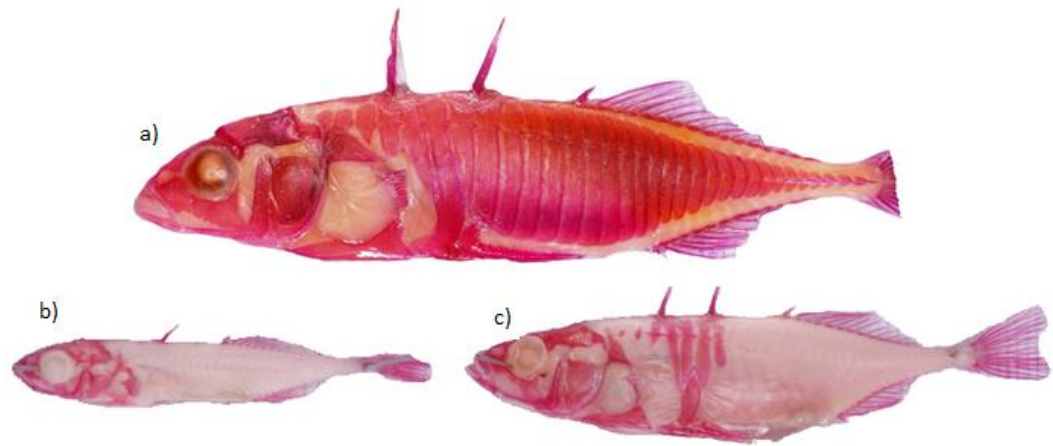


Figure 1: a) Complete b) plateless and c) low morph stickleback after being stained with alizarin red S in order to highlight the differences in bony armour.

Although the genetic basis for the reduction of armour appears to be similar in the majority of populations, it is highly unlikely that a low-morph population founded all other populations through migration. Instead, it is more likely that the alleles which control for the reduced phenotypes are present at low levels within the marine population (Colosimo et al., 2005). This low frequency is thought to be maintained by hybridisation that may occur between anadromous and freshwater stickleback in coastal streams and lakes during the breeding season (Colosimo et al., 2005). Ectodysplasin-A (*Eda*) has been found responsible for the high variation in plates (Colosimo et al 2005; Barrett, 2010), with mutations in the *Eda* gene affecting skeletal structure and scale formation in a diversity of species such as zebrafish, mice and even humans (Harris et al., 2008; Song et al., 2009; Hill et al., 2002). In stickleback, two complete *Eda^C* alleles results in a complete morph phenotype, whereas those with two low *Eda^L* alleles tend to be low morphs, with heterozygous hybrids resulting in low, partial or complete morphs (Marchinko, 2009). Additionally, linkage disequilibrium is thought to occur, with genes at four additional loci

contributing in addition to *Eda* to minor variation in plate size and number (Colosimo et al., 2005).

There have been several different hypotheses as to the selective advantage of armour reduction in anadromous and freshwater stickleback. For example, it has been suggested that the different habitats found in freshwater compared to marine environments may require higher body flexibility, or a change in swimming performance (Colosimo et al., 2005). Low calcium levels have also been described as a contributing factor to reduced armour coverage in freshwater stickleback, with low levels inhibiting full skeletal development (Giles, 1983; Bell et al., 1993). Lastly, predators differ greatly between marine and freshwater environments, with a correlation noted in many studies between predator density and presence of armour traits. Those from marine and freshwater environments with a large number of piscivorous fish and birds are often heavily armoured, in comparison to those with aquatic insect predators present which tend to be low plated (Marchinko et al., 2009; Reimchen and Nosil, 2002; see Chapter 3).

Piscivorous fish, such as the brown trout (*Salmo trutta*) and salmon (*Salmo salar*), along with a variety of bird species, commonly feed on stickleback. Reimchen (1991) observed two phases that occurred during predation events by trout: pursuit and, if successful, manipulation. The 'pursuit' stage involves three potential outcomes: prey rejection (the trout veers away from the stickleback); prey escape (stickleback evades capture) or capture (trout catches the stickleback in its jaws). If captured, there are again three potential outcomes in the manipulation stage, when the trout tries to swallow the stickleback: prey rejection (the stickleback either escapes or is released and not pursued further); prey escape (the stickleback escapes and reaches safety before being recaptured by the trout) or eaten (the stickleback is swallowed by the trout).

During the manipulation stage, the probability of the prey escaping and the chance of oesophageal abrasion for the predator is greatly increased as the size of stickleback approaches the gape-limit of the trout. As with many piscivorous predators, it is advantageous for trout to swallow the stickleback headfirst. Spines, fin rays and the operculum would flatten during headfirst swallowing, whereas they may cause the stickleback to lodge in the oesophagus if swallowed tail-first. Additionally, stickleback regularly try to escape once caught by a predator using 'strong body flexures and thrusts from the caudal fin' (Reimchen, 1991). If orientated headfirst, any attempts to escape would only serve to propel the stickleback further into the mouth of the trout. On the other hand, a tail-first orientation would propel the stickleback out of the trout's mouth, greatly increasing the chance of escape for the prey. Because of this, trout preying on stickleback tend to attack from a headfirst orientation. If this fails, trout often try to manipulate the stickleback into the correct alignment. As the size of the stickleback increases to the maximum gape limit of the trout, the handling time increases substantially, producing greater opportunity for the stickleback to escape (Reimchen, 1991).

Reimchen (1991) also observed a large increase in the number of injuries amongst the largest stickleback present within his study. This increase in injury number could be correlated to the higher chance of escape compared to those of a smaller size, due to the gape limit of predators. Alternatively, this could also be related to the age of the prey – larger individuals would tend to be older and therefore would have had more opportunity to acquire injuries from failed predation attempts. In his study, Reimchen (1991) established that the majority of stickleback escapes were due to rejections by the trout in both the pursuit and manipulation phase, which was in turn strongly correlated to body size of the stickleback. This rejection by predators due to the size and presence of armour traits on the stickleback prey is widely accepted as one of the main causes of the adaptive radiations in stickleback.

One such radiation of three-spined stickleback occurs on the island of North Uist, an island in the Outer Hebrides, off the coast of Scotland. Approximately 16,000 years ago, numerous lochs of varying depth, pH, nutrient levels, availability of resources and presence of predators were colonised by marine stickleback with the retreat of the covering ice sheet (Magalhaes et al., 2016). With so many ecological variables between lochs, North Uist allows for in-depth studies of the potential adaptive radiation occurring within stickleback.

As previously discussed, trout are known to frequently prey on stickleback where their habitats overlap (Reimchen, 1991), and it is well established that there are populations of trout in North Uist lochs. Although they are considered to be one of the main predators of stickleback on the island, it is so far unknown to what extent this occurs due to the logistical difficulty in studying predation in aquatic environments without using destructive methods such as stomach content analysis. As previously described, predation can be an important factor in generating adaptive radiations, and it is therefore important to establish predation rates within North Uist lochs. Chapter 2 focuses on the use of wax model stickleback to estimate attack rates by trout in different lochs on North Uist. It is hypothesised that the stickleback models will be left with tooth imprints in lochs which trout are present, with a higher frequency of bites being observed in those lochs with a higher density of trout.

The objective of Chapter 3 is to establish whether selection is occurring within generations of stickleback in various environmental conditions. If morphological changes are occurring, this suggests that the population of stickleback on North Uist is still undergoing a radiation. A morphological comparison of two samples of a single cohort of stickleback collected six months apart (before and after winter) is used to quantify selection on armour traits that exist with and without predatory trout present. It is predicted that, in those lochs with trout present, stickleback will survive overwinter if they have larger armour traits i.e. spines and number of plates, whereas those with

fewer defenses against predation will be selected against and removed from the population.

Lastly, the objective of Chapter 4 is to establish how genetically related the North Uist lineage of stickleback are to other populations. Genetic sequences for individuals on North Uist are added to sequences from a previous study by Mäkinen and Merilä (2008) in order to look at patterns of relatedness across the stickleback species. Compared to three different lineages (European, Black Sea and trans-Atlantic) it is predicted that the sequences from North Uist populations will be most closely related to the European lineage. As North Uist populations are thought to be undergoing adaptive radiation, it is also predicted that the North Uist freshwater populations will be further separated from the marine European lineage.

Chapter 2: Investigating Predator-Prey Relationships Between Stickleback and Trout Using Model Replicas

2.1 Introduction

The interactions between predators and prey are very important in driving the population dynamics of individual species, structuring ecological communities and driving the evolution of various attack and defence strategies (Drossel et al., 2001; Yoshida et al., 2003). However, except in some large mammals where predation can be directly observed (Creel et al., 2014; Trainor et al., 2014; Foster et al., 2013), it can be difficult to estimate predation rates in natural settings. Cryptic, nocturnal, aquatic species and those with a large range may be very difficult to find and observe for long periods of time.

With modern genetic techniques, it is now possible for many species diets to be analysed using non-invasive techniques such as DNA barcoding in faecal samples (Jumabay-Uulu et al., 2013; Pastor-Beviá et al., 2014; Brown et al., 2014). Although such techniques are highly effective for undertaking dietary studies, the logistics of retrieving faecal samples from some terrestrial and the majority of aquatic species do not facilitate the use of such techniques.

An alternative method is the use of destructive sampling methods, where DNA barcoding or visual analysis of remains can be carried out (Jo et al., 2013; Zeale et al., 2010; Rougerie et al., 2011). However, removal from a population of large numbers of individuals, particularly apex predators, can have serious repercussions for the functioning of ecosystems. In order to prevent this, some studies have been carried out with aquatic species using stomach flushing instead of destructive sampling, however this is only possible with larger species with a high survival rate after capture (for example, Barnett et al., 2010). The importance of understanding interactions between predators and

prey within an ecosystem cannot be overstated, so it is important to develop methods that reduce the need for destructive sampling on a large scale.

The use of replica models is a common method of quantifying predation, in particular within reptiles, amphibians and small rodents, but there have only been limited previous studies carried out within aquatic environments (Thompson et al., 2000; Caley and Schluter, 2003). This method involves producing models of a prey species from a malleable material that will record an impression if attacked by a predator. Replicas are inexpensive and easy to produce in large numbers, and often allow the distinction between multiple potential predators (Hinman et al., 1997). On the other hand, some predator impressions may be difficult to distinguish, particularly if the study requires fine-scale identifications (i.e. down to species). Additionally, rates of attack gained from such a study may not reflect actual levels of predation. This is due to the fact that replicas, regardless of accuracy, will not have many of the characteristics and behaviours of real prey, such as predator avoidance and defence (Low et al., 2014). Despite these drawbacks, model replicas are often considered a viable method for assessing relative rates of predation across varying environments.

The aim of this study was to establish the viability of using model replicas to investigate the predator-prey relationship between stickleback and trout in different environmental conditions on North Uist. More than half of North Uist is covered by water (fresh, salt or a mixture of fresh and tidal salt water), with countless different environmental conditions found on the island. There is a large variation in pH associated with varying concentrations of alkaline metals such as: magnesium, sodium and calcium. Adult mortality of stickleback in acidic lochs is high, with few reaching more than one year old, whereas those in alkaline lochs can be found up to three years old. This difference in life expectancy, in addition to the many environmental factors that vary between lochs, has resulted in a divergence of mean body size between populations (MacColl et al., 2013).

Brown trout (*Salmo trutta*) are the most common predatory species found across the island, which are known to feed on stickleback (MacColl & Aucott 2014). Other predators do also occur such as: European eels (*Anguilla anguilla*), arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*) and sea trout (*Salmo trutta trutta*), as well as a few invertebrate predators such as water beetles (*Dytiscus semisulcatus*) (MacColl et al., 2013).

2.2 Methods

2.2.1 Wax Models

Stickleback replicas were made using paraffin wax coloured brown by combining it with melted brown Crayola crayon. Plaster of Paris moulds were taken of frozen anadromous and freshwater stickleback in order to produce the wax replicas. The models were evenly divided into 'big' and 'small' categories: the big models moulded from a large, heavily plated marine stickleback, and the small models moulded from a smaller, un-plated freshwater stickleback. The big models were approximately 6.5cm in length, with 0.4m long dorsal spines. The small models were approximately 3.5cm in length, with no dorsal spines present. Before the paraffin wax was poured into the moulds, fishing line was passed dorso-ventrally through the mould to attach the replicas to anchoring lines, and a polythene tail was inserted at the posterior end. Once the wax was poured and the mould had cooled, excess wax was removed and the polythene tail was trimmed to a suitable length. Control wax pieces with a broad conical shape of a similar size to the models, but lacking any obvious resemblance to stickleback, were made using the same method, using the bottom of a 50ml falcon tube as a mould. These were used to establish whether trout will attack any neutrally buoyant object.

2.2.2 Model Lines

Eight wax models were attached to each 40m anchoring line made of green garden twine. The eight models (four big and four small) evenly spaced along the twine, weighed down with fishing weights at set intervals of 2m (Figure 2),

with 11m of twine either side to allow the twine to be secured to either side of the shore. The different models were set to be at either 10cm or 20cm from the bottom of the loch, determined using a random number generator, as it was unknown if depth would affect predation rate. Pictures were taken of each model, and its string and position number were recorded to allow a comparison after they had been set in the lochs. Figure 1 illustrates how the lines were set once underwater. As the models were made of paraffin wax, they floated once in the water.

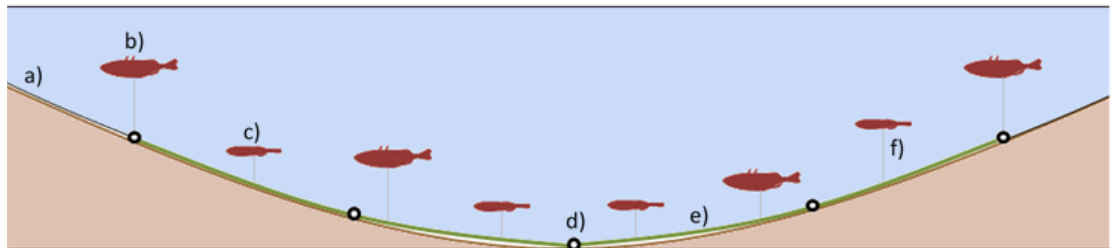


Figure 2: Layout of the model lines underwater: a) string attached to buoyancy aid b) large stickleback model c) small stickleback model d) fishing weights e) twine f) transparent fishing line.

Each line was attached to a buoyancy aid on either end, to prevent loss and to aid their removal after the soaking period. Transparent rather than opaque fishing line was used to attach the model fish to the twine, as this was thought to give a more realistic visual of a floating stickleback.

Model lines were placed in thirteen different lochs (see Table 1), strung across narrow bays, in water 0.5-2m deep. Great care was taken to ensure that the wax models were not scratched during the setting process. The ends of the lines were then anchored on dry land at either end. Lines were left in place for 24 hours. Two of the lochs selected for sampling (BUAI and CHRU) were known (from gill netting surveys) not to contain any trout or other predatory fish. A third loch (CEIT) was thought unlikely to contain trout because of its small size and lack of salmonid breeding habitat. The remaining lochs were known to contain resident populations of brown trout of varying densities. Three of these (DUIN, SGEA and TROS) are also accessible to migratory salmonids and

potentially to other marine predatory fish. Brown trout density in lochs containing trout was estimated as a fifty-year average of number of trout caught per angler per hour in fly fishing competitions by the North Uist Angling Club (MacColl & Aucott 2014). Wax shapes were added to a repeat line in SGEA, due to its high attack rate during its first sampling, alongside two anadromous and two freshwater models, to establish whether trout would attack any shape. A repeat line was also placed in nan Strùban to see if the attack rate would remain similar.

Table 1: Names and locations of lochs in which lines of model fish were set in April/May 2016. Trout density is estimated as number of trout caught per angler per hour.

Name	Code	Coordinates	Number of Lines Set	Trout Density (CPUE)
Fada	FADA	57°36'N 7°12'W	1	0.80
Scadavay	SCAD	57°35'N 7°14'W	1	1.29
Sgealtair	SGEA	57°35'N 7°12'W	2	0.07
Tormasad	TORM	57°33'N 7°19'W	1	0.22
a'Bharpa	BHAR	57°34'N 7°17'W	1	0.26
Chadha Ruaidh	CHRU	57°36'N 7°12'W	1	0.00
Maighdein	MAIG	57°35'N 7°12'W	1	0.57
nan Strùban	STRU	57°34'N 7°21'W	2	0.51
na Buaile	BUAI	57°38'N 7°11'W	1	0.00
Ceithir Eileanan	CEIT	57°39'N 7°26'W	1	Unknown
na Moracha	MORA	57°34'N 7°16'W	1	2.29
Duin	DUIN	57°38'N 7°13'W	1	0.10
Trosavat	TROS	57°35'N 7°25'W	1	0.11

2.2.3 Analysis

After the lines were collected, the wax models were immediately checked under a microscope for signs of predation. Bite marks were usually easily distinguishable from scratches or other marks sustained on rocks etc. whilst

the models were in the lochs, however they were only counted as attacked if they had at least two consecutive holes (tooth marks). Additionally, the position of the bites on the body were recorded. In order to remove observer bias, the presence or absence of trout in each loch was unknown to the person checking the models until after they had been checked for attacks. In order to establish if there was any correlation between CPUE data and estimated attack rate from this study, data was analysed in GenStat (Payne et al., 2009) using Generalised Linear Models. GLM's were carried out in GenStat to establish patterns within the data (see Table 2). The probability of attack (0 or 1) was modelled as a function of size (length) of model, distance from loch bed and position on string, using binomial errors and a logit link function.

In addition to the visual checks, a waterproof camera was placed in Sgealtair during the second line that was set, due to its high attack rate during the first. Due to the visibility within the loch and the depth of the water, it was only possible to film one model close to the shore at a time. The cameras did not manage to capture a trout attacking the models, however trout were visible within Sgealtair in the same location as the models.

2.3 Results

Models were recorded as attacked in all the lochs in which trout are known to be present (twelve out of fifteen lochs), with BUA1, CHRU and CEIT having no recorded attacks. 46 out of 120 (38.3%) models were attacked, with the majority of these being small models (60.9%) rather than big models (39.1%). The number of attacks on an individual line ranging from 1 to 7, however this was insignificant in a chi square test ($\chi^2_2 = 2.38$, $N=46$, $p<0.95$).

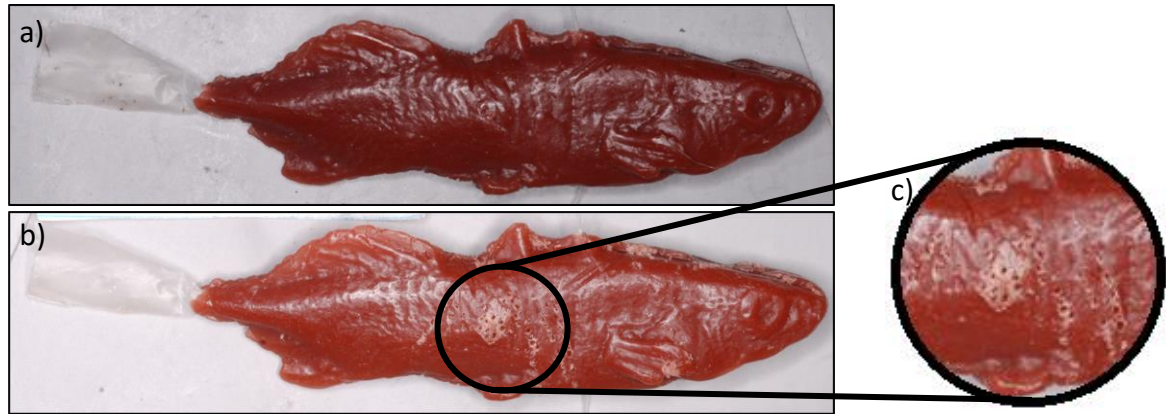


Figure 3: Model stickleback 12.8 a) before and b) after the line was set in DUIN. Trout tooth marks are visible along the body of b), shown in close up in c).

The ‘shapes’ were removed from the analysis due to the small number present in this exploratory study. All four shapes in SGEA were attacked, compared to only three of the stickleback models being attacked on the same line.

Table 2: Generalised linear model of the probability that models were attacked. All values were fitted into the same model; the shapes were removed from this analysis due to the small number used.

Parameter	Wald Statistic	d.f.	P. Value
Length (Excluding Tail)	6.00	1, 112	0.01
Distance from Centre of String	0.38	1, 111	0.54
Depth	1.21	1, 111	0.27
Position	0.40	1, 111	0.53
Length (Exc. Tail) * Location	1.353	1, 111	0.25
Length (Exc. Tail) * Distance from Lake Bed	0.642	1, 111	0.42

In a model of average attack rate in each loch, neither trout density ($F_{1,12}$, 1.262, $P=0.287$) nor location ($F_{1,12}$, 0.099, $P=0.753$) had a significant effect on attack rate. Attack rate was calculated as the percentage of stickleback attacked per string (Figure 3). However, when lochs that are connected to the sea (DUIN,

SGEA and TROS) were removed from the analysis, trout density had a significant effect on attack rate ($F_{1,9}$, 9.57, $P=0.017$).

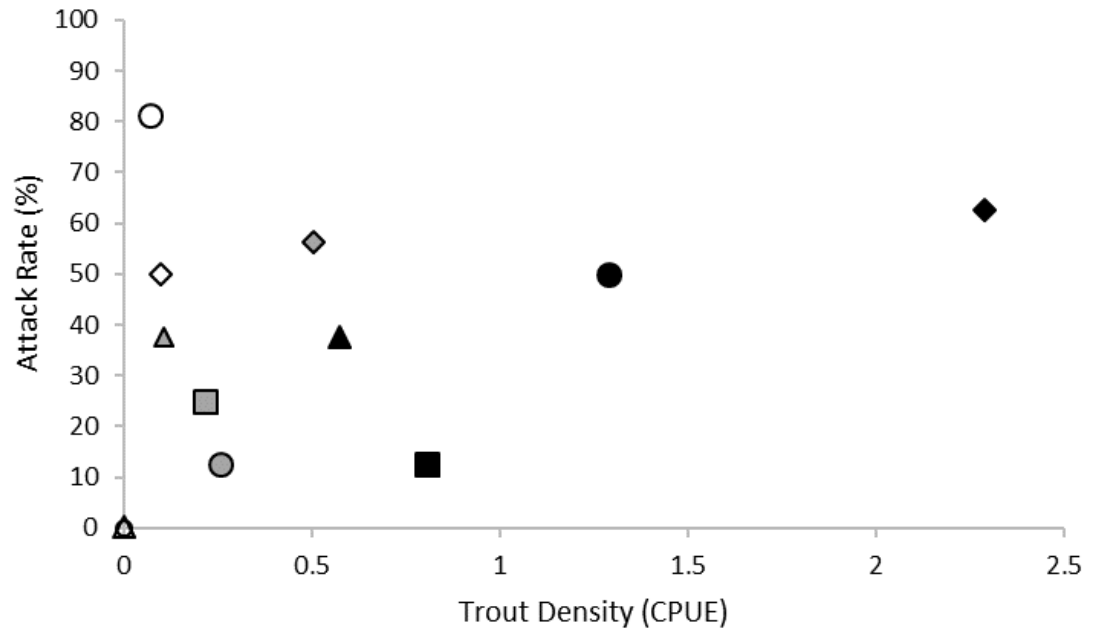


Figure 4: Attack Rate (%) of models in each loch against Trout Density (CPUE): CHRU $\triangle$; BUAI $\square$; SGEA $\circ$; DUIN $\diamond$; TROS $\triangle$; TORM $\square$; BHAR $\bullet$; STRU $\diamond$; MAIG $\blacktriangle$; FADA $\blacksquare$; SCAD $\bullet$ and MORA $\blacklozenge$. Attack rates for STRU and SGEA are the mean rates of the two lines set in each loch. Trout density of CEIT is unknown, therefore has not been included.

The location of bites on the body of the stickleback models were compared, either as 'mainly front' or 'mainly back', as bites tended to be present on both. The majority of attacked models (78.0%) had bites on both the front and back of the model, with the large majority of these models being attacked mainly on the front half ($X^2_2 = 5.48$, $N=41$, $p<0.05$). Again, the additional shapes were removed from the main analysis (Table 2 and Figure 4).

2.4 Discussion

The aim of this study was to establish the viability of using replica models of stickleback to gain an insight into predator-prey relationships in the lochs on North Uist. The results show that stickleback (or prey of the same size and

shape) are commonly attacked in all lochs on North Uist where trout are known to be present.

Firstly, although there are a number of potential predators found in some lochs such as eels (*Anguilla Anguilla*), there is strong evidence to suggest that the predators which attacked the replicas were trout. All of the imprints left on the models were similar (see Figure 2) and were small, deep indents suggesting teeth marks. Due to the presence of dorsal and pelvic spines on the majority of stickleback, trout prefer to approach potential prey items headfirst, so the spines fold down and the risk of choking is reduced (Reimchen, 1991; see Chapter 1). This attack behaviour which has been observed in trout and other species, matches with the large number of bite marks that were seen on the main body of the replicas, significantly more often towards the head end. This suggests the bite marks are due to the presence of predatory fish. Secondly, two of the lochs where lines were placed (BUAI and CHRU) are known to contain stickleback but no trout. Lines set in these two lochs, along with CEIT where the absence of trout is suspected but unconfirmed, were the only lines not to receive any attacks. This also supports the idea that brown trout are one of the main predators of stickleback on North Uist.

The smaller, freshwater stickleback models were preyed upon significantly more than larger models, suggesting that they are generally preferred as prey by trout. This could be due to several factors including the fact that trout have been found to prey on smaller food items in order to reduce handling time and risk of choking (Reimchen, 1991; Sánchez-Hernandez and Cobo, 2015; see Chapter 3).

The shapes were added as controls at a late stage to determine whether it was a particular shape or simply the presence of a novel shape that attracted attention from predators. As all of the shapes tested in this study were attacked, this could suggest that trout test all unfamiliar objects as potential prey. Alternatively, it could be the experience of many trout that most objects

of that size found in the water column are stickleback, resulting in the trout assuming the shapes are also prey. As these controls were only tested in SGEA, with a high mean attack rate of 81.3%, a wide range of different shapes would need to be tested in different lochs of both high and low attack rate in order to establish whether the attack rate of control shapes in lochs is consistent with that of the models.

Attack rate and estimated trout density did not correlate in this study when all lochs were taken into account. However, once the lochs that are connected to the sea were removed from the analysis, this became a significant correlation. This could be due to the fact that many salmonid species migrate into freshwater for breeding. The Atlantic salmon (*Salmo salar*), for example, lays eggs in freshwater stream beds, where juveniles remain until they migrate downstream to marine waters in the late spring (Metcalf and Thorpe, 1990). Over twenty different species of marine fish have been known to enter freshwaters in the Outer Hebrides (Campbell and Williamson, 1979), potentially drastically increasing or decreasing predator density in these lochs at the time of the study from the CPUE estimates used here. Further study would allow for the effect of seasonal changes in predator density, due to migratory behaviours, to be determined.

Attack rates measured in this study may not reflect the mean attack rate across the entire loch. First of all, lines were only set in one location in each loch. Trout may be present in different densities in different habitats within the loch, as well as during different times of the year. Lochs on North Uist can reach extremely large sizes, e.g. SCAD, with habitats and environments varying throughout the loch. Therefore, the single lines set in this study may not be representative of the whole loch. Distance from the loch bed was not significant to the rate of attack, which may be due to the fact that the lines were all set in relatively shallow water (0.5-2m), or because the distances used (10 and 20cm) were not significantly far apart enough to cause a difference in

predation. Further work would be required to establish whether location or depth plays any role on rate of attack, due to higher density of predatory trout.

Although the exploratory study appears to suggest a possible method for determining relative trout density in a loch, there are also several factors that may cause differing attack rates than if live stickleback were used as prey. Wild stickleback in the same habitat as trout will, for example, have both a learned and a genetic behavioural response which is carried out upon sight of a predator. There is a known correlation between density of predators and strength of this anti-predator response, which also extends to general aggressiveness and boldness behaviours (Wund et al., 2015). It is possible that due to a heightened response behaviour in predator-rich environments, successful attacks may be proportionally less frequent than in predator-poor lochs. Secondly, Hagen and Gilbertson (1972) found that mean spine length of stickleback was longer in populations sharing habitat with piscivorous predators. Moodie (1972) also found stickleback remains in the gut of trout were significantly shorter than those expected from a representative sample. Due to the practicalities of making a wax model stickleback, the spines may not have been proportional to those standard in each loch tested, either reducing or increasing the probability of a trout attacking. Lastly, a study by Stuart et al. (2012) looked at the effect of visual contrast on predation behaviour: whether avian predators attacked indiscriminately upon detection, or whether there is a behavioural decision made. Although visual contrast was not looked at in this study, it is unlikely that visibility of the wax models matched that of a live stickleback in the lochs, potentially decreasing or increasing the chance of attack by a predator.

As this was an exploratory study, only one line was set in the majority of sampled lakes, with two exceptions (SGEA and STRU). In order to increase the power of this study, more replicates would be needed in each loch, in different locations, times and conditions. Further study may reveal more subtle patterns

in the predator-prey relationship between trout and stickleback, such as the effect of season or water visibility.

Although a commonly used method among reptiles, amphibians and small mammals, the use of model replicas in aquatic environments is relatively uncommon for studying predator-prey interactions. Frequently used to study aposematism or the trade-off between sexual selection and conspicuousness, many studies using model prey have produced similar results, with attack rates significantly higher in certain replicas as hypothesised by ecological theory (Husak et al., 2006; Watson et al., 2012; Vervust et al., 2007). Antiapostatic selection can often occur, where rare or unfamiliar prey is predated proportionally more often than common prey (Lindström et al., 2001). A previous study of attacks on amphibian models found this occurring, with novel phenotypes of prey items being attacked more frequently (Noonan and Comeault, 2009). It is possible that a similar effect may be occurring with the models, due to the differences between real and model stickleback, however this could still allow for relative densities to be estimated.

This exploratory study suggests that this method would be suitable for further in-depth study, suggesting a reliable method for estimating relative trout density. By increasing the sample size in various locations, times, weather conditions and even seasons, a far clearer picture of relative predation rates for North Uist stickleback in different lochs could be gained. Not only does predation rate have a tremendous effect on behaviour, it plays a vital role in the evolution of a prey species. As discussed in Chapter 3, the presence or absence of trout predation for three-spined stickleback (studied across their entire range for their rapid divergence) could be a significant factor in their rapid evolution.

Chapter 3: Selection on Phenotypic Traits in the Presence of Differing Predation Regimes

3.1 Introduction

Schluter (2000) defined adaptive radiation as 'the differentiation of a single ancestor into an array of species that inhabit a variety of environments and that differ in traits used to exploit those environments'. In order to distinguish this process from other forms of divergence, Schluter (2000) established a set of criteria which must be detected within a set of populations in order for it to be regarded as an adaptive radiation. Firstly, 'common ancestry' must be shared by all species in the radiation. Secondly, there must be a 'phenotype-environment correlation': a strong association between the morphological and physiological traits present and the specific environments in which they are found. This implies that any phenotypic differences must have a genetic basis, and not simply be a plastic response to the environment experienced during development. Thirdly, 'trait utility' must be evident by the trait consistently demonstrating the ability to improve an aspect of an individual's life (i.e. survival, fecundity).

Schluter's phenotype environment correlation suggests that differences between adaptive radiations are due to natural selection arising from diverse ecological conditions, when specific phenotypes have significantly higher survival or reproductive rates than others (Schluter, 2000). Natural selection acts by changing the mean distribution of phenotypes. This change is often towards the most rapid rise in mean population fitness, rather than the highest possible fitness potential of a population (Schluter, 2000). Alternatively, the variation in radiations might arise not by natural selection, but simply from genetic drift or founder effects sampling the ancestral phenotypes randomly. In this case, radiations of populations cannot be considered adaptive (Schluter, 1996).

Three-spined stickleback (*Gasterosteus aculeatus*) are found across the northern hemisphere, in marine and freshwater environments (Taylor et al., 2006). They have repeatedly radiated into diverse morphologies following colonisation of freshwater environments from the sea. Armour is particularly variable between stickleback radiations, and is often assumed to be the result of divergent natural selection (Barrett et al., 2008). This armour comprises a number of elements (spines, plates and pelvis), all of which vary greatly between populations, even in close geographical proximity.

Unlike many other fish, stickleback lack scales, instead having bony lateral plates which protect the body from injury during predation and buttress the dorsal spines. The pelvic fins are also modified into a spined pelvic girdle, and in addition stickleback have three dorsal spines. All spines can be locked outwards when the stickleback is attacked, and these, plus the lateral plates, provide protection against predation (Reimchen, 1992; Bell et al., 1993). Marine stickleback, the ancestors of freshwater populations, are typically large, with long spines in relation to their length and have between 30 to 36 lateral plates along each side of the body. This is known as a complete morph. In contrast, derived freshwater stickleback tend to have fewer plates, smaller spines and sometimes reduced pelvises (Barrett et al., 2008).

Several hypotheses have been proposed to explain this variation in armour among stickleback populations. It has been observed that marine and lake habitats with a high abundance of piscivorous fish and birds often contain heavily armoured stickleback, compared to those with few or no predators. Dorsal and pelvic spines both serve the purpose of increasing the size (effective diameter) of the stickleback. This makes it more difficult for gape-limited fish and birds to swallow them, and increases the chance of injury to the predator (see Chapter 1, Marchinko, 2009). This is consistent with the observation that predators often feed on prey smaller than would be expected based on those present (Hansen et al., 2014). It is thought that up to 90% of attacks on

stickleback by predatory fish fail, mainly due to the spines (Reimchen, 1992). Thus, in the presence of gape-limited predators, it is beneficial for stickleback individuals to have large spines. On the other hand, in the absence of such gape-limited species, insects tend to be the dominant predator. Unlike birds and fish, insects are not gape-limited, instead capturing juvenile stickleback by holding on to their spines (Miller et al., 2015). Insects predominantly prey on juvenile individuals, as larger stickleback tend to be stronger and therefore often escape predation. Whilst in the juvenile stage, individuals with reduced armour may also have a growth advantage, reaching the size threshold to escape predation more quickly than slow growing individuals with more robust armour (Barrett et al., 2008).

Alternatively, it has been hypothesised that reduction of armour may occur irrespective of the risk of predation. Giles (1983) proposed the 'calcium limitation hypothesis', in which the concentration of calcium within an environment greatly affects development of armour. It has also been suggested that the number of lateral plates will decrease where there is a high probability of escape, such as areas with a high availability of shelter, or where prey and predator swimming speeds are closely matched (Reimchen, 1992). Reimchen and Nosil (2002) also found evidence of selection on a short-term basis due to seasonally changing ecological pressures. A stickleback population on the Queen Charlotte Islands, western Canada, was found in the presence of both gape-limited avian piscivores and grappling insect predators. Over fifteen years, there was evidence of selection for an increase in spine number during the winter when birds were the main predator presence, and selection for decreased spine number during the summer when the main predators present were the insects (Reimchen and Nosil, 2002).

The aim of this chapter is to investigate natural selection on armour traits in lochs on the Scottish island of North Uist, and whether predation might play a role in this. After the last deglaciation, a large number of the 180 lochs on North Uist were colonised by marine stickleback. Geographically isolated from each

other for approximately 16,000 years, phenotypic and genetic divergence has been widely recorded between these populations (Magalhaes et al 2016). Between these lochs there is strong variation in environmental factors, particularly pH and alkaline metals such as sodium, magnesium, potassium and calcium. These metals are key in many physiological processes including osmoregulation and bone formation (MacColl et al., 2013). The lochs also vary greatly in levels of predation, competition and resource availability, all thought to play a role in causing divergence between isolated populations (Magalhaes et al., 2016).

Stickleback on North Uist are found in the presence of both trout and insect predators, including the larvae of Dytiscus water beetles and dragonflies. These predators are often found in the same loch however, as trout also prey on the insects and greatly reduce their density, lochs either contain primarily trout and a few insects, or no trout and an abundance of insects. Although both are thought to play a role in the adaptation of populations (i.e. robust armour or a lack of armour traits), any adaptations occurring in a population with both predators' present would be overwhelmingly due to the presence of trout.

To detect selection on these armour traits during a single generation, stickleback collected in the summer (August 2015) from five lochs with contrasting predation regimes (with and without trout) were compared with matching stickleback samples collected the following spring (April/May 2016), at the beginning of the breeding season and before recruitment of the new cohort. Armour trait differences between these samples can therefore only be due to natural selection or growth. The latter possibility was controlled for by examining the relationship between armour traits and overall size.

The main hypothesis for this study is that there will be phenotypic differences in armour traits within the single cohort between the two sampling periods, depending on the presence of trout or insects as the main predators. Lochs in which trout are present will show signs of an increase in armour, with those

most heavily armoured individuals surviving between the two sampling periods. On the other hand, if insects are present in large numbers, there will be a higher survival rate in less armoured individuals. For the North Uist adaptive radiation to fully satisfy Schluter's (2000) criteria, it is also expected that natural selection should favour reduced armour most strongly in populations with least armour, giving rise to a positive correlation between armour and the magnitude of selection.

3.2 Methods

3.2.1 Data Sampling

Fish were sampled from five lochs (Figure 5) in August 2015 and April/May 2016 (Table 1), using unbaited minnow traps set in water approximately 0.5-2m deep along the shoreline (Gee traps, Dynamic Aqua, Vancouver, Canada) and left for approximately 24 hours. Three of the lochs had insects but no trout and the other two had trout but few insects (Magalhaes et al., 2016). Once the traps were collected, all individuals from each loch were humanely killed as per Schedule 1 of the Animals (Scientific Procedures) Act 1986, by an overdose of MS222 anaesthetic, before being stored in 70% ethanol.



Figure 5: Map of North Uist with the location of the sampled lakes, the drawings indicate the presence of either insects or trout as the main predators in each lake.

Table 3: Name, code, location and number of samples collected from each of the sampling sites on North Uist.

Lake	Code	Coordinates	Number of Fish	
			August 2015	May 2016
na Buaille	BUAI	57.647980, - 7.196565	68	71
Ceithir Eileanan	CEIT	57.578412, - 7.256497	67	66
na Reival	REIV	57.605683, - 7.496097	12	14
Scadavay	SCAD	57.584570, - 7.235863	68	43
Tormasad	TORM	57.563128, - 7.315407	14	27

3.2.2 Fish Staining

As the stickleback had been stored in 70% ethanol for transport from North Uist, they were re-hydrated in varying concentrations of ethanol to distilled water: 70% ethanol -> 50% ethanol -> 30 ethanol -> 0% ethanol. The fish were then fixed in a 10% buffered neutral formalin for 24 hours, before being soaked in distilled H₂O for 3 hours. Individuals were rinsed twice in distilled H₂O in order to remove any residual formalin. The stickleback were then soaked in a 1% KOH solution for 1 hour and incubated in a bleaching solution overnight (5ml of 33% H₂O₂ in 1L KOH). Finally, in order to stain the bone, the fish were then added to alizarin red S stock solution and 1% KOH (1:50) before being stored in 40% isopropyl alcohol (propan-2-ol).

3.2.3 Armour Analysis

Once stained, the left side of each fish was photographed. Images were scaled and measured using the software tpsDig232 (Rohlf, 2016). The continuous traits shown in Figure 6 below were measured for each stickleback.

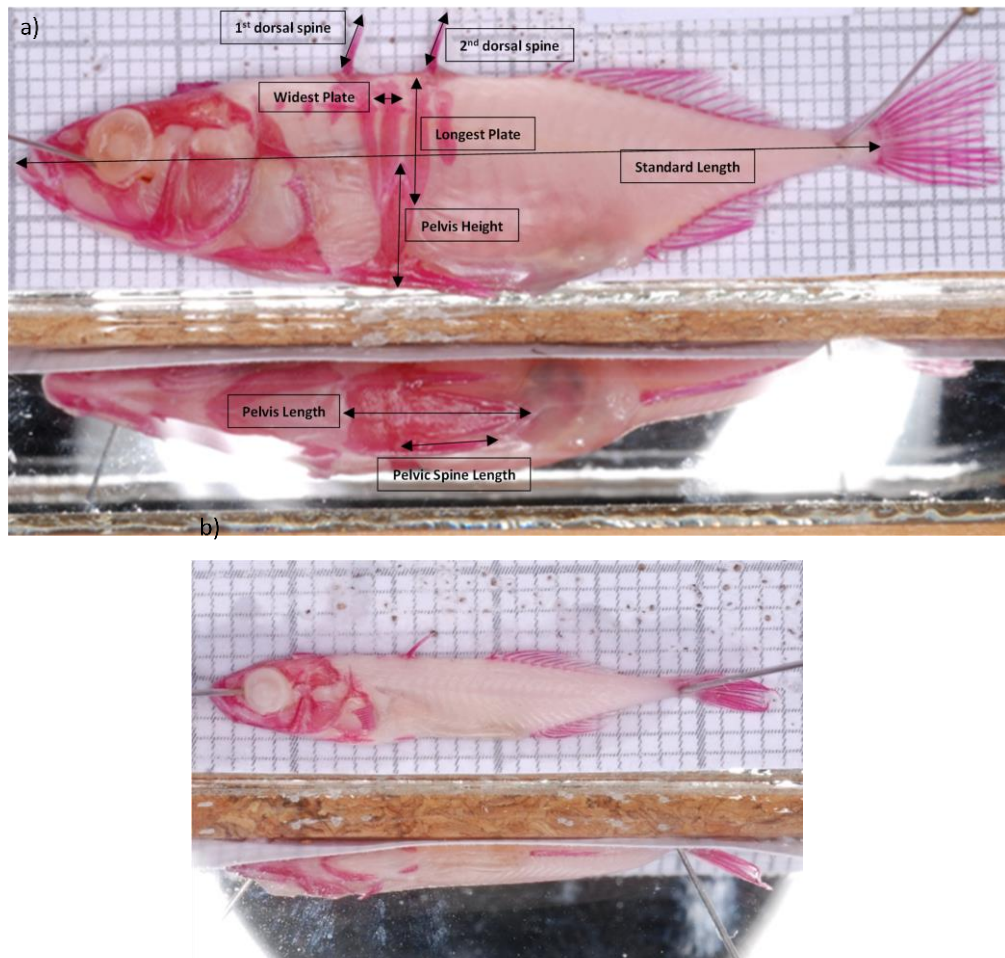
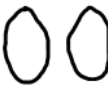


Figure 6: Photographs of two phenotypic extremes within the study from a) BUI and b) SCAD, showing the measurements taken using tpsDig232.

In addition, the following meristic traits were recorded for comparison: number of plates, number of dorsal spines, presence of pelvic spine and pelvis score. The latter followed Bell et al. (1983), although their system was adapted for use in this study (see Table 4), as no stickleback were found with pelvic spines but without a full pelvis.

Table 4: The scoring system used to quantify pelvic variation of three-spined stickleback in this study, adapted from Bell et al. (1983).

Ventral View	Lateral View	Pelvic Score
		3
		2
		1
Not Present	Not Present	0

Distributions of meristic traits (e.g. number of plates and number of spines) before and after potential armour trait selection were compared using contingency tables. Continuous traits were analysed by comparison of the allometry of such traits with standard length by fitting GLMs in Genstat with 'year' and the log of standard length as explanatory variables. Under the null hypothesis of no selection, it is predicted that there would be no change in the form of this relationship between the two samples from a population (i.e. before and after selection). If selection had occurred then it is expected that the slope or especially the intercept of the allometric relationship would differ between samples. For example, if fish with relatively shorter spines, given their body length, were more common in the spring sample, this would indicate selection for reduced armour over winter. The magnitude and direction of selection were estimated as the difference in trait value before and after selection (meristic traits), or the difference in the intercept of the allometric relationship with length (continuous traits).

3.3 Results

Bony armour characteristics of 450 individuals from 5 different populations were quantified throughout this study. Between the five lochs, there were obvious phenotypic differences (see Table 5). For example, stickleback from REIV were consistently larger, with a longer pelvis and dorsal spine than the other populations, appearing more ‘heavily armoured’ than other populations. On the other hand, individuals from SCAD and TORM tended to have fewer plates and dorsal spines, and spines were shorter on average. Stickleback from SCAD also have a lower pelvis score and a smaller pelvis.

Table 5: Mean values of continuous and meristic traits from all individuals of stickleback sampled from each loch (BUAI, CIET, REIV, SCAD, TORM). GLM’s were carried out in GenStat on each trait, using ‘Loch’ as the explanatory variable.

Trait	Mean Trait Value					F Statistic	d.f.	P Value
	BUAI	CIET	REIV	SCAD	TORM			
Standard Length	27.68 ± 0.50	28.51 ± 0.61	47.49 ± 1.84	27.28 ± 0.46	28.39 ± 114	0.73	1, 448	0.392
No. of Plates	3.45 ± 0.20	3.56 ± 0.05	5.81 ± 0.03	0.05 ± 0.03	0.17 ± 0.08	285.24	1, 448	<0.001
No. of Spines	2.99 ± 0.01	3.00 ± 0.00	3.00 ± 0.00	2.04 ± 0.04	2.51 ± 0.09	351.27	1, 448	<0.001
Longest Spine	2.37 ± 0.04	2.42 ± 0.05	3.79 ± 0.03	1.38 ± 0.04	1.30 ± 0.01	151.89	1, 448	<0.001
Pelvis Score	1.96 ± 0.08	2.98 ± 0.26	3.00 ± 0.00	1.06 ± 0.05	2.85 ± 0.08	15.81	1, 448	<0.001
Pelvis Length	2.53 ± 0.16	4.44 ± 0.12	10.01 ± 0.08	1.47 ± 0.07	3.72 ± 0.22	1.54	1, 448	0.216

Individual traits were also analysed for differences within lochs between the two sampling periods, August 2015 and April/May 2016 (Table 6). There was evidence of selection for reduced armour in all three of the lochs where trout were absent. Longest spine and widest plate in SCAD, longest spine, pelvis length, pelvis height and widest plate in CEIT, and pelvis height and longest plate in REIV were all smaller after winter relative to fish length.

Table 6: Results of analyses of selection on meristic and continuous armour traits of three-spined stickleback in five lochs on North Uist, in winter 2015 to 2016. P values indicate the effect of Year on the model.

Trait		BUAI	CEIT	REIV	SCAD	TORM
No. of Plates	P Value	0.45	0.10	0.89	***	***
	Wald Statistic	0.57	2.82	0.02	***	***
	Coefficients	0.33	-0.21	-0.04	***	***
		± 0.43	± 0.12	± 0.30		
No. of Spines	P Value	0.23	**	**	0.92	0.86
	Wald Statistic	1.45	**	**	0.01	0.03
	Coefficients	0.02	**	**	0.09	-0.04
		± 0.02			± 0.09	± 0.21
Longest Spine	P Value	<0.001	<0.001	0.13	0.21	0.76
	Wald Statistic	126.30	97.5	2.48	1.57	0.09
	Coefficients	-0.42	-0.43	-0.33	-0.12	0.04
		± 0.04	± 0.04	± 0.21	± 0.09	± 0.12
Pelvic Spine Present	P Value	0.13	0.30	**	0.78	0.07
	Wald Statistic	2.32	1.11	**	0.08	3.39

Trait		BUAI	CEIT	REIV	SCAD	TORM
Pelvic Spine Length	Coefficients	-0.14	-0.02	**	-0.01	0.15
		± 0.09	± 0.02		± 0.02	± 0.08
	P Value	0.12*	0.30	0.84	0.55	0.33
	Wald					
	Statistic	2.41	1.11	0.04	0.36	0.96
	Coefficients	-0.39	-0.06	-0.06	0.035	0.24
		± 0.25	± 0.06	± 0.27	± 0.06	± 0.24
	P Value	0.15	0.35	**	0.11	0.07
	Wald					
	Statistic	2.08	0.89	**	2.57	3.39
Pelvic Score	Coefficients	-0.25	-0.05	**	0.17	0.30
		± 0.17	± 0.05		± 0.10	± 0.16
	P Value	0.52	0.01	0.56	0.51	0.07
	Wald					
	Statistic	0.42	6.40	0.30	0.45	3.41
	Coefficients	-0.23	-0.30	-0.14	0.09	0.65
		± 0.350	± 0.12	± 0.24	± 0.14	± 0.35
	P Value	0.18	0.01	0.04	0.20	0.60
	Wald					
	Statistic	1.79	7.78	4.84	1.68	0.28
Pelvis Height	Coefficients	-0.35	-0.30	-0.49	0.11	0.11
		± 0.27	± 0.107	± 0.22	± 0.09	± 0.20
	P Value	0.08	0.32	0.01	0.10	0.23
	Wald					
	Statistic	3.10	1.00	8.39	2.70	1.52
	Coefficients	-0.35	-0.30	-0.49	0.11	0.11
		± 0.27	± 0.107	± 0.22	± 0.09	± 0.20
	P Value	0.18	0.01	0.04	0.20	0.60
	Wald					
	Statistic	1.79	7.78	4.84	1.68	0.28
Longest Plate	P Value	0.08	0.32	0.01	0.10	0.23
	Wald					
	Statistic	3.10	1.00	8.39	2.70	1.52

Trait		BUAI	CEIT	REIV	SCAD	TORM
Widest Plate	Coefficients	-0.13	-0.08	-0.71	0.10	0.17
		± 0.07	± 0.08	± 0.25	± 0.06	± 0.14
	P Value	<i>0.02</i>	<i>0.01</i>	0.16	***	***
	Wald Statistic	5.53	7.04	2.06	***	***
	Coefficients	-0.06	-0.19	-0.10	***	***
		± 0.03	± 0.07	± 0.07		
Degrees of Freedom		1, 138	1, 132	1, 25	1,110	1,40

*Individuals with no pelvic spine were removed from this analysis.

**Traits were either the same value between both years or not present for all individuals in the population.

*** Plates only present on a few individuals from the entire population therefore sample size was too small for analysis.

Figures 7 and 8 below illustrate the relationship between spine length and plate width against log standard length. The diagrams in the bottom right hand corner of each graph illustrate the main predator present in the loch (insect or trout).

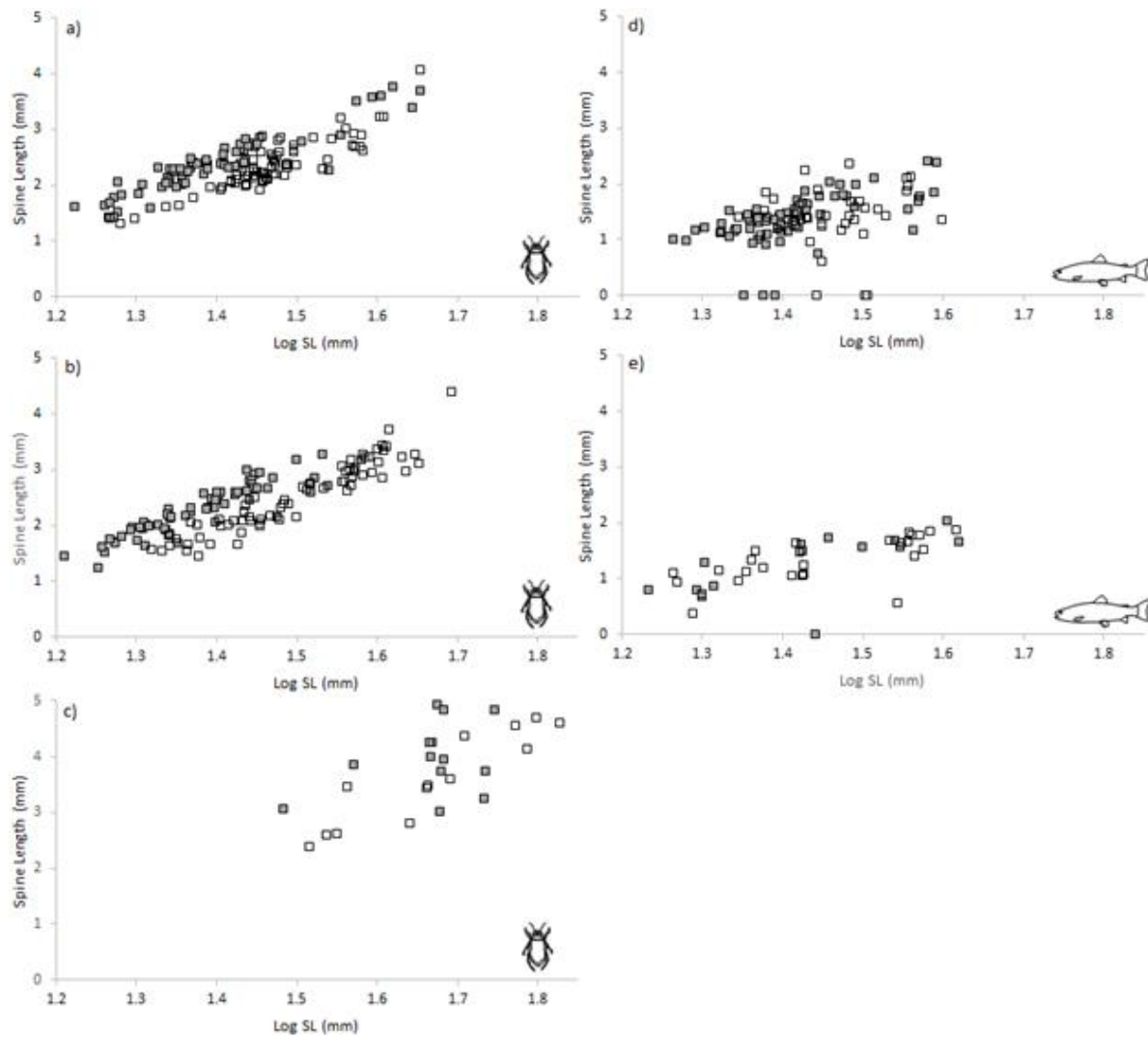


Figure 7: Dorsal Spine Length against log (standard length) for three-spined stickleback from five lochs on North Uist, a) BUAI b) CEIT c) REIV with insects as the main predators, and d) SCAD and e) TORM, with trout as the main predator present, during August 2015 ■ and April/May 2016 □.

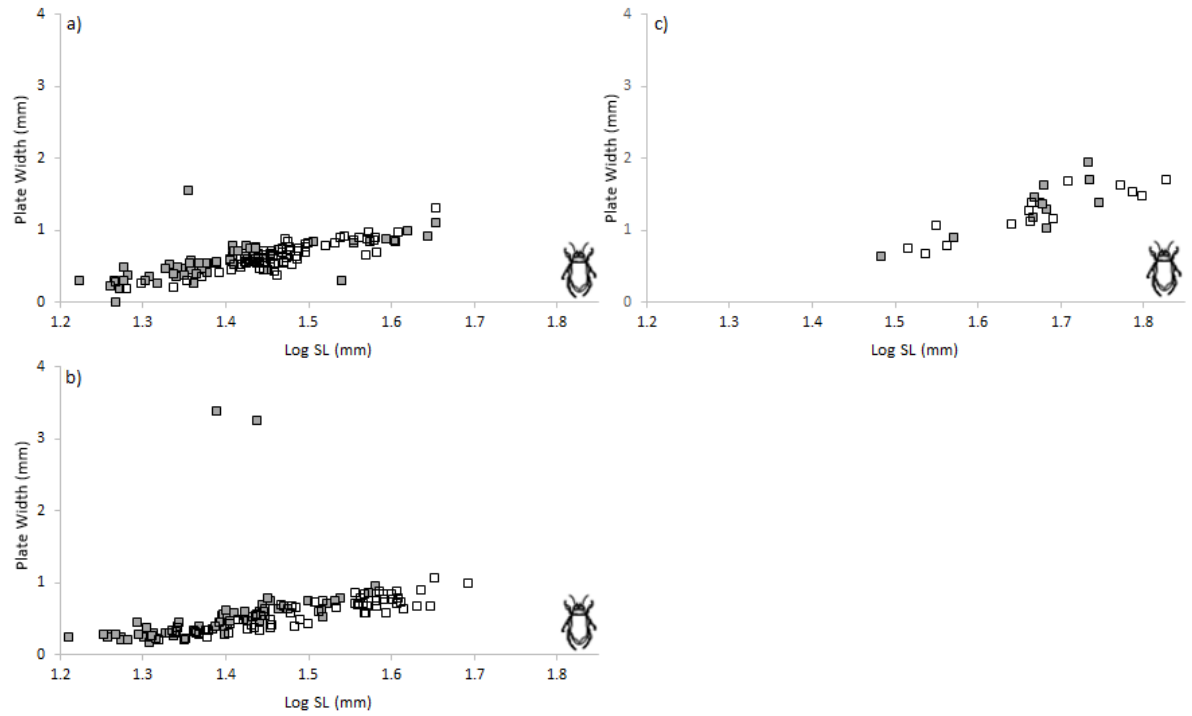


Figure 8: Plate Width against logStandard Length for three-spined stickleback from three lochs on North Uist, a) BUAI b) CEIT c) REIV with insects as the main predators. SCAD and e) TORM, with trout as the main predator present, did not have plates and therefore were not included in the analysis. Two samples were taken, during August 2015 ■ and April/May 2016 ■.

The magnitude of selection against mean trait value was insignificant (Figure 5 and 6), however they are both negative values. This illustrates that for both dorsal spine length ($r_p = -0.66$, $n=5$, $p=0.23$) and pelvis height ($r_p = -0.40$, $n=5$, $p=0.51$), selection is strongest in those populations with the most armour (REIV, CEIT and BUAI).

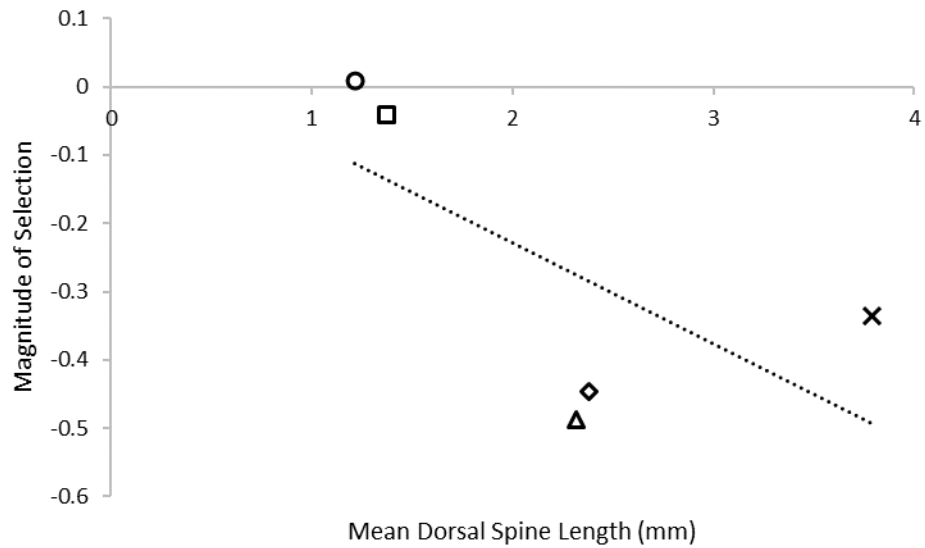


Figure 9: Magnitude of Selection on Mean Dorsal Spine Length for the five lochs sampled in North Uist: BUAI $\diamond$; REIV $\times$; TORM $\circ$; CEIT $\triangle$ and SCAD $\square$. Magnitude of Selection was calculated using the Y values at the mean value of Log Standard Length for each loch.

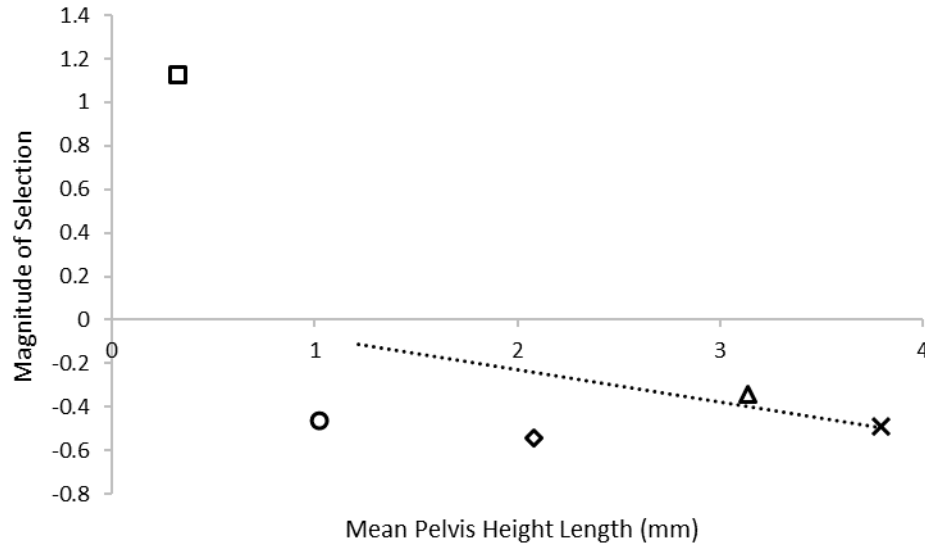


Figure 10: Magnitude of Selection on Mean Pelvis Height for the five lochs sampled in North Uist: BUAI $\diamond$; REIV $\times$; TORM $\circ$; CEIT $\triangle$ and SCAD $\square$. Magnitude of Selection was calculated using the Y values at the mean value of Log Standard Length for each loch.

3.4 Discussion

Between the two sampling periods, there were significant differences in trait values for three out of the five lochs: BUAI, CEIT and REIV. In these lochs, several armour traits in spring 2016 were significantly smaller relative to the length of the stickleback than in the previous summer, suggesting that there is selection occurring on these traits. All three of these lochs have insects as the main predators, suggesting that stickleback with less armour are more easily able to avoid predation. This is consistent with previous observational and experimental studies (Marchinko, 2009; Miller et al., 2015; Barrett et al., 2008; Gross, 1978).

There was no evidence for selection on armour in either SCAD or TORM, both of which have trout as their main predator. The magnitude of selection was strongest in REIV, BUAI and CEIT. The lochs on North Uist are all thought to have been colonised at roughly the same time (Magalhaes et al, 2016). This means that in order for the stickleback in SCAD and TORM to have lost armour so quickly, there must have been a stronger magnitude of selection in these lochs. Selection against armour is thus expected to be strongest in populations with the least armour. However, Figures 9 and 10 illustrate a negative correlation between magnitude of selection and mean trait value. This shows that there is a stronger magnitude of selection acting upon BUAI, CEIT and REIV (populations with the largest mean trait values), implying that there may be other factors than predation acting on selection in TORM and SCAD. This suggests that contemporary selection is acting alternatively to the historical selection which produced the observed phenotypic differences between populations.

There are several potential reasons why there were no significant differences in armour found between the two sampling periods in TORM and SCAD. Although trout are present in the lochs, they may be too small to regularly consume adult stickleback and thus fail to cause detectable selection within the

populations in this time frame. A more likely scenario is that there is an alternative selective agent that has a stronger effect than predation on these particular populations. Another commonly hypothesised cause of selection for armour traits is the availability of dissolved calcium. Calcium is an important component of bone, but it is unclear whether it is obtained directly from the environment, or through the diet. Experimentally reducing calcium availability has been shown to reduce the growth of juvenile stickleback, although not to affect their armour traits (Spence et al., 2012). On North Uist, Loch Scadavay ('SCAD') is known to have particularly harsh conditions, being acidic and with low levels of calcium, which could result in the lack of armour found in the lochs. This could also be the result of a general lack of resources, low levels of specific nutrients (e.g. phosphorus) or toxic levels of other elements (e.g. transition metals) (Sanchez et al., 2005). Selection towards less armour would be expected in such cases, which is not seen in this study. It is possible that an equilibrium has been reached within the lochs between the two selective agents, with the presence of trout selecting for increased armour traits and the lack of nutrients selecting for decreased armour.

The absence of apparent selection occurring within SCAD and TORM could also be due to countervailing selection. This occurs when factors affect selection in opposing directions during different times of year or different life stages (Pemberton et al., 1991). For example, if a trait enhances survival during the juvenile stage then inhibits success as an adult or vice versa, this often results in there being no net selection overall (e.g. Schluter and Smith, 1986; Coltman et al., 1999; Pemberton et al., 1991). In SCAD and TORM, it is possible that the trout, which tend to be small, especially in SCAD, do cause selection on juvenile stickleback but do not eat adult stickleback in sufficient numbers to cause selection on them (MacColl & Aucott, 2014). Selection for low armoured phenotypes may be occurring during the adult stage due to factors previously mentioned (i.e. water quality, nutrient availability, levels of toxins).

SCAD and TORM are also both large populations. Whether in the presence or absence of any selective agents, the high occurrence of mutations possible within the gene pool may have allowed stickleback in these lochs to become low-plated rapidly. REIV, BUAI and CEIT, however, are relatively small populations, reducing the opportunity for such mutations to occur, resulting in a 'waiting time' for mutations to become prevalent within the populations.

Overall, the results suggest that there is evidence of selection occurring within the freshwater lochs of North Uist. There is evidence that this may drive adaptive radiation due to predation in some lochs, however not in all of them. In reference to the criteria for adaptive radiation developed by Schluter (2000), 'common ancestry' is shared by all the stickleback on North Uist, with full-plated marine ancestors invading the freshwater lochs of the island approximately 16,000 years ago. In BUAI, CEIT and REIV there is evidence for the 'phenotype-environment correlation' criteria set by Schluter (2000), with a reduction of armour present in all lochs with insect predators. However, this is not the case for stickleback in the presence of trout (SCAD and TORM). Lastly, 'trait utility' is demonstrated in BUAI, CEIT, and REIV as the opportunity for insect predators to hold on to the armour is reduced, therefore increasing the survival rate of low plated individuals in these lochs. All of the criteria for an adaptive radiation, according to Schluter (2000) are met within some populations of stickleback within North Uist.

Although there is evidence to suggest so, more study is required to determine if adaptive radiation is occurring due to predation on North Uist stickleback. In addition to, or perhaps as well as, predation, there are many other factors which may be affecting selection and radiation in stickleback including: founder effects, water quality and nutrient availability.

Chapter 4: Genetic Radiations of Three-Spined Stickleback Since the last Glacial Retreat

4.1 Introduction

The distribution of genotypes in space and time is the result of both present processes and past history (Hewitt and Butlin, 1997). With the advance of molecular techniques, present processes such as drift, dispersal, selection and mating are now commonly studied. However, it is equally important to study historical events which have had an effect on populations in order to gain a full understanding. For example, is the homogenous nature of a population because of environmental selection or due to founder effects (Hewitt, 1999).

Parallel to these advances, paleo-climate studies have become increasingly important to our understanding of such past events, giving further strength to hypotheses regarding the causes of current genetic structures (Hewitt, 1999). Climatic oscillations during the Pleistocene have been a major factor effecting the present genetic structure of species across Europe (Mäkinen et al., 2006). Ice core analyses have established that average temperatures changed across a range of 10-12%, with ice sheets expanding and retracting across Europe (Hewitt, 1999). Species and populations reacted to these oscillations in a variety of ways: dispersing to new locations, surviving in refugia or even becoming extinct (Hewitt, 2000). During warmer periods, refuge populations were able to recolonise areas with glacial retreat. Previous studies have established that during the last ice age, most species currently distributed throughout Europe were found in refugia to the South, mainly in the Iberian and Italian peninsula and the Balkans (Hewitt, 1999).

Nuclear ribosomal genes (rRNA) and complete mitochondrial DNA (mtDNA) have been used to understand phylogenetic relationships in many diverged lineages. However, it is much more common for single mitochondrial genes to

be used to assess low-level taxonomic relationships (Farias et al., 2001). The ancient mitochondrial cytochrome *b* (cyt-*b*) gene is found in nearly all eukaryotes, and many prokaryotes. It has been widely used in order to study divergence, due to its ease of alignment (Takehana et al., 2003) and is one of the most widely sequenced genes across vertebrate species (Johns and Avise, 1998).

Over the last twenty years, there has been an increase in the study of protein-coding sequences. As a result, it is now being widely accepted among the scientific community that positive selection for advantageous mutations plays a role in the emergence of new phenotypes (Rech et al., 2014). Cyt-*b* contains both highly conservative and highly variable regions, allowing for the gene to be used in a range of studies, from ancient to population level divergences (Farias et al., 2001). Within related species, the amino acid sequence in cyt-*b* is usually highly conserved, however, nucleotides can be highly variable even within a species (Céspedes et al., 1998).

The three-spined stickleback (*Gasterosteus aculeatus*) is a well-studied example of a species which survived within a glacial refuge and then recolonised with the last glacial retreat approximately 16,000 years ago. Marine stickleback colonised many freshwater environments across the northern hemisphere, resulting in both phenotypic and genomic differences between populations due to the large variations in environmental factors (Magalhaes et al., 2016).

The aim of this study is to establish a radiation history for the stickleback sampled from across Europe, the Atlantic and eastern United States. Using data obtained from a previous study (Mäkinen and Merilä, 2008), it is known that there are three major lineages, trans-Atlantic, European and the Black Sea. Sequences from North Uist populations were added to these sequences from Mäkinen and Merilä (2008) as a part of this study.

4.2 Methods

4.2.1 Stickleback Sampling

Due to time restrictions during this thesis, DNA sequences extracted during previous research were used. Stickleback from North Uist and an Alaskan outgroup were sampled from lochs in 2014, using unbaited minnow traps set in water approximately 0.5-2m deep along the shoreline (Gee traps, Dynamic Aqua, Vancouver, Canada) and left for approximately 48 hours. DNA extraction had been carried out on eighty-one individuals using caudal fin clips, and the cytochrome-*b* gene was amplified using standard PCR protocols (Robertson et al., 2016).

To determine how the North Uist populations are related genetically to other populations of stickleback, fifty-six additional sequences were downloaded from GenBank, with haplotypes from three major groups; European, Black Sea and trans-Atlantic populations (Mäkinen and Merilä, 2008). Sequences taken from Mäkinen and Merilä (2008) were all unique haplotypes, therefore the frequency of each haplotype in a population cannot be taken into consideration within this study. Sequences from North Uist and Genbank were then combined into a single dataset for analysis (Table 7).

Table 7: Number of sequences and unique haplotypes within each lineage. Some unique haplotypes are shared between lineages.

Lineage	Number of Sequences	Number of Unique 'Long' Haplotypes
North Uist	61	29
European	50	50
Trans-Atlantic	9	9
Black Sea	8	8
Total	128	100

For the purpose of this study, the North Uist samples are included as part of the European lineage, due to their very close sampling location to three sites sampled by Mäkinen and Merilä (2008): MEA, QUI and END. All these locations are shown in Figure 11.



Figure 11: a) Map showing locations of populations sampled throughout Europe and the Black Sea b) Inset map of the coast off of Maine, USA (Google Maps, 2017). North Uist populations, along with MEA, are in the white box.

4.2.2 Sequence Data Analysis

Sequences were aligned and edited manually in BioEdit 7.2.6. (Hall, 1999). Due to higher mutation rates occurring in non-coding regions the genome than coding regions, nucleotides were cut at two differing lengths. ‘Short’ nucleotide sequences contained only the coding region of *cyt-b* (949bp), and ‘long’ nucleotide sequences also containing non-coding nucleotides (1367bp). The ‘short’ sequences were then translated in to amino acids, again using BioEdit

(292 amino acids). Nine sequences were removed from the 'long' nucleotide data set due to missing base pairs in North Uist sequences.

4.2.3 Phylogenies and Networks

Phylogenetic trees were produced using PhyML 3.0 (Guindon et al., 2005) for all three sequence sets (long, short and amino acids) with duplicate haplotypes removed. Networks were produced using NETWORK 5.0.0.1 (Fluxus-engineering, 2017).

4.3 Results

Phylogenetic trees were produced for all three data sets; however these were not supported by bootstrapping, with almost all branches failing to reach a confidence level of 95%. An example phylogenetic tree for amino acid sequences is illustrated below (Figure 12). The Alaskan outgroup (Broads 1) is clearly separated from the rest of the sequences which appear to be one population. There is evidence that suggests there is a genetic separation occurring with FAIK populations.

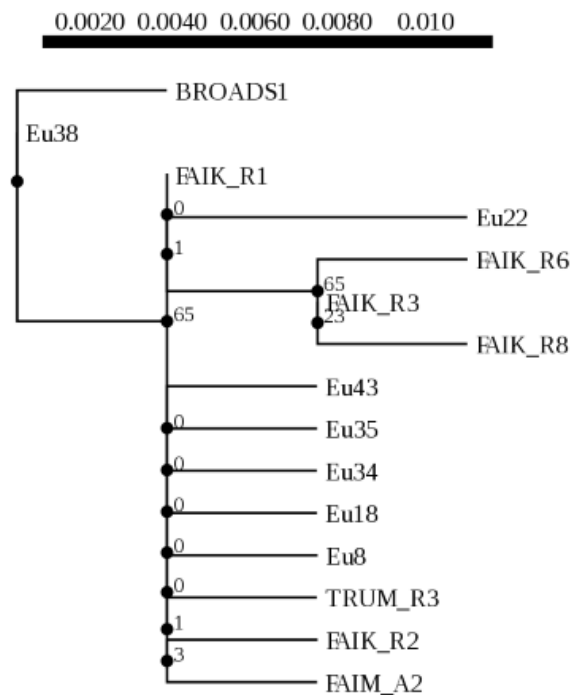


Figure 12: Phylogenetic tree of unique amino acid sequences for three-spined stickleback. The numbers on the branches are the results of the bootstrapping.

The networks below (Figures 13, 14 and 15) show the genetic changes that have occurred within *cyt-b* of stickleback populations across three main lineages: Atlantic, Black Sea and European. The addition of the North Uist populations into the EU lineage is reflected by the placement of North Uist populations in the networks. The number of unique haplotypes decreased significantly between the nucleotide and amino acid sequences. With 75 unique haplotypes in the 'long' nucleotide sequence, 64 in the 'short' nucleotide sequence and only 15 different mutations in the amino acid sequence, the vast majority of changes in the nucleotide sequence are synonymous.

The 'long' sequences used in this study (Figure 13, Table 8) contain nucleotides from both the coding and non-coding regions of the *cyt-b* gene. Overall, 75 unique haplotypes were identified from 128 individuals sampled, suggesting three main radiations: 'B', 'C' and 'E'. These are characterised by the presence of multiple nodes from a single haplotype. Broads 1 is, as expected for an outgroup, 37 mutations away from the main cluster of haplotypes, the majority

of which are only 1 to 3 mutations from the most similar sequence. In addition to Broads 1, an additional radiation consisting of TRUM and DUIN populations is also significantly separated from the main group. These populations have 49 nucleotide mutations between their most closely related sequence.

The 'short' nucleotides network (Figure 14, Table 9) consists of only the coding sequence from the *cyt-b* gene, with 67 unique haplotypes identified from 137 total sequences. Illustrating a similar pattern as seen in Figure 13, two main radiations are present: 'A' and 'E'. Again, the Broads lineage is most closely related to the At lineage, with 37 nucleotide mutations between the two. All other haplotypes are, again, only a maximum of 3 mutations from their next closest sequence.

Table 8: Key for the ‘long’ nucleotide network (Figure 13).

Code	Haplotype Location	Code	Haplotype Location	Code	Haplotype Location
A	FAIK, FAIM, OBSE	BS4	SIV	EU16	MEA
B	FAIK, FAIM, OBSE, TRUM, DUIN, VIS, RIT, BUG, KLU, WUP, STE, ROG, LEU	DU1	DUIN	EU17	ORR, MYR
C	OBSM, STE, HAK, MYR, PUL, KAR, MEA, ISL, NEV, BUG, SLU, BAR, IND, TVA, ORR	DU2	DUIN	EU18	HUN
D	FAIM, OBSM, TRUM, DUIN	DU3	DUIN	EU19	RUD, SEY, FOR
E	TRUM, MAI, RIT	DU4	DUIN	EU20	LIP
F	TRUM, DUIN	DU5	DUIN	EU21	TET, SLU, ALM
G	CHO, ALM, KUB, SIV	DU6	DUIN	EU22	MEA
H	OBSE, IND, BAR	FK1	FAIK	EU23	TVA
I	FAIK, LEU, ALE, WIL, HUN	FK2	FAIK	EU24	ONE
J	FAIK, DUIN	EU1	SEY, FOR, VAL	EU25	TVA
K	HUN, RUD	EU2	GRE	EU26	VAL
L	OLE, BOR, OLE	EU3	LIP	EU27	LAG
M	TRUM	EU4	HAK	EU28	LAG
N	MAI	EU5	WUP	OE1	OBSE
O	FAIM, OBSM	EU6	ALE	OE2	OBSE
AT1	MAI	EU7	OLE	OM1	OBSM
AT2	MAI	EU8	STE	TR1	TRUM
AT3	MAI	EU9	REN	TR2	TRUM
AT4	MAI	EU10	RIT	TR3	TRUM
AT5	MAI	EU11	ONE	TR4	TRUM
BR1	BROADS	EU12	PUL	TR5	TRUM
BS1	MAN	EU13	HAK	TR6	TRUM
BS2	KUB	EU14	RIT	FM1	FAIM
BS3	KUB	EU15	NEV		

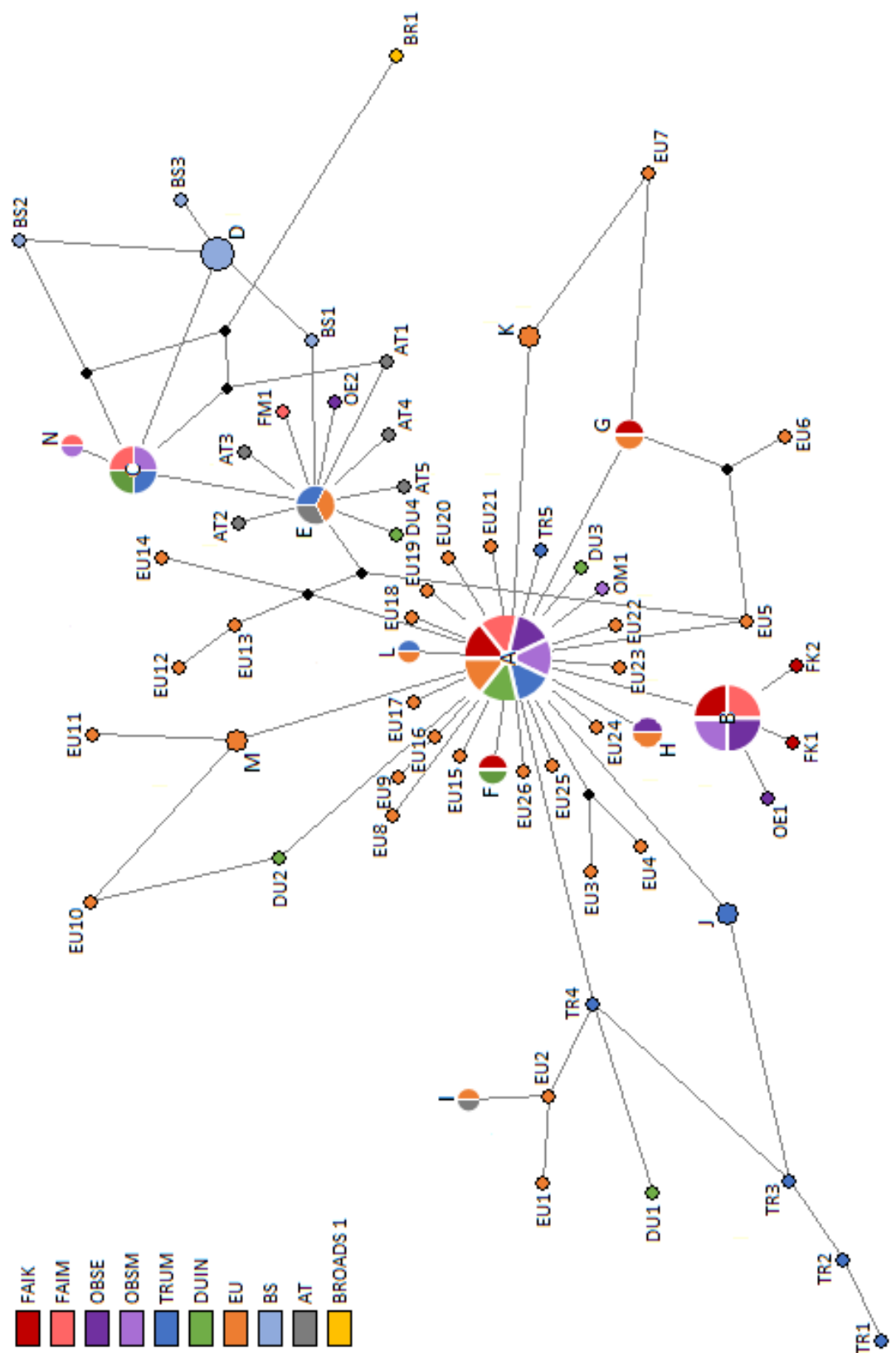


Figure 14: 'Short' nucleotide network, produced using nucleotide sequences from the coding region of *cyt-b*.

Table 9: Key for the 'short nucleotide network (Figure 14).

Code	Haplotype	Code	Haplotype
A	FAIK, FAIM, OBSE, OBSM, TRUM, DUIN, STE, HAK, MYR, PUL, KAR, MEA, ISL, NEV, BUG, SLU, BAR, IND, TVA, ORR, VIS, RIT, KLU, WUP, ROG, LEU	EU6	ONE
B	FAIK, FAIM, OBSE, OBSM	EU7	ORR
C	FAIM, OBSM, DUIN	EU8	MEA
D	CHO, ALM, KUB, SIV, KUB	EU9	HAK
E	TRUM, RIT, MAI	EU10	SEY, FOR, VAL
F	FAIK, DUIN	EU11	GRE
G	FAIK, LEU, ALE, WIL, HUN	EU12	LAG
H	OBSE, IND, BAR	EU13	LAG
I	MAI	EU14	VAL
J	TRUM	EU15	MEA
K	OLE, BOR	EU16	PUL
L	TRUM, HAK	EU17	RUD, SEY, FOR
M	HUN, RUD	EU18	LIP
N	FAIM, OBSM	EU19	LIP
AT1	MAI	EU20	HUN
AT2	MAI	EU21	TVA
AT3	MAI	EU22	ALE
AT4	MAI	EU23	TET, SLU, ALM
AT5	MAI	EU24	ORR, MYR
BR1	BROADS	EU25	NEV
BS1	SIV	EU26	WUP
BS2	KUB	FK1	FAIK
BS3	MAN	FK2	FAIK
DU1	DUIN	FM1	FAIM
DU2	DUIN	OE1	OBSE
DU3	DUIN	OE2	OBSE
DU4	DUIN	OM1	OBSM
EU1	TVA	TR1	TRUM
EU2	ONE	TR2	TRUM
EU3	STE	TR3	TRUM
EU4	REN	TR4	TRUM
EU5	PUL	TR5	TRUM

The amino acid network (Figure 15, Table 10) illustrates that these sequences are much more highly conserved than nucleotide sequences, with 65.7% of individuals sharing identical amino acids between all populations except the Broads 1 outgroup. Only 15 unique sequences were identified out of 137 sequences. All sequences have only one mutation in the amino acid sequence between nodes, apart from a freshwater population WUP 'EU1', in which there are two. The amino acid sequence of Broads 1 is one mutation different from EU2 (BAR and IND, marine sampling sites from the North West of Russia).

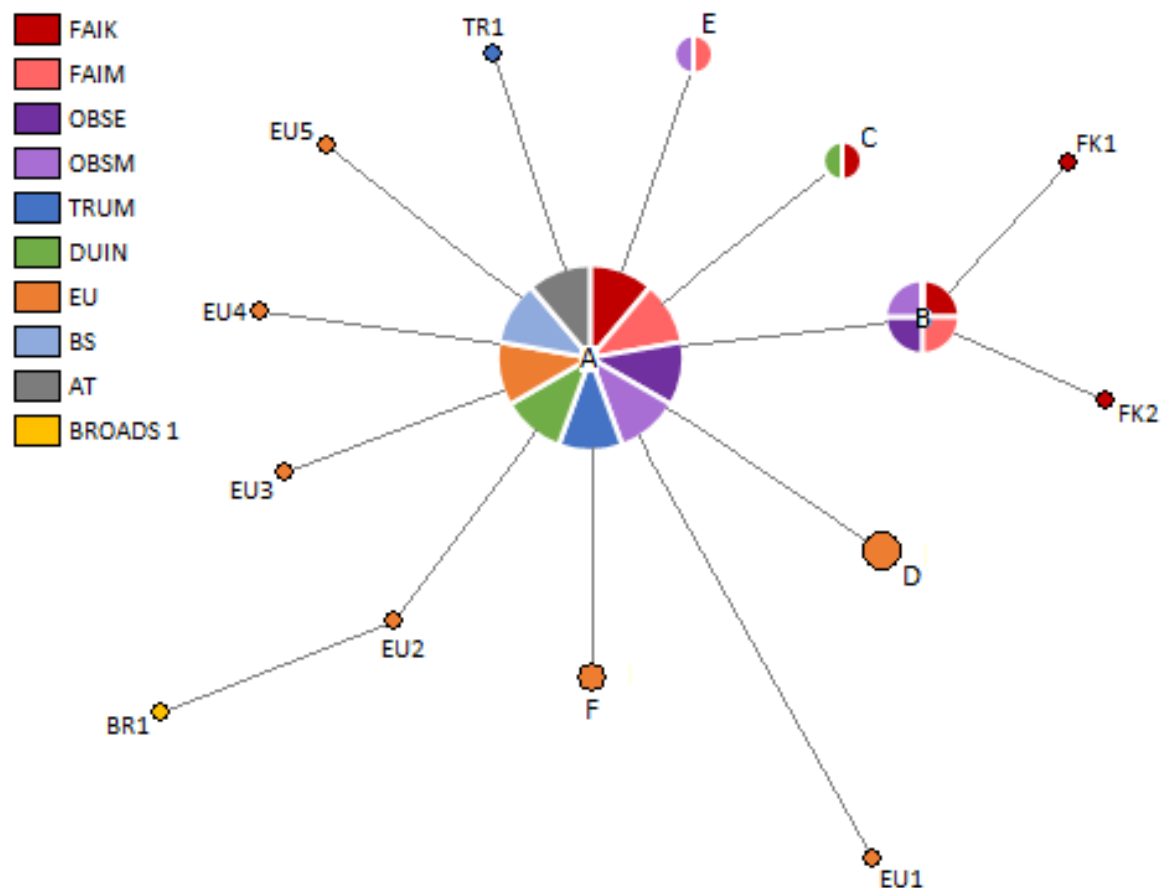


Figure 15: Amino acid network, translated from nucleotide sequences from the coding region of cyt-b.

Table 10: Key for the amino acid network (Figure 15).

Code	Haplotype
A	FAIK, FAIM, OBSE, OBSM, TRUM, DUIN, STE, HAK, MYR, PUL, KAR, MEA, ISL, NEV, BUG, SLU, BAR, IND, TVA, LIP, ORR, VIS, RIT, LEU, ONE, OLE, BOR, KLU, WUP, HUN, RUD, SEY, FOR, ROG, ALE, WIL, TET, ALM, VAL, LAG, MAI, QUI, CHA, RIT, CHO, KUB, SIV, MAN
B	FAIK, FAIM, OBSE, OBSM
C	FAIK, DUIN
D	HUN, RUD, SEY, FOR, VAL, GRE
E	FAIM, OBSM
F	TVA, LAG
BR1	BROADS
EU1	WUP
EU2	MEA
EU3	REN
EU4	NEV
EU5	ALE
FK1	FAIK
FK2	FAIK
TR1	TRUM

4.4 Discussion

Analysis of all three networks show that some areas have a high genetic diversity, whereas other areas have produced only minimal numbers of haplotypes. North Uist individuals, as part of the EU lineage, illustrate a more genetically diverse population than other lineages. Mäkinen and Merilä (2008) also found that the EU lineage is six-eight times more genetically diverse than the trans-Atlantic and Black Sea lineages.

Mäkinen and Merilä (2008) hypothesised that during the ice age, stickleback survived in two different refugia: in the Black Sea and the Atlantic. The complete separation of the Black Sea lineage from all but the Atlantic haplotypes suggests that marine stickleback colonised Black Sea locations. It also suggests that there has been no hybridisation between the two lineages since they became geographically isolated at the beginning of the last ice age. With EU haplotypes being related to the At lineage, it is highly likely that long

distance dispersing populations at the northern limits of the Atlantic refuge expanded into new habitats as the ice sheet retreated.

When an ancestral population founds multiple new colonies, as has occurred across the northern hemisphere, founder effects can lead to severe population bottlenecks with reduced allelic diversity and heterozygosity (Hundertmark, and Van Daele, 2010). Reduced genetic diversity in the Black Sea populations may be responsible for the low number of haplotypes observed.

Reduction in genetic diversity caused by a small founder population can be counter-acted by a rapid population expansion (Zenger et al., 2003) or by multiple invasions (Genton et al., 2005). Genetic diversity has even been shown to increase above ancestral levels after bottlenecks in populations of flies (Wade and Goodnight, 1998). The high genetic diversity found within the European lineage suggests that there may have been a rapid population expansion after colonisation. The freshwater populations separated by geographical barriers may have maintained high genetic diversity, allowing for the large EU radiation seen in the networks. Within both nucleotide networks, there is evidence of several North Uist sequences being more closely related to trans-Atlantic and Broads individuals. TRUM, DUIN, OBSM and FAIM are all anadromous populations, which come into freshwater to breed, and therefore have frequent opportunity to hybridise with marine individuals (Brawand et al., 2014).

Within the European lineage, there are several smaller radiations that appear to have occurred after the original European colonisation. Northern European populations seem to have common nucleotide mutations within the non-coding sequence of *cyt-b*, suggesting that there is little gene flow between the north and south. There is also evidence that FAIK/OBSE populations and TRUM/DUIN populations have begun to radiate even further from this northern population.

Synonymous mutations within the nucleotide sequence are those which do not alter the corresponding amino acid sequence. As seen by the significant reduction in unique sequences once the nucleotides were translated into amino acids, most mutations were synonymous. The amino acid network illustrates that the EU/North Uist populations are the only lineage to have gained amino acid mutations away from the main cluster 'A', except for the Broads outgroup. This suggests that widespread adaptive radiation may be occurring within EU populations.

Amino acid mutations differ in their effect on an individual. Some mutations will be deleterious (meaning that it is removed from the population due to reduced survival or reproduction) or neutral (having no effect and remain at a low level within the population). However, if a mutation reaches a high frequency in a population, this suggests that it is favourable in some way to the environment and has increased reproduction or survival in its carrier (Dobzhansky, 1973). If these changes in amino acid sequence were due solely to other factors such as genetic drift over time, you would expect approximately equal numbers of changes across the other lineages (Black Sea and Atlantic), however this is not observed. As discussed in Chapter 3, there is evidence that adaptive radiation is occurring within stickleback populations on North Uist, and therefore it is highly possible that this is also occurring within other EU populations. Reduced heterozygosity due to founder effects may be preventing such rapid divergence within the Black Sea lineage.

Further genetic analysis of stickleback, particularly freshwater populations, in both European and Black Sea lineages would allow for confirmation of this theory. A common property of adaptive radiations is the repeated evolution of similar phenotypes, which occurs when an ancestral group repeatedly colonises similar environments (Wund et al., 2008). Connecting environmental variables, such as predation regime, with these genetic changes would also allow help to illustrate potential adaptive radiations.

Chapter 5: Conclusions

The main focus of this thesis has been the subject of adaptive radiations. Wax model fish have been used to estimate the rates of predation by trout within stickleback populations on North Uist. Two samples from the same cohort of stickleback were taken six months apart (before and after winter) and armour traits were analysed in order to quantify selection in populations with and without the presence of predatory trout. Lastly, the nucleotide and amino acid mutations within populations were analysed for evidence of adaptive radiations occurring.

The use of model prey in order to establish predation rates is a relatively new concept, with only a small number of studies using such techniques, particularly within aquatic environments (Husak et al., 2006; Kuchta, 2005; Noonan and Comeault, 2009). Although this was only an exploratory study, the results were very promising, with the attack rate by trout on the stickleback models correlating with CPUE estimated trout density. This suggests that this could be a reliable way of estimating relative trout predation rates within closed environments, instead of using destructive sampling methods such as stomach content analysis.

Although seemingly successful, one major drawback to this particular study is the lack of repetition. Most lochs only had one line set in it, giving a very low sample size and therefore little power to the study. To strengthen the reliability of this method, more lines would need to be set in a larger number of lochs with different variables i.e. location within loch, season, time of day, exposure time. If the data still suggests a correlation with the CPUE trout density, this would strongly support the use of this method to estimate relative trout density. This known density would allow further study of the effects of predation on adaptive radiations of stickleback, such as that carried out in Chapter 3. A higher attack rate on replica stickleback would imply a higher

magnitude of selection in those lochs where predation plays a factor in natural selection.

Additionally, the models themselves could be improved and adapted to answer a variety of questions. This study used only 'big' and 'small' models to represent a complete and a low plated morph, however there is a wide range of phenotypes other than the two used here. For example, by keeping the same body size, but altering the spine prominence, gape limit of predatory fish could be tested. Colouring the throats of stickleback red, as though they were males in breeding condition, would allow for questions of fitness trade-off to be studied. As discussed in Chapter 3, countervailing selection can occur when selection acts in different directions depending on age or season (Schluter and Smith, 1986). Introducing a range of body sizes with the same level of armour would allow for such theories to be tested in lochs that do not appear to have selection acting on them.

The analysis of morphological traits from the same cohort of stickleback, both before and after winter, showed evidence for selection against armour traits due to predation in three of the lochs (BUAI, CEIT and REIV). On the other hand, SCAD and TORM did not appear to follow this pattern. As illustrated in Chapter 2, predatory trout are present within both TORM and SCAD, which adaptive theory suggests could cause selection for robust armour traits (Marchinko, 2009). However, individuals from these two populations have greatly reduced armour, and do not show evidence for selection in either direction. This implies that the radiation is not adaptive or is due to an alternative selective agent.

In order to clarify the reason for this reduction in armour, much further work would be required. Freshwater habitats contain many additional environmental variables which may affect selection of a population, for example: pH; temperature; alkaline metal accessibility; competition and resource availability (Magalhaes et al., 2016). Such variables would need to be measured or controlled in a lab environment in order to establish if any of these

correlated with changes in morphological traits. Although there is CPUE data of trout density on several lochs on the island, the use of model lines such as those tested in Chapter 2, would allow for lochs of unknown trout density to be studied. Vervust et al. (2007) has previously used model replicas of the Italian wall lizard *Podarcis sicula* to illustrate morphological adaptations of populations under different predation regimes. This method could be adapted for use in aquatic environments for the study of stickleback.

Alternatively, radiations are often non-adaptive and therefore do not correlate with any environmental variables. However, it is believed that all lochs on North Uist were colonised during the same glacial retreat approximately 16,000 years ago. The rapid loss of armour traits in CAD and TORM infers a historically high magnitude of selection, as genetic drift or founder effects usually only cause such significant effects over a much longer period of time (Marroig and Cheverud, 2004). Similarly, in order to confirm the presence of adaptive radiations due to predation in BUAI, CEIT, REIV and any other lochs, these variables would need to be analysed to disregard other factors. Larger sample sizes and the study of additional lochs would assist in gaining a more accurate view of morphological radiations occurring on North Uist.

Another way to help establish the type of radiation occurring is to look at the genetic code of these populations. As discussed in Chapter 4, there is evidence of adaptive radiations occurring on North Uist due to the presence of mutations in the amino acid sequences of *cyt-b*. The presence of an amino acid mutations at high frequencies within a population suggests that it is somehow advantageous to the individuals, otherwise it would have been removed or remain at low levels (Dobzhansky, 1973).

In order to improve this study, genetic and morphological data could be combined. Any genetic radiations identified could then be tested for selection using the method used in Chapter 3, as it is highly likely that morphological changes will also be present. Variables within habitats such as pH, availability

of resources and predation rate could be tested to establish which selection agent is causing each adaptive radiation.

The overall aim of this thesis has been to identify the presence of adaptive radiations within three-spined stickleback populations. Evidence suggests that there is selection for reduction in armour traits in lochs with insects as the main predator, and there is strong genetic evidence of adaptive radiations throughout Europe and North Uist. Further study, obtaining relative predation rates using the model replicas used in Chapter 2 would allow for an in depth look at these patterns throughout the stickleback habitat.

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