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Morphological and Genetic Variation of Three-spined Stickleback (*Gasterosteus aculeatus*) in a Hybrid Zone

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Master of Research

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2 Acknowledgements

Firstly, I would like to give my sincerest gratitude to my supervisor Dr Andrew MacColl, for his guidance throughout my Masters and for giving me the opportunity to learn from and work alongside him. Further, I would also like to express my gratitude to the MacColl lab, with a special thanks to Megan Barnes and Ben Fisher for their willingness to assist me in any way they could throughout my research project.

3 Abstract

The mechanisms that contribute to evolutionary divergence between populations are difficult to discern, but may be suggested by patterns in the distribution of morphological traits, alleles and genotypes. Hybrid zones across ecotones and population variation across environmental gradients can be especially useful in this regard. Here, I analyse changes in morphological traits such as armour plating, standard length and body shape, as well as genetic variation such as the allele frequency of the well-known Eda locus, across environmental gradients within a hybrid zone and between systems. I show the lack of a pattern in morphological traits across the hybrid zone, but very strong genetic structure. In addition, I observe allele frequencies of chromosomal inversions across environmental gradients to show a tight link between the chromosome XI inversion and pH in freshwater systems.

4 Introduction

4.1 Hybrid zones

Hybrid zones between divergent populations offer the unique opportunity to study how genetic and morphological differentiation is maintained in the face of gene flow (Barton and Hewitt, 1985). Gene flow counteracts divergence unless a form of reproductive isolation or other factors which prevent gene flow are present (Abbott et al., 2013), therefore understanding these factors are key to evolutionary ecology (Funk et al., 2006). Reproductive isolation can be separated into prezygotic isolation, where selection acts to prevent zygote formation (McKenzie et al., 2016, Dean et al., 2021, McKinnon et al., 2004), and postzygotic isolation, where selection acts to reduce zygote fitness (Schluter and Conte, 2009, Szymura and Barton, 1991) such as via transgressive segregation, where hybrids experience extreme phenotypes not suited to their local environment, or the asynchronous emergence of larvae (Rogers and Bernatchez, 2006). The maintenance of divergence within a hybridising zone depends on the mechanisms of gene flow reduction balancing out the homogenisation of gene flow (Barton, 2001, Felsenstein, 1981, Morjan and Rieseberg, 2004).

Secondarily, hybrid zones are useful in the study of adaptation because they provide us with a natural system from which to observe divergent evolution. Adaptations that experience strong selection in one population will be distributed across a gradient in the hybrid zone as they become less common and less useful, creating an interesting opportunity to observe allele frequencies as they rise and diminish (Brodie et al., 2002, Barton and Hewitt, 1985, Vines et al., 2016).

For example, a previous study showed that the fire-bellied toads *Bombina bombina* and *B. variegata* hybridized across a 3-4km range, displaying clear clines in traits and alleles across this distance (Szymura and Barton, 1991). According to theory, hybrid zones slow or even prevent ecological speciation due to gene flow between the populations and the reduction of linkage disequilibrium between distinguishing genes (Abbott et al., 2013). In contrast, linkage disequilibrium between strongly divergent alleles can arise through hybridization between the divergent populations, resulting in the maintenance of divergence (Rundle and Nosil, 2005, Ravinet et al., 2018). Therefore, previous studies have suggested that divergence is dependent on the increase of large allele frequency differences between populations and the increase of linkage disequilibrium between divergent alleles (Felsenstein, 1981, Barton, 2001, Vines et al., 2016). For example, a study focussing on the clines in alleles between two three-spined stickleback (*Gasterosteus aculeatus*) populations showed how linkage disequilibrium between loci can force clines in alleles to share slopes or centres, which will have a substantial effect on gene flow (Vines et al., 2016), as well as presenting the roles of selection in promoting and inhibiting speciation.

Here, I describe the morphological and genetic variation within a compact, high density hybrid zone between two divergent populations of three-spined stickleback (*Gasterosteus aculeatus*), freshwater and anadromous, on the Scottish island of North Uist. I describe patterns of variation in morphological phenotypes and a small number of adaptive genomic loci, before going on to explore associations between allele frequencies and environmental and morphological variation that might shed light on the adaptive significance of the allelic variation.

More specifically, I investigate morphological variation such as armour plating, standard length and shape, as well as genetic variation including the Eda genotype, mitochondrial lineage, and chromosome I and XI inversions. Further, I investigate the possibility of heterozygote deficiency within the hybrid zone and the extent of hybrid fitness.

4.2 The Eda gene

The Eda gene is the main genetic determinant of lateral armour plating in three-spined stickleback (Colosimo et al., 2004, Archambeault, 2019, Leinonen et al., 2012) and is responsible for the difference in plate count and plate size between divergent freshwater and marine stickleback populations (Jones et al., 2012, Archambeault et al., 2020). The Eda locus has two main alleles Eda^{L} and Eda^{C}, which when homozygous give rise to low (~7 plates) and completely plated (~30 plates) phenotypes respectively, but the heterozygous genotype expresses a partial plating (~11 to ~28) which results in an intermediate phenotype between the low and complete plate morphs (Colosimo et al., 2004). The adaptation of the reduced, low plated phenotype is widespread and repeatedly evolved in freshwater systems from the ancestral completely plated marine populations (Colosimo et al., 2004) and remains a divergent gene differentiating freshwater and marine three-spined stickleback populations (Jones et al., 2012). A study on three-spined stickleback in western Ireland reported that divergent selection between lateral armour plate morphs maintains reproductive isolation within a hybrid zone (Ravinet et al., 2015), and it is likely that similar divergent selection is present in Hosta stream.

Despite the fact that strong selection on Eda in freshwater has been clearly demonstrated (Barrett et al., 2008), the nature of the adaptation that different alleles confer remains unclear. The variation in plating phenotype caused by the different alleles likely has consequences for protection from predation, but the strength of selection on the Eda^{L} allele in freshwater, in the absence of predators, suggests that other mechanisms are in play. One possibility is that Eda has 'pleiotropic' effects, either directly due to its own variation, or because of variation in other, tightly linked loci. Eda also shares a haplotype with BAFF (TNFsf13b) (Colosimo et al., 2005, Rodríguez-Ramírez et al., 2023), a major immune system gene, and there is evidence that the expression of some immune genes in stickleback is associated with Eda genotype (Robertson et al., 2017). BAFF has also been linked to metabolic variation and the control of obesity in mice and humans (Chan et al., 2021). This suggests the possibility that the Eda haplotype might be linked to metabolic variation in stickleback. Here, I observe plate morphology and Eda genotype across a hybrid zone to observe any selection pressures as well as identify any mismatches between Eda genotype and plate phenotype. Further, I investigate the effects of Eda on metabolism in three-spined stickleback via the hepatosomatic index to uncover patterns of selection.

4.3 Mitochondrial lineage

Mitochondrial DNA (mtDNA) is used as a phylogeographic marker due to the absence of recombination and its rapid rate of mutation (Mäkinen and Merilä, 2008, Beck et al., 2022), and was long assumed to be selectively neutral, but increasing evidence suggests that mitochondrial variation may have adaptive consequences (Ballard and Youngson, 2015). For example, studies show how diet can affect the frequency of various *D. melanogaster* mitochondrial haplotypes, suggesting that environmental conditions can select for advantageous mitochondrial haplotypes due to differing fitness benefits (Aw et al., 2018, Pichaud et al., 2013). In the context of three-spined stickleback, the mitochondrial lineage can be used to distinguish between descendants of major clades (Johnson and Taylor, 2004). Around the UK and Scotland, there are two lineages present: the trans-Atlantic and the European lineage (Fang et al., 2018, Mäkinen and Merilä, 2008, Ravinet et al., 2013). These two lineages diverged from the older, pacific lineages 60,000 years ago (Fang et al., 2018), and experience secondary contact around the British Isles, the European lineage having migrated north from an Iberian refugium, while the trans-Atlantic lineage migrated northeast from an eastern-American refugium after the last glacial period 15,000 years ago (M. Barnes and A.D.C. MacColl, unpublished data). Previous research has suggested that the European lineage has a greater affinity for freshwater adaptation than the trans-Atlantic lineage (Dean et al., 2019), which is of particular interest when studying marine and freshwater divergence in a hybrid zone as we might observe adaptational biases towards fish of the European lineage or other patterns of selection across the hybrid zone. Here, I observe patterns of

mitochondrial DNA variation within a three-spined stickleback hybrid zone to make inferences about the nature of selection.

4.4 Chromosomal Inversions

Chromosomal inversions are of recent interest to evolutionary biologists as they often hold adaptations that are important for the fitness of the organism (Dean et al., 2019, Kirkpatrick and Barton, 2006). A chromosomal inversion is a section of the chromosome that is inverted, changing the direction of the DNA and preventing alignment during the recombination stage (Sturtevant, 1921). This means that genes within the chromosomal inversion are protected from being shuffled during sexual reproduction in heterozygotes. Natural selection may favour sets of adaptive genes that cluster within such inversions, resulting in a small section of the chromosome that can explain much about an organisms evolutionary history as well as the selection pressures they currently face. It is because of these factors that chromosomal inversions are an evolutionary mechanism which can cause divergence. For example, the yellow monkeyflower, *Mimulus guttatus,* exhibits two geographically widespread ecotypes which differ by a chromosomal inversion responsible for an adaptive annual/perennial life-history shift as well as reproductive isolation between the ecotypes (Lowry and Willis, 2010). Additionally, a previous study that set out to identify loci under divergent selection between marine and freshwater populations of three-spined stickleback across the northern hemisphere (Jones et al., 2012), found that many locations fall within chromosomal inversions, further supporting the hypothesis that inversions are pivotal in stickleback divergence. It is hypothesised

that ancestral marine three-spined stickleback utilised the chromosomal inversion evolutionary mechanism when colonising freshwater systems, particularly on chromosomes I, XI and XXI (Jones et al., 2012, Roberts Kingman et al., 2021). Previous studies have shown that loci within these inversions are key differences between marine and freshwater populations (Jones et al., 2012, Roberts Kingman et al., 2021).

The inversion on chromosome I contains the ATP1a1 gene, which codes for a membrane-bound enzyme which transports Na^+ and K^+ ions across cell membranes. In the context of fish, this enzyme is important in the osmoregulatory function in varying salinities due to its role in pumping Na^+ ions out of cells (Kaplan, 2002). Furthermore, previous studies have shown that selection on this gene is related to the salinity of the environment (Hohenlohe et al., 2010) and this gene has been observed in high frequencies in numerous freshwater three-spined stickleback systems (Jones et al., 2006). The inversion on chromosome XI contains the KCNH4 gene, which codes for a potassium voltage-gated channel which transports K^+ ions out of a cell. This movement of positive ions occurs after an action potential and aims to return a cell to a resting state (Hodgkin and Huxley, 1952). In the context of fish, this ion channel is suggested to affect osmoregulatory function and KCNH4 expression in three-spined stickleback gills changes depending on the salinity of the water (Taugbøl et al., 2014). A previous study quantified this hypothesis by observing the change in armour plating as well as the change in allele frequencies across lochs of varying pH and calcium ion concentrations. They found that genetic adaptations were selected from standing genetic variation, where fish from acidic lochs had

accumulated alleles of low frequency in the marine population, whereas the genetic composition of fish from alkaline lochs remained similar to their ancestral marine counterpart (Haenel et al., 2019). Here, I examined the pattern of variation of chromosomal inversions I and XI in a hybrid zone, coupled with variation across wider environmental gradients to make inferences about putative patterns of selection.

4.5 Heterozygote Deficiency

Heterozygote deficiency is a phenomenon which can occur as a result of hybridization, where there are fewer heterozygotes than expected by chance. Heterozygote deficiency in a hybrid zone can give a lot of information on the dynamics between the populations as well as the nature of the divergent selection since it can be accounted for by contrasting pre- and post-zygotic mechanisms, (McKenzie et al., 2016). These evolutionary mechanisms include (post-zygotic) reduction in hybrid viability, where hybrids experience a reduced fitness, and (pre-zygotic) assortative mating, where individuals choose to reproduce within their population, reducing the number of heterozygotes (McKenzie et al., 2016). It is possible to differentiate between prezygotic and postzygotic isolation by testing the population for Hardy Weinberg equilibrium in a subset of young and old fish (Schluter et al., 2010). The Hardy Weinberg equilibrium is a formula which uses allele proportions within a population to predict the number of heterozygotes expected by chance without selection pressures acting on the heterozygotes (Mayo, 2008). If the prediction is not aligned with the observed heterozygote count, this implies that there is selection acting on the heterozygotes. If there is a deficit of heterozygotes in the young fish as well as the old fish this

suggests prezygotic isolation as the deficit is present from young ages. However, if the heterozygotes are only absent in older fish, this suggests that the heterozygotes are experiencing a decrease in fitness as they are not maturing to a similar length as homozygous fish or they are dying before becoming older.

There are mixed results for selection on the partially plated phenotype in three-spined stickleback, as heterozygote deficiency has been observed in a handful of freshwater systems (McKenzie et al., 2016, Jones et al., 2006, Marchinko et al., 2014). Previous studies attributed the lack of heterozygous fish to disruptive selection pressures on partially plated fish (Schluter et al., 2010), breeding behaviour (Jones et al., 2006), or schooling behaviour (Dean et al., 2019). The Hardy Weinberg equilibrium can be used to detect the deficit of heterozygote phenotypes or genotypes in hybrid zones, however the cause of the deficit cannot be determined by Hardy Weinberg calculations alone.

The hepatosomatic index can be used to investigate the condition or energy status of a fish. For example, a study on the effects of various diets on *Oreochromis niloticus* (Nile tilapia) revealed a significant difference in hepatosomatic index, concluding that the addition of maltose to fish diet can improve fish condition (Ighwela et al., 2014). In this case, hepatosomatic index can infer selection pressures on heterozygotic fish indicating fish condition and energy status. Here, I use the Hardy Weinberg equilibrium to test for heterozygote phenotype and genotype deficiency within a hybrid zone and I also investigate the evolutionary mechanisms behind the lack of hybrids using the hepatosomatic index as a proxy for fitness (Chellappa et al., 1995, Wootton et al., 2006, Campbell and Love, 1978).

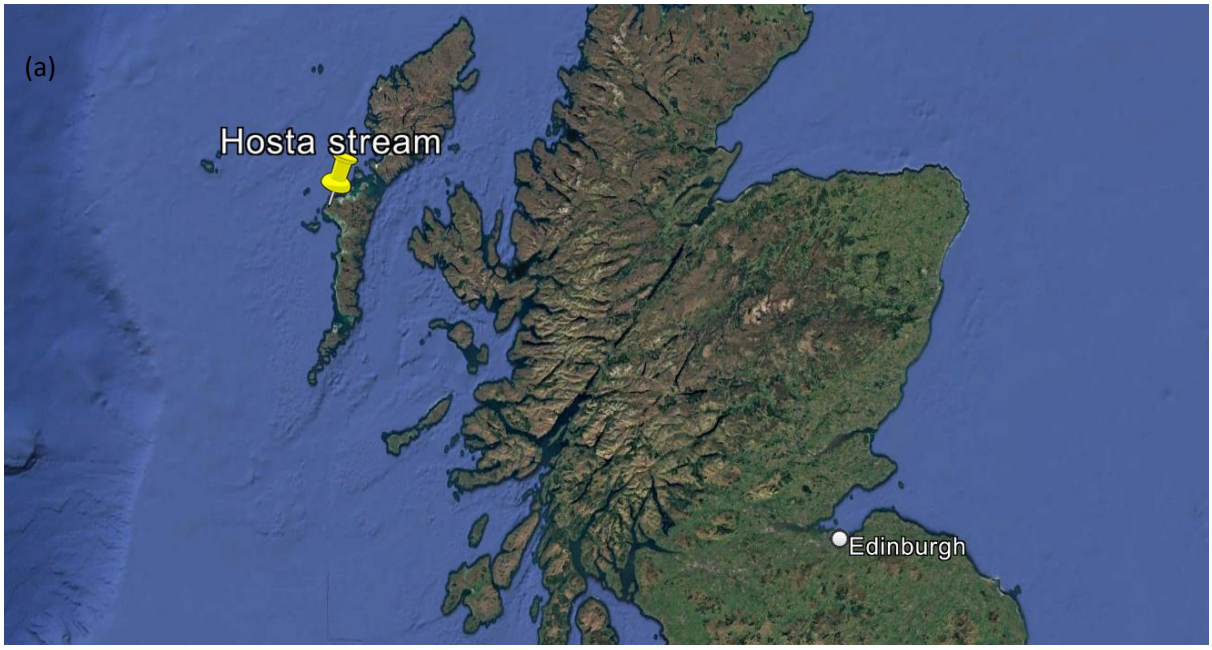
4.6 Study Species

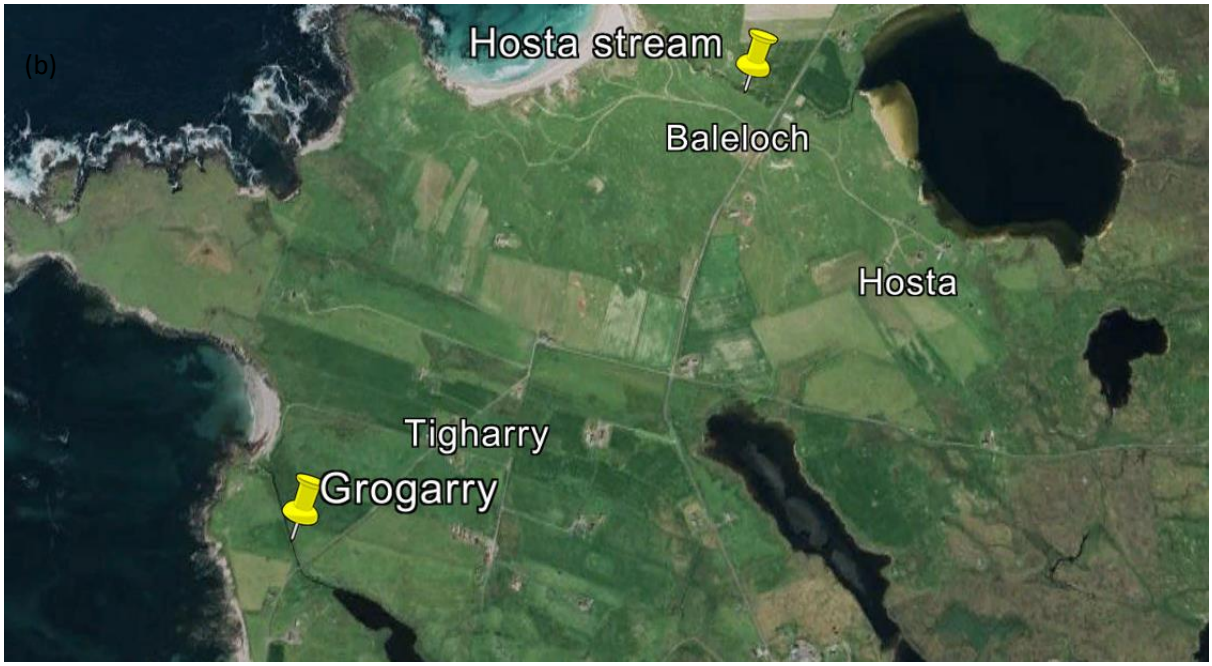
Three-spined stickleback are ideal for studying evolutionary ecology as they display a fast reproductive rate and a short generational time (Reid et al., 2021), which aids their capability for rapid evolution and adaptation. Further, the repeated adaptation to freshwater environments (Colosimo et al., 2005, Glazer et al., 2014, Schluter et al., 2004) provides the opportunity to study parallel evolution and hybridization between freshwater and ancestral marine populations and achieve a greater understanding of naturally occurring ecological speciation (Magalhaes et al., 2016, Magalhaes et al., 2021). For example, Schluter et al. (2004) used the comparison of four independently evolved freshwater three-spined stickleback populations to reveal shared genetic biases, discovering that adaptations in lateral plate number and body shape evolved in parallel. A study such as this is only possible due to the three-spined sticklebacks ability to adapt rapidly and the repeated colonisation of freshwater systems.

5 Method

5.1 Study System

Data were collected mainly from Hosta stream located on the west coast of North Uist, Scotland (Figure 1). It is inhabited by resident freshwater three-spined stickleback as well as seasonally accommodating migrating marine three-spined stickleback who use the stream as a breeding site. Hosta stream is around 1m in width, and runs approximately 1km through sand dunes from Loch Hosta to the Atlantic Ocean. Hosta stream is unusual in the high density of three-spined stickleback found there, which makes for an ideal study site. Due to the repeated migration and reproduction of marine three-spined stickleback in Hosta stream, resident freshwater three-spined stickleback vary greatly in physical traits such as armour plating, shape and size as well as genetics (Begum 2021 unpublished PhD thesis).




(b)
Hosta stream
Baleloch
Hosta
Tigharry
Grogarry


(c)
Loch
1
2
3
4
5
6
7
8
9
10
Baleloch

Figure 1. (1a) North Uist, Scotland, (1b) Hosta stream and Grogarry ditch (marine), (1c) Hosta stream and Loch with sampling sites 1 to 10 and the Loch site plotted, (1d) North Uist, Scotland further sampling sites for pH and chromosome XI inversion correlation.

5.2 Sample collection

Stickleback were caught using wire mesh, "Gee's" minnow traps during the breeding period from 28th April to 20th May 2023 as well as 24th April to 15th May 2024. Traps were set from the bank at regularly spaced sites in pools in the stream and left overnight. Sites 1 to 10 were within Hosta stream, whereas site 11 was located on the far side of Loch Hosta. Anadromous stickleback were sampled from a nearby site, Grogarry ditch, a drainage channel that runs from Loch Grogarry to the sea, where there is no evidence of hybridisation with

freshwater stickleback. These anadromous stickleback were used as the nearest reference pure marine population. The Grogarry sample will be referred to as 0m or Grog hereafter. At all sampling sites, juvenile stickleback smaller than 20mm standard length were released. At each site the sample of fish were euthanised by submersion in an MS222 solution (200mg of MS222 in 500ml of water, buffered with 400mg Sodium Bicarbonate powder to prevent an imbalance of pH), resulting in overdose, followed by mechanical destruction of the brain to confirm death. The fish were tagged and stored in 70% ethanol solution. Fin clips of the caudal and two pelvic fins were stored in 96% ethanol solution for DNA extraction.

Firstly, 242 Three-spined stickleback were sampled from 11 sites across Hosta stream and loch as well as Grogarry (sample A, 2023), North Uist, Scotland (57”32.2’N, 7”40.4’W), ranging from 261m (close to the bottom of the stream where it runs out across a sandy beach, and the lowest point in the stream with a regular stickleback population to 1302m from the sea, on the far side of the loch itself from the stream outflow (Figure 1). This sample includes 20 fish from Grogarry, the reference marine population.

A second Hosta sample set (sample B, 2024), totalling 137 three-spined stickleback, was taken at random from the middle section of the stream (sites 4 to 9), from which standard length (mm) and armour plate phenotype were measured as well as fin clips taken for armour plate genotyping.

Additionally, 82 three-spined stickleback, 30 completely plated, 29 low plated and 23 partially plated phenotypes (sample C, 2024), were sampled non-randomly for plate phenotype from Hosta stream from the middle section of the stream (sites 4 to 9) for dissection and hepatosomatic index calculations.

Finally, 6-10 three-spined stickleback were sampled from 17 freshwater lochs in North Uist (sample D, 2024) as well as pH measurements taken using a pH probe submerged at around 1 metre depth. The lochs and streams sampled were: Loch a'Bharpa, Loch Olabhat, Loch na Reivil, Loch Sgadabhagh, Loch Trosavat, Loch Bhrusda, Loch Fada, Loch na Maighdein, Loch Sanndaraigh, Olabhat stream, Trosavat stream, Bharpa stream, Loch a'Chadha Ruaidh, Loch Hosta, Loch Scolpaig, Loch nam Magarlan and Loch na Gearrachun.

5.3 Morphology

Sample A stickleback were prepared for bone staining via incubation in 1% KOH solution, bleached in hydrogen peroxide and KOH solution and finally stained using Alizarin red solution (Peichel et al. 2001) to visualise external bony morphology and stored in 40% isopropyl alcohol. This facilitated the accurate counting of the lateral armour plates and designation of plate morph (complete, partial, low, (Colosimo et al., 2005)) which is a key difference between the divergent marine and freshwater ecotypes. Low plated fish displayed lateral armour plating that did not extend to the caudal peduncle. Partially plated fish display plates that extend to the caudal peduncle, including a caudal keel, however the

row of plates is discontinuous. Lastly, completely plated morphs display a continuous set of lateral armour plating that extends fully to the caudal peduncle, also including a caudal keel. Once stained, the left flank of each fish was photographed on a 1mm grid for image analysis.

To investigate variation in morphology, a digitized landmark system was used to compare and quantify variation in the sample A fish. The landmarks used followed Svanbäck and Eklöv (2003), but were adjusted to provide a more specialised morphometric analysis for the divergence between marine and freshwater three-spined stickleback. The changes include the addition of landmarks at the base of each of the dorsal spines, landmarks on the posterior and anterior points of the pelvic girdle (Aguirre, 2009) as well as landmarks on various bony plates unique to three-spined stickleback (Albert et al., 2008). Overall, 25 landmarks were recorded from each sampled fish using the program tpsdig232 (Rohlf, 2015). These landmarks were then exported, arranged and orientated using Procrustes superimposition, before undergoing principle component analysis using MorphoJ to produce a visualisation of any differences in shape that occur between the armour plate morphs (Klingenberg, 2011).

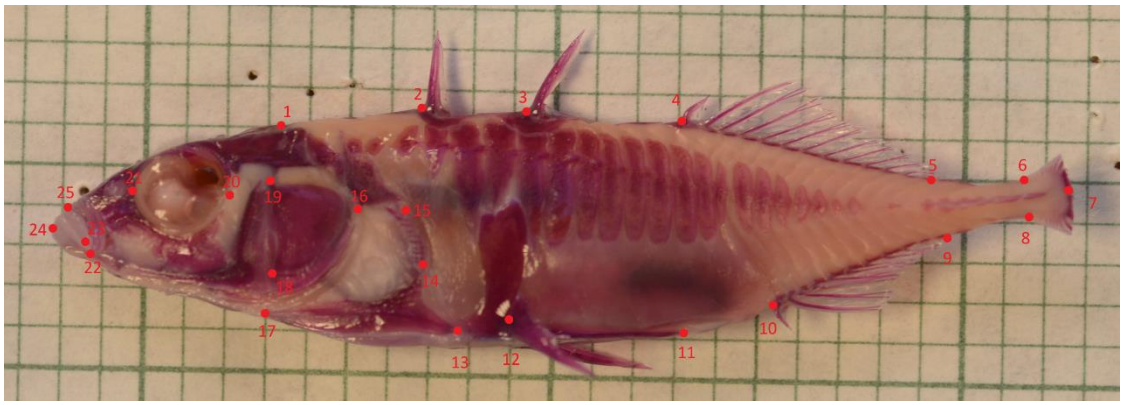


Figure 2. A three-spined stickleback stained using Alizarin red with the locations of the 25 digitized landmarks for morphological shape variation analysis.

Additionally, standard length (the length of a fish measured from tip of snout to tip of caudal peduncle), plate count, dorsal spine length, the largest lateral plate length and pelvic plate length were also measured at 11 sites across Hosta stream and loch and used to describe morphological variation in Hosta hybrid zone.

Two further samples of three-spined stickleback were collected in 2024 (24th April to 15th May) and used for comparative analysis of length and armour plating and Hardy Weinberg calculations for heterozygote deficiency (sample B), and to investigate the link between lateral plating phenotype, Eda and hepatosomatic index (sample C).

5.3.1 Hardy Weinberg equilibrium and Hepatosomatic Index

The Hardy Weinberg equation is used to observe allele or phenotype proportions within a system. By comparing observed proportions with proportions expected by the Hardy Weinberg equilibrium, we can deduce selection pressures on particular phenotypes or alleles. In this case, the Hardy Weinberg equation was used to predict counts of partially plated fish as well as Eda genotype in sample B and check for heterozygote deficiency using the following equation:

$$p^2 + 2pq + q^2 = 1$$

Where p = completely plated homozygous frequency, and q = low plated homozygous frequency, $2pq$ = partially plated heterozygous frequency.

Hepatosomatic index is used as an index of an organism's stored energy by taking into account the liver weight as a proportion of the fish's overall weight and controlling for weight differences in sexual organs between sexes. This is often used as a proxy for fish fitness (Chellappa et al., 1995, Wootton et al., 2006, Campbell and Love, 1978) which offers important information about selection pressures within a hybrid zone. 82 fish from sample C were euthanised and dissected to measure the liver weight, gonad weight, adipose tissue weight and total weight. Hepatosomatic index (HSI) was calculated from the total, liver, adipose and gonad weights of each fish, according to the following equation: HSI = liver weight/somatic weight, where somatic weight is the overall weight of the fish minus the weight of the gonads.

5.4 Statistical analysis

5.4.1 Morphological analysis

Linear models, correlation tests and t-tests in R (Rstudio, 2020) were used to test relationships between morphological measurements.

Samples from 2023 (sample A) and 2024 (samples B and C) were significantly different in standard length, and so were not able to be pooled together for an increased sample size.

Similarly, the sampling method for samples B and C differed in that sample C was targeting equal numbers of armour plate phenotypes, whereas sample B required the random sampling of the population to be an accurate representation of natural plate phenotype proportions. This means that they cannot be pooled together for an increased sample size.

5.4.2 Morphometric analysis

In order to compare overall body shape of three-spined stickleback a morphometric approach was used. This involved a Procrustes superimposition to standardize orientation, size and position of the fish images, followed by principal component analysis to determine differences in body shape between morphs using MorphoJ (Aguirre and Bell, 2012, Klingenberg, 2011).

5.5 Genetics

5.5.1 DNA extraction method

DNA extraction followed the Qiagen DNeasy 96 Blood & Tissue Kit (2023) DNA extraction protocol for animal tissue.

5.5.2 The Eda gene

The Ectodysplasin gene (Eda) is a gene which determines the degree of armour plating in three-spined stickleback. There are two key alleles; Eda^{L} (low) and Eda^{C} (complete) which when homozygous produce low and completely plated phenotypes respectively and a partially plated phenotype when heterozygous. It is well known as a divergent gene between anadromous and freshwater ecotypes (Colosimo et al., 2005, Jones et al., 2012). We genotyped fish from 11 sample sites across Hosta stream and loch, as well as a Grogarry sample (marine), using the previously identified Stn382 microsatellite marker (Colosimo et al., 2005). PCR conditions were as follows: 93°C 3 min, 95°C 30 s, 56°C 30s, 72°C 30s, 5 cycles of 94°C 30s, 50°C 30s, 72°C 30s, 35 cycles of 90°C 30s, 50°C 30s, 72°C 30s, followed by 72°C 10 min, then cooled to 4°C. This marker produces a single band of 153bp product indicating a homozygous Eda L genotype, a single band of 218bp product indicating a homozygous Eda C genotype, or a double banded result indicating a heterozygous Eda CL genotype. Electrophoresis gel was 2% agarose at 100V for 30 minutes. We tested for Hardy-Weinberg equilibrium across the population as well as in small fish (<35mm) in order to determine the cause of any heterozygote deficit and the significance of deviations was tested by chi-squared test.

5.5.3 Mitochondrial lineage

Stickleback on North Uist belong to one of two major mitochondrial clades: trans-Atlantic (At) or European (Eu) (Mäkinen and Merilä, 2008, Dean et al., 2019). Sample A fish were genotyped at cytochrome b to obtain their mitochondrial haplotype, using a restriction enzyme assay (Barnes M., 2023 unpublished). PCR conditions were as follows: initial denaturation at 94°C for 3 minutes followed by 36 cycles: denaturation at 94°C, 30 seconds; annealing at 60°C, 30 seconds; extension at 72°C, 1 minute. Then a final extension at 72°C for 5 minutes. The protocol produces two results per sample, one at the ND4 region and one at the ND5 region, where an agreement between the two can be deemed a sound result. The ND4 region was cut using Hind*III*, resulting in a single band at 625 bp for trans-Atlantic lineage and double bands at 442 and 183 bp for European lineage. The ND5 region was cut using Pst*I*, resulting in double bands at 397 and 219 bp for trans-Atlantic lineage and a single band at 616 bp for European lineage fish. Electrophoresis gels were 2% agarose and ran for 30 minutes at 100V. We were then able to quantify variation in the frequency of the two haplotypes across the hybrid zone.

5.5.4 Chromosomal Inversions

Finally, sample A fish were assayed for chromosomal inversions on linkage groups I and XI using a PCR genotyping protocol (Peichel and Roesti 2022). For chromosomal inversion analysis, previously developed primers were used to amplify indels with F_{ST} of ~1 between freshwater and marine inversion orientations. PCR conditions were as follows: denaturation at 95°C, 1 minute 30 seconds; 94°C, 30 seconds; annealing at 58°C, 30 seconds; extension at 72°C, 30 seconds. Repeat steps 2-4, 4 more times; 90°C, 30 seconds; 58°C, 30 seconds; 72°C, 30 seconds; Repeat steps 6-8, 29 more times; Then a final extension at 72°C for 5 minutes. Electrophoresis gel was 2% agarose at 100V for 1 hour. These PCR assays produce single bands for homozygous freshwater alleles (242bp and 376bp for chromosome I and XI inversions respectively) and homozygous marine alleles (191bp and 359bp for chromosome I and XI inversions respectively), and double bands for heterozygous freshwater and marine alleles. Previous work on North Uist (Haenel et al., 2019) has suggested an association between pH and allele frequency at the chromosome XI inversion. Therefore, in addition to genotyping stickleback from Hosta hybrid zone and anadromous fish from Grogarry, we sampled 6-10 three-spined stickleback from 17 North Uist lochs with varying pH, totalling 156 fish, and assayed the chromosome XI inversion of these fish.

6 Results

During the breeding period in 2023, 221 three-spined stickleback were collected from Hosta stream and Hosta loch, and a further 20 fish from Grogarry (marine). These fish were measured to obtain standard length, digitally landmarked for geometric analysis, phenotyped for armour plate morph and genotyped for the Eda gene. Additionally, 137 fish (sample B) were collected from Hosta stream in 2024, the following year, and genotyped for the Eda gene for Hardy Weinberg analysis and testing for heterozygote deficiency. A subset of 176 three-spined stickleback of the 2023 Hosta stream and loch sample (sample A) were also genotyped at cytochrome b to determine their mitochondrial haplotype, and for the inversions on chromosome I and XI. Lastly, a further 82 fish were collected from Hosta stream between 400m and 900m (sample C), and subsequently dissected to obtain the liver weight, adipose tissue weight and gonad weight.

6.1 Hosta environmental gradients

Salinity, water temperature and pH of Hosta stream were significantly negatively correlated with distance from the sea (Fig. 3, salinity; r = -0.65, t_9 = -2.60, P = 0.029, temperature; r = -0.84, t_9 = -4.65, P = 0.001, pH; r = -0.78, t_9 = -3.70, P = 0.005)

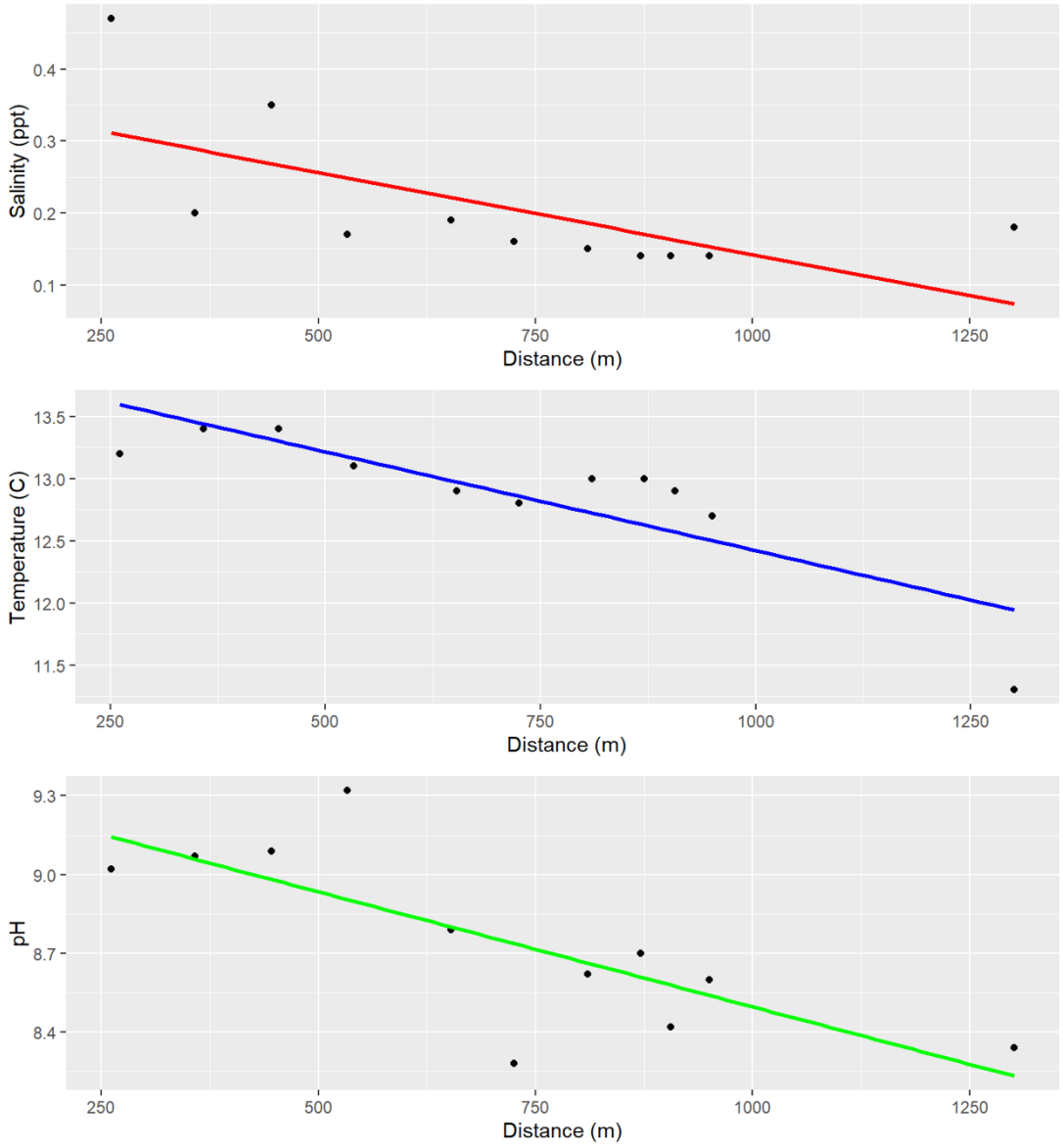


Figure 3. Salinity, water temperature and water pH across 11 sites on Hosta stream and loch.

6.2 Morphological Variation

6.2.1 Armour plating and standard length

There was considerable morphological variation in the hybrid zone in all recorded measurements. All three armour plate phenotypes were distributed throughout the stream. There were more low plated than partially plated and completely plated fish (Fig. 4). All of the sampled anadromous fish from Grog were completely plated. Anadromous fish from Grog were longer than fish in the hybrid zone ($t_{17.1} = 12.9$, $P < 0.001$), with a mean length of 68.7mm compared with 45mm for Hosta fish, but there was no consistent variation in length across the hybrid zone itself (Fig. 5; $r = 0.15$, $t_{82} = 1.42$, $P = 0.16$).

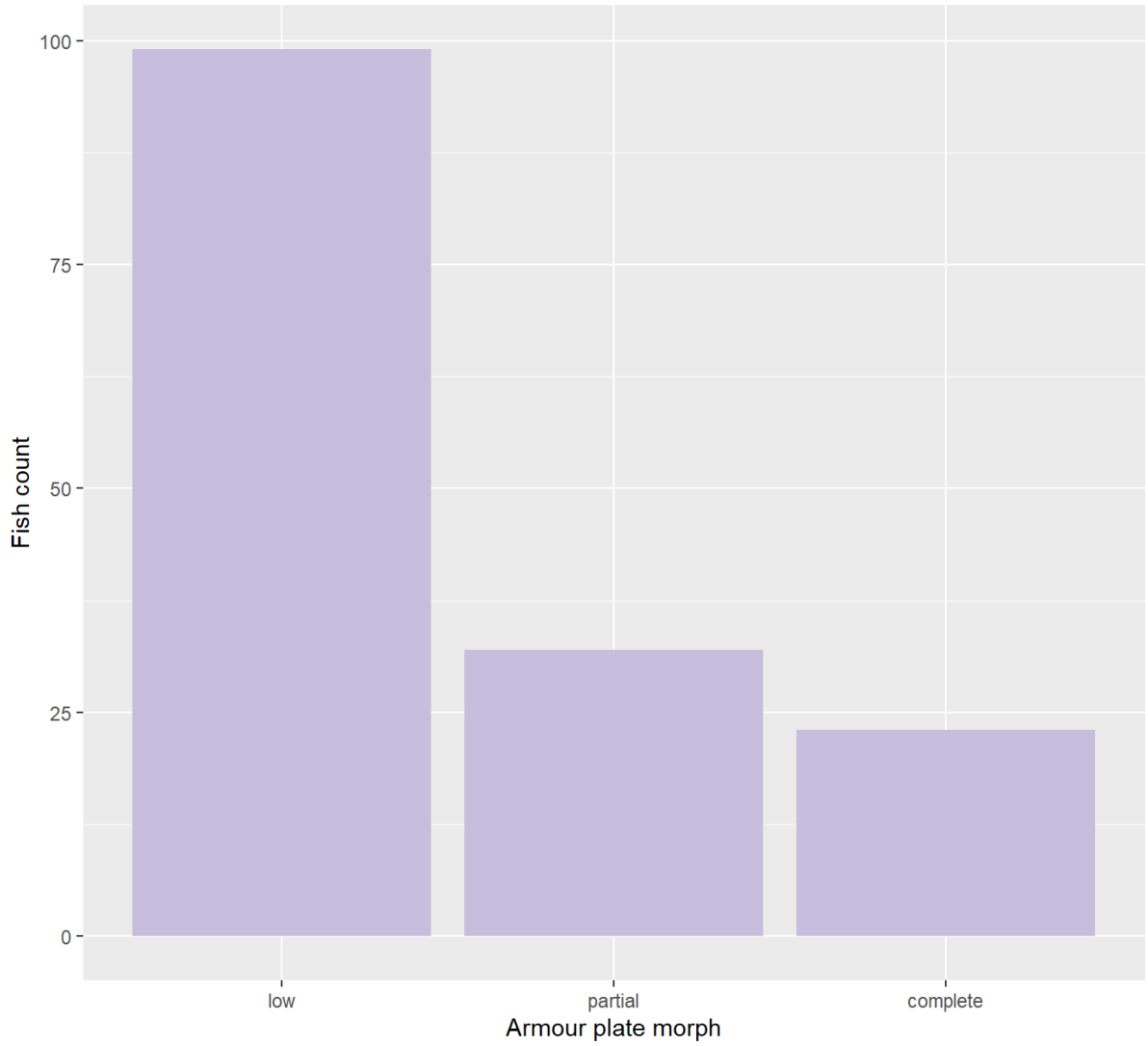


Figure 4. Counts of each armour plate morph, low plated, partially plated and completely plated in 228 three-spined stickleback across the entire length of Hosta stream and loch.

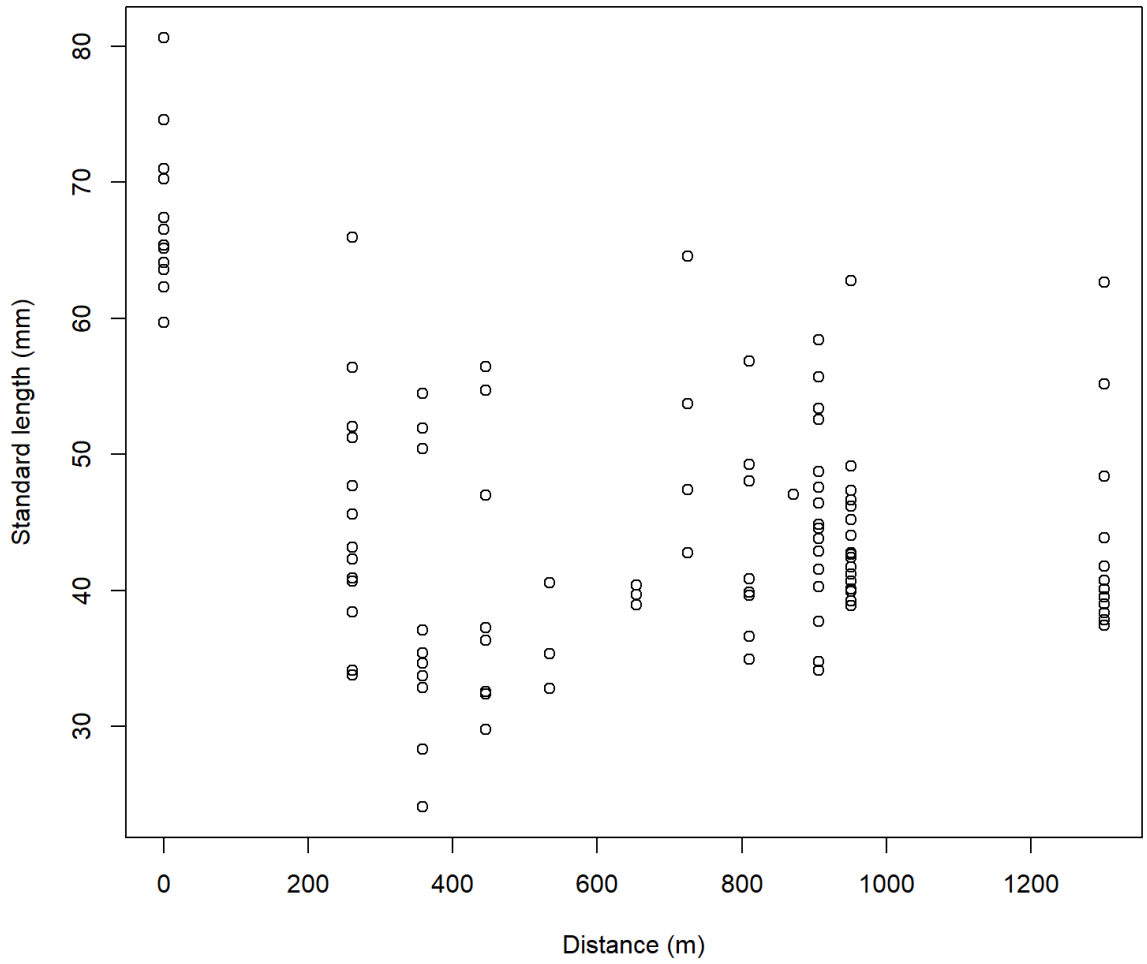


Figure 5. The lengths of 228 sample A three-spined stickleback across Hosta hybrid zone and loch, including Grogarry sample (marine), which was not used in linear models or correlation tests involving the standard lengths of fish across the distance of the hybrid zone.

Lateral armour plate count within the hybrid zone shows a significant negative correlation with distance from the sea (Fig. 6; $r = -0.23$, $t_{206} = -3.46$, $P < 0.001$). Low plated morph fish had between 4 and 9 armour plates, with a mean of 6 armour plates (Fig. 7). Completely plated morph fish displayed between 30 and 33 armour plates and a mean of 31. Completely plated fish from Grogarry had significantly more plates than completely plated fish from Hosta stream and loch ($t_{23.3} = 3.2$, $P = 0.004$). The mean completely plated fish from Hosta displays 31.3 plates, whereas the mean completely plated fish from Grogarry displays 32.6. Partially plated morph fish display a much greater number of plates than low plated morphs ($t_{16.3} = -13.92$, $P < 0.001$), more closely resembling a completely plated morph, with a mean of 24 armour plates. Additionally, the range of plate number in partially plated fish was much greater than in both low and completely plated morphs, from as few as 11 armour plates to as many as 32. Overall, plate count was an accurate predictor of armour plate morph and there are significant differences between the three armour plate morphs and the plate counts (Fig. 7; $F_{2,93} = 796.3$, $P < 0.001$). Excluding grog and Hosta loch, there remains a significant negative correlation between armour plate count and distance from sea within the hybrid zone itself ($r = -0.146$, $t_{186} = -2.00$, $P = 0.046$).

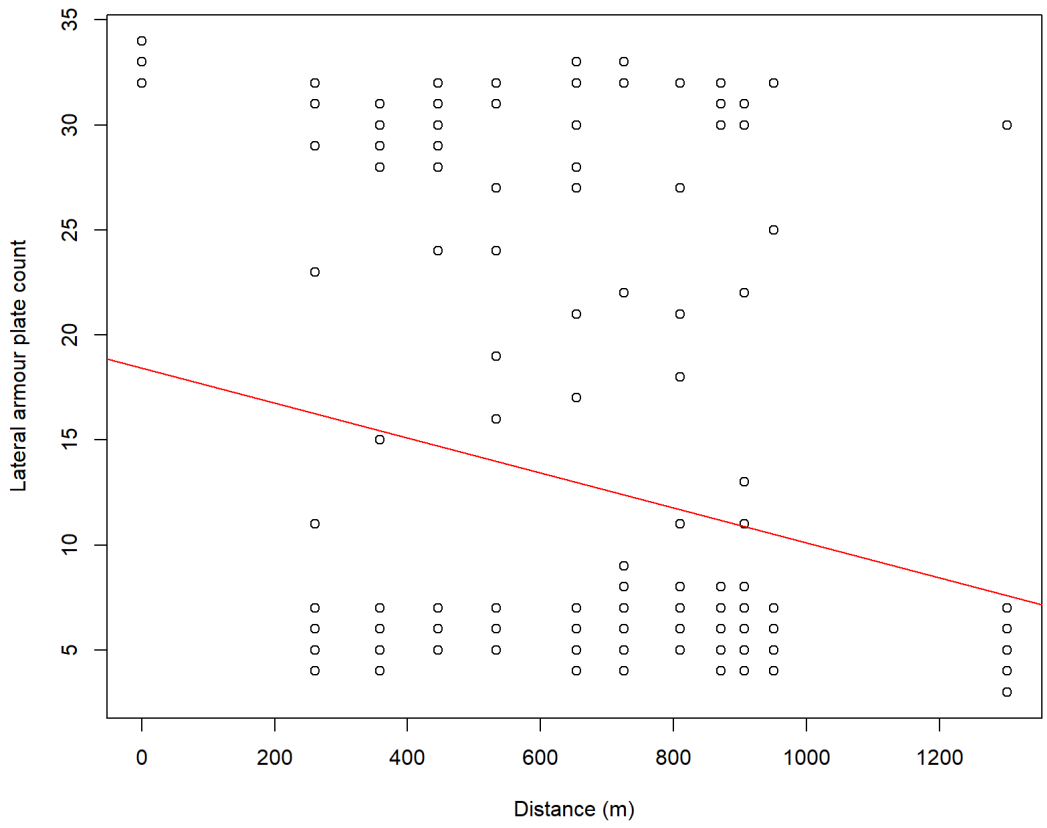


Figure 6. lateral armour plate count of 241 sample A three-spined stickleback against distance across Hosta hybrid zone. Data points overlap, but all are accounted for in the linear model. Red line indicates a significant negative correlation. Grogarry sample (0m, marine) not included in the linear model.

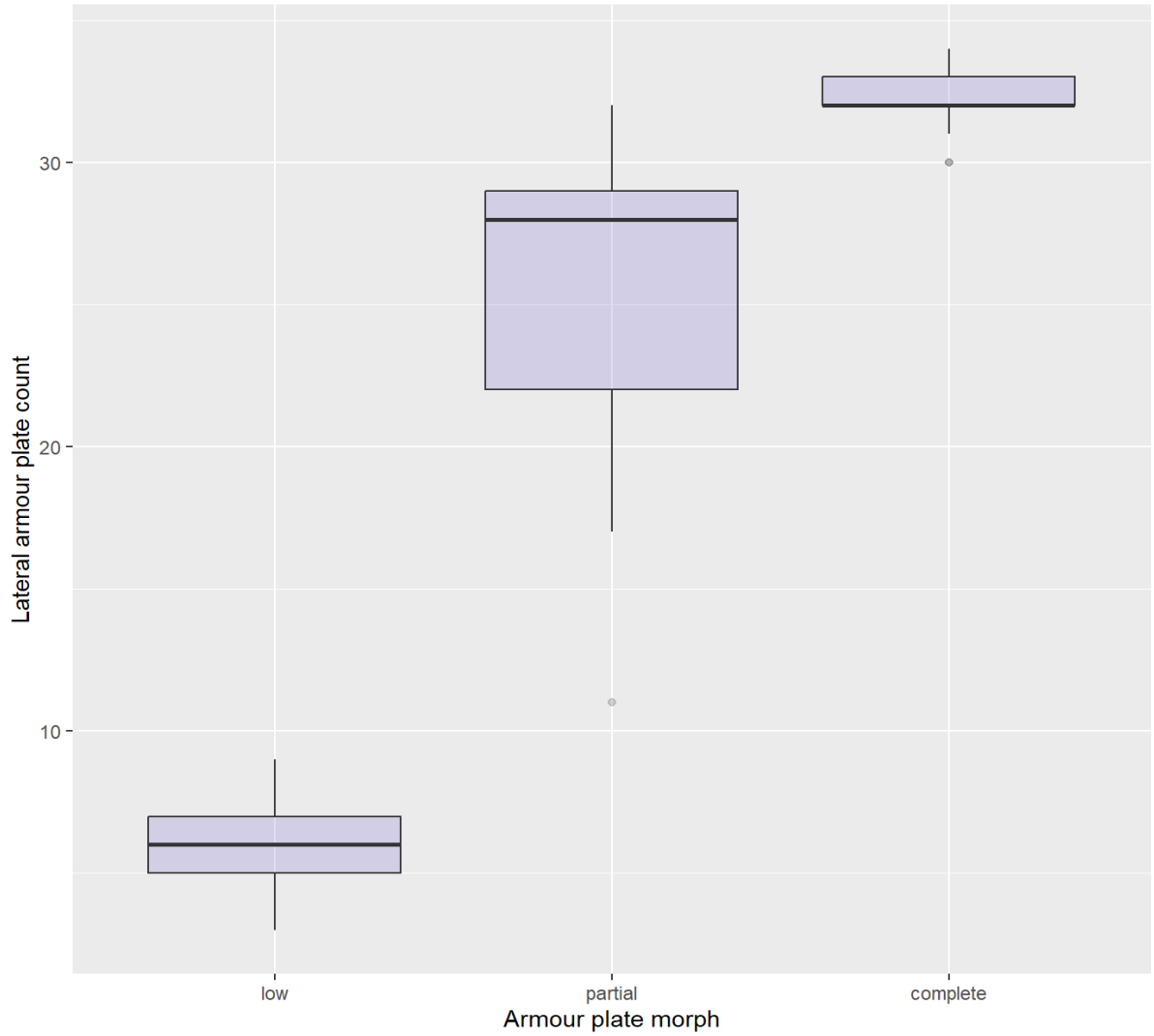


Figure 7. The plate counts of 241 sample A fish in Hosta stream, Hosta loch and Grogarry (marine) from each plate morph category: low plate, partial plate and complete plate.

The standard lengths of three-spined stickleback are not significantly different between the armour plating phenotypes (Fig. 8; $F_{2,94} = 0.706$, $P = 0.496$). With average mean standard lengths of 41.6mm, 44.2mm and 43.5mm for partially plated, low plated and completely plated morphs respectively. Notably, partially plated fish are smaller on average than low and completely plated phenotypes and there is a complete lack of partially plated fish larger than 50mm (fig. 8). There were no significant correlations between dorsal spine length (Fig. 9; $r = 0.017$, $t_9 = 0.051$, $P = 0.960$), largest lateral plate length (Fig. 9; $r = 0.196$, $t_9 = 0.601$, $P = 0.563$) or pelvic plate length (Fig. 9; $r = -0.143$, $t_9 = -0.435$, $P = 0.674$) and distance from the sea within Hosta hybrid zone.

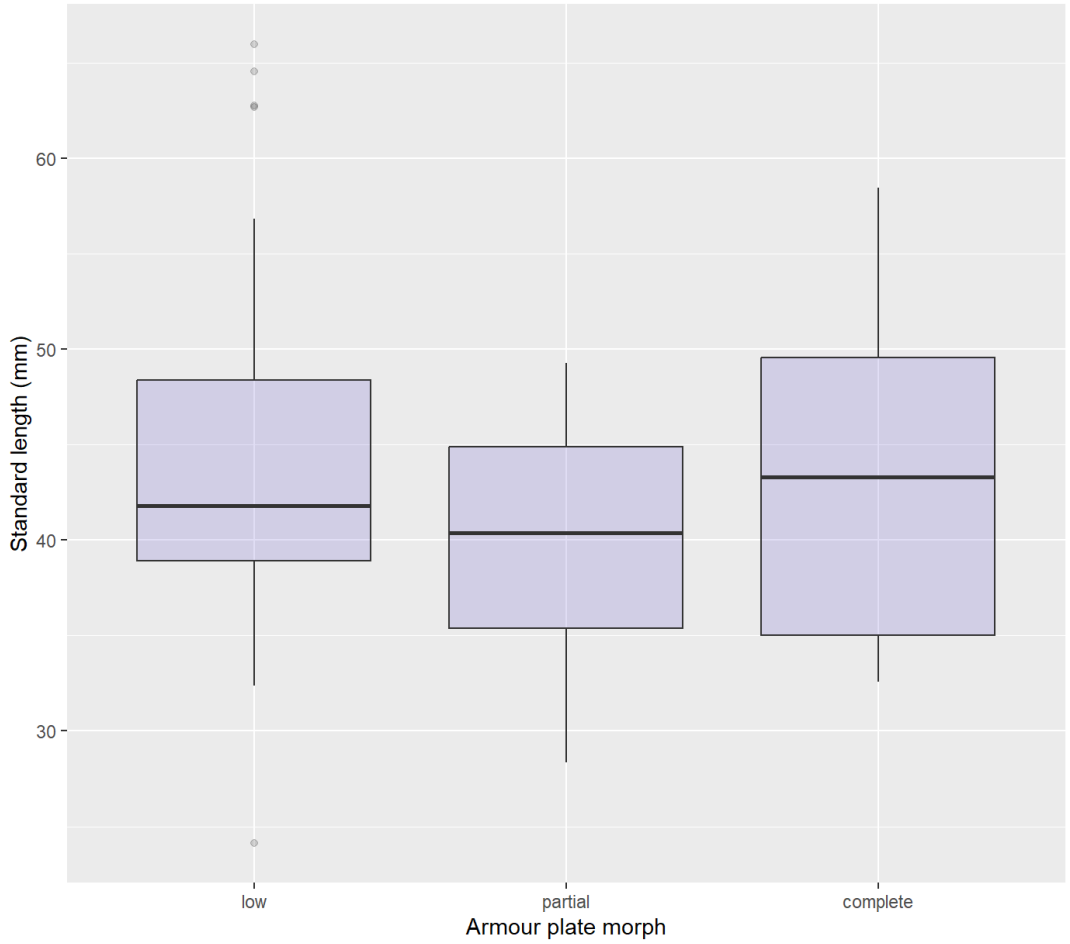


Figure 8. The standard lengths of each of the plate morphs, partially plated, low plated and completely plated three-spined stickleback in Hosta stream and Loch, excluding Grogarry (228 fish, sample A). The thick black line within each box represents the median standard length and the whiskers represent the minimum and maximum standard lengths, with potential outliers excluded and marked with hollow circles.

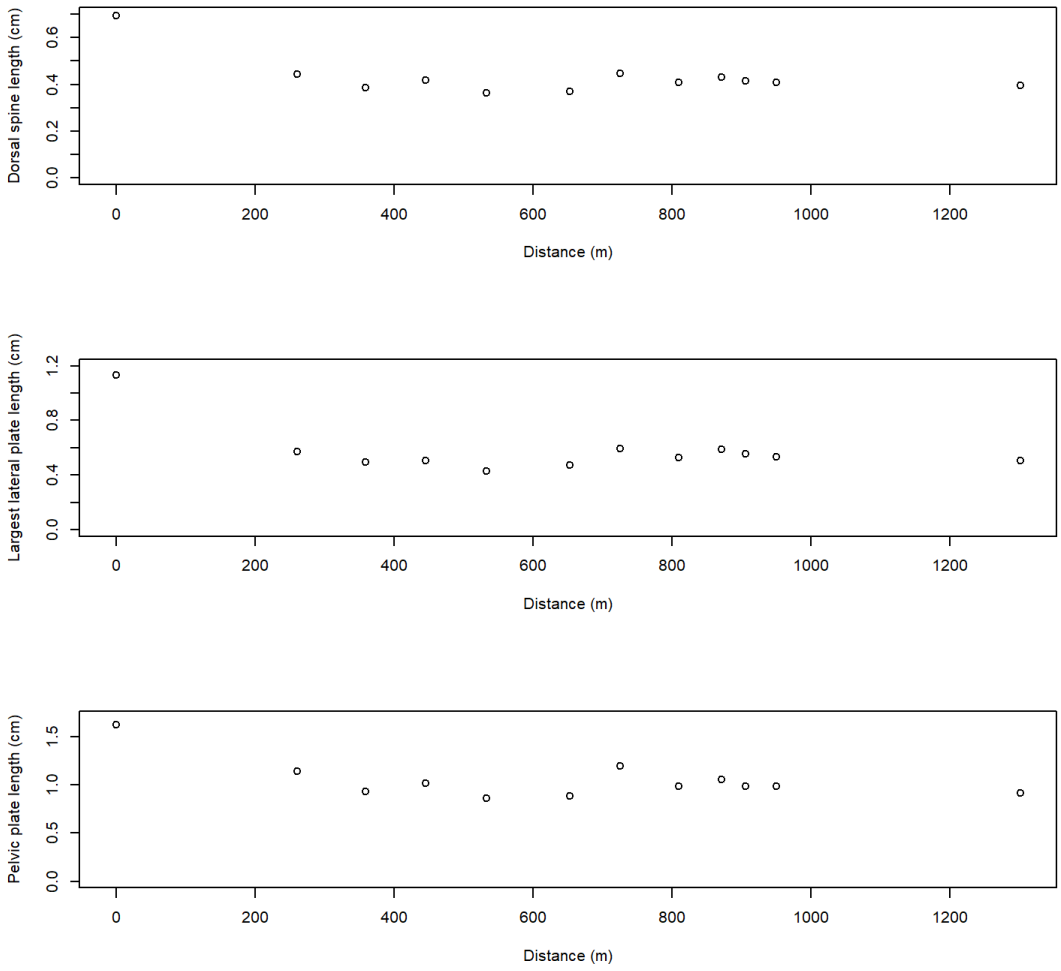


Figure 9. Differences in armour between sampling sites in 108 sample A three-spined stickleback. (a) Dorsal spine length, (b) Largest lateral plate length, and (c) pelvic plate length against distance from the sea within Hosta hybrid zone and loch (sample A). Grog (0m, marine) not included in models.

6.2.2 Morphological shape

Fish body shape was not significantly different among the armour plate morphs (Procrustes ANOVA: $F_{2,92} = 0.84$, $P = 0.857$). Similarly, fish body size, estimated from the centroid size of the landmarks, was not significantly different among the armour plate morphs (ANOVA: $F_{2,101} = 0.25$, $P = 0.782$) within Hosta stream. Principle components 1 and 2 explain 26% and 19% of the variance respectively. Principle component 3 appears to display the twisting effect that is a result of the preservation method and as such is not included. There was little variation in body shape within Hosta hybrid zone, however there seems to be a significant difference between Grog and Hosta fish shapes (Procrustes ANOVA: $F_{3,92} = 1.98$, $P < 0.001$).

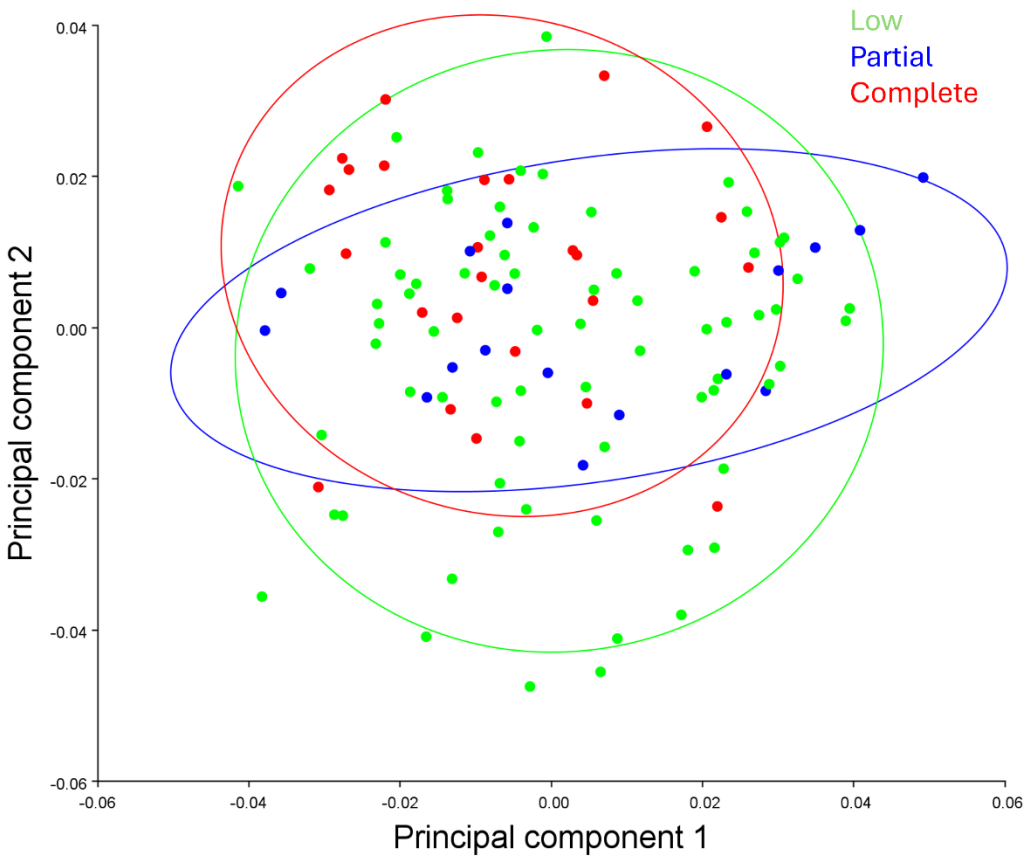


Figure 10. Variation in body shape among plate morphs of 117, sample A, three-spined stickleback from Hosta stream and loch across the first two PCs on 25 digitised landmarks. Data points and 95% confidence ellipses are colour coded to represent the three armour plate morphs, low, partial and complete as green, blue and red respectively.

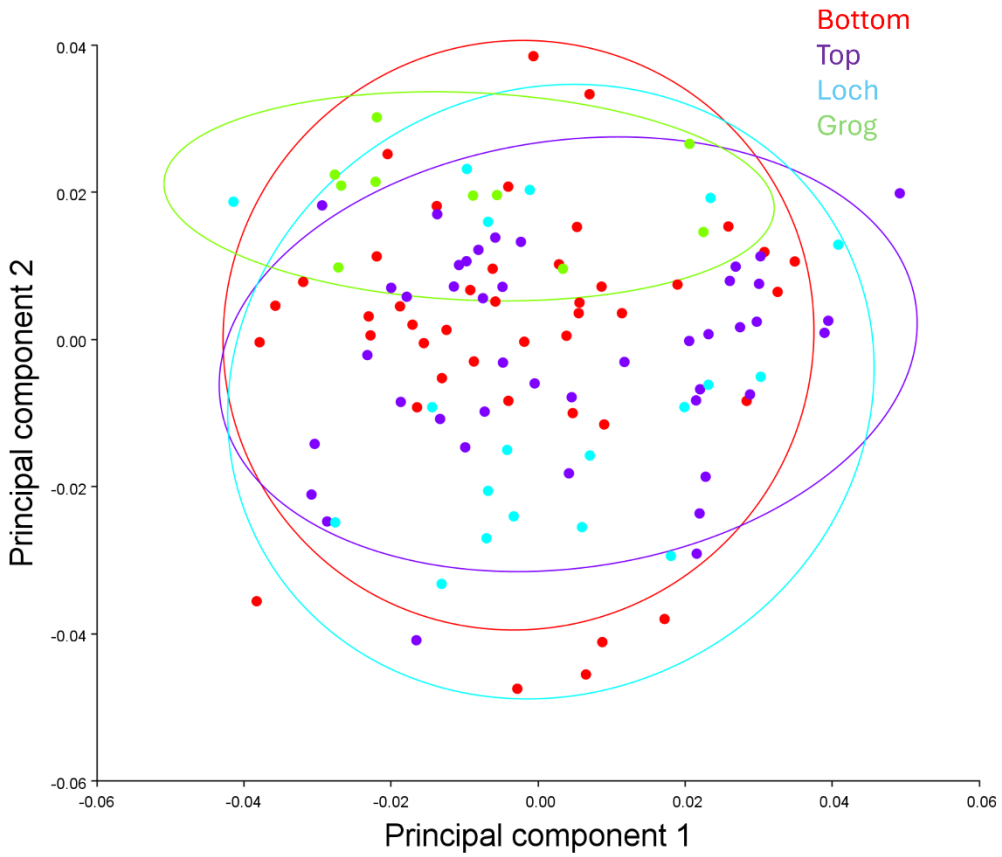


Figure 11. Variation in body shape among 117 sample A three-spined stickle-back from Hosta hybrid zone, split into bottom of the stream (sites 1 – 5, red) and top of the stream (sites 6 – 10, purple), Hosta loch (Loch, blue) and the anadromous population from Grogarry (Grog, green) across the first two PCs on 25 digitised landmarks. Data points and 95% confidence ellipses are colour coded to represent the 4 distance groups previously mentioned.

6.3 Genetic variation

The frequency of Eda C alleles followed a significant negative trend from the start of Hosta stream at 261m to Hosta Loch at 1300m from the sea (Fig. 12; r = -0.72, t_9 = -3.09, *P* = 0.013). Plate morph ratios (Figure 3) were not in Hardy Weinberg equilibrium and showed a deficit of partially plated fish within Hosta stream (χ^2 = 61.86, df = 2, *P* > 0.001). This heterozygote deficit was not observed in fish smaller than 35mm in standard length (χ^2 = 0.198, df = 2, *P* = 0.906). However Eda genotypes, rather than armour plate phenotype, did not show the same heterozygote deficit, either in all fish (χ^2 = 2.07, df = 2, *P* = 0.356) or small (<35 mm) fish (χ^2 = 0.2, df = 2, *P* = 0.9).

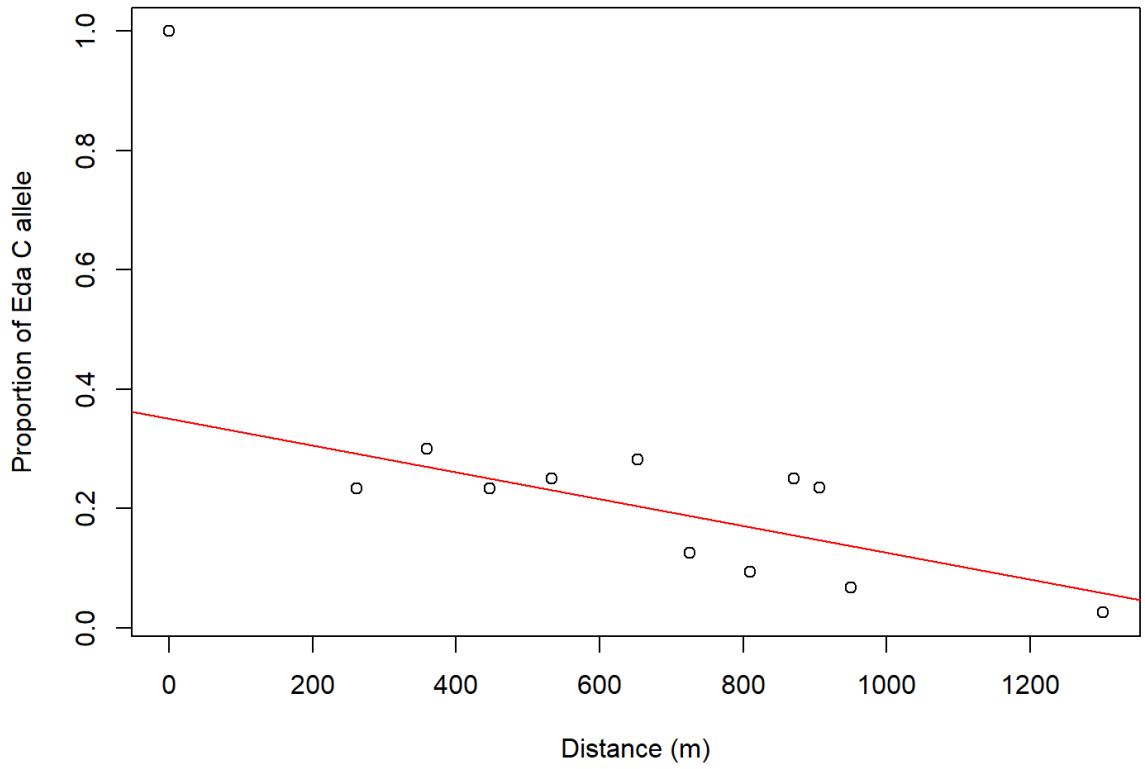


Figure 12. The proportion of Eda completely plated alleles "C" across Hosta hybrid zone (260 – 1000m), Loch (1300m) and Grog (0m, marine) in 186 three-spined stickleback. Red line indicates significant correlation. Grog sample (0m) not included in linear model.

Sex, standard length and the Eda genotype were incorporated into a linear model to determine the factors associated with hepatosomatic index. The results (Table 1) show that sex is the most significant factor for explaining hepatosomatic index, which increases with length in females (Fig. 13; r = 0.48, t_{38} = 3.39, *P* = 0.002), but not males (Fig. 13; r = 0.03, t_{39} = 0.19, *P* = 0.853). None of the model terms involving Eda, either as a main effect, or in interaction, had any association with hepatosomatic index.

Table 1. The statistical relationships between hepatosomatic index and sex, standard length (SL) and Eda genotype, for 81 three-spined stickleback in the Hosta stream hybrid zone on North Uist, obtained from generalised linear model with normal errors and identity link function (sample C).

Term	F	df	P
Sex	85.75	1,77	<0.001
SL	30.99	1,77	<0.001
Sex:SL	7.35	1,77	0.008
Eda:SL	0.48	2,75	0.627
Sex:Eda	0.73	2,73	0.312
Eda	2.73	2,71	0.967
Eda:Sex:SL	1.73	2,69	0.186

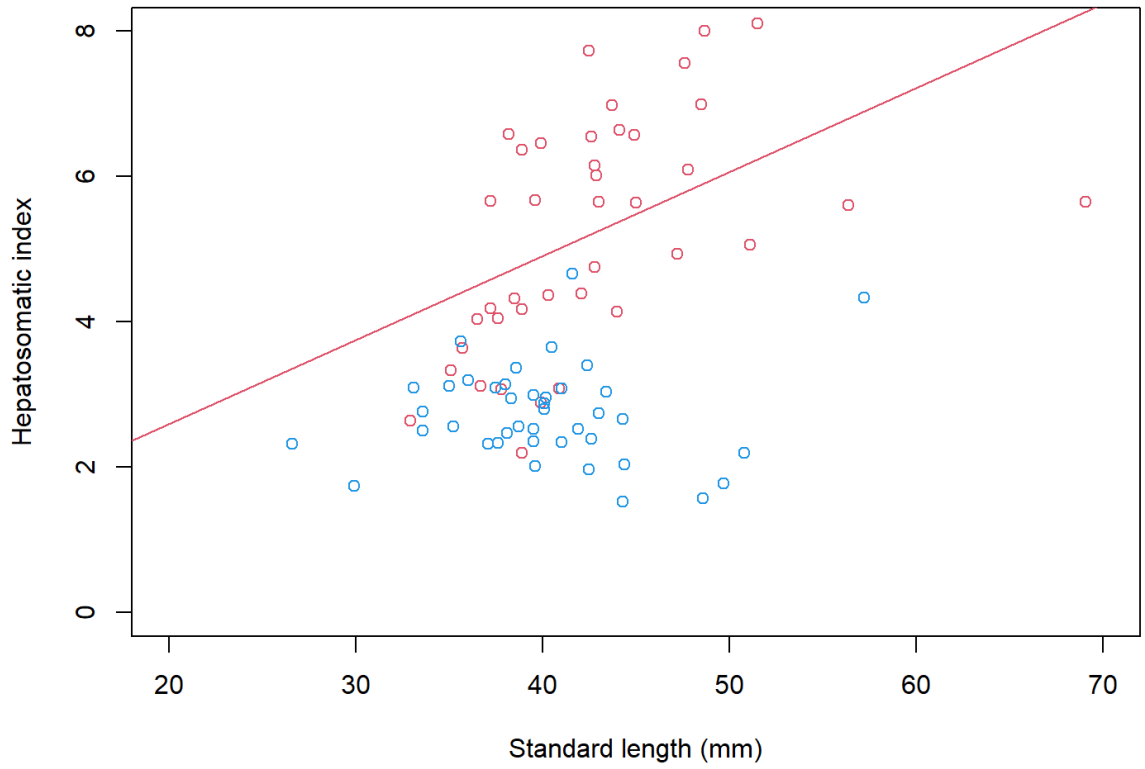


Figure 13. Hepatosomatic index against standard length (mm) for 81 three-spined stickleback in Hosta stream (sites 4-9, sample C). Red and blue points indicate female and male three-spined stickleback respectively. Red line shows significant positive correlation between female hepatosomatic index and standard length. No significant trend observed in males.

6.3.1 Mitochondrial variation

The majority of fish in the stream were of the Eu mitochondrial lineage, despite higher frequencies of At fish in the anadromous population from Grogarry and in Loch Hosta. From 700m upwards, the trans-Atlantic lineage was present in small numbers, reaching as high as 25% at 825m and 1300m. There was a significant correlation between the proportion of trans-Atlantic lineage fish and the distance from the sea (Fig. 14; $r = 0.724$, $t_9 = 3.145$, $P = 0.012$). Grog fish (0m, marine) were removed from the model, but the Grog sample consisted of 50% European and trans-Atlantic lineage fish.

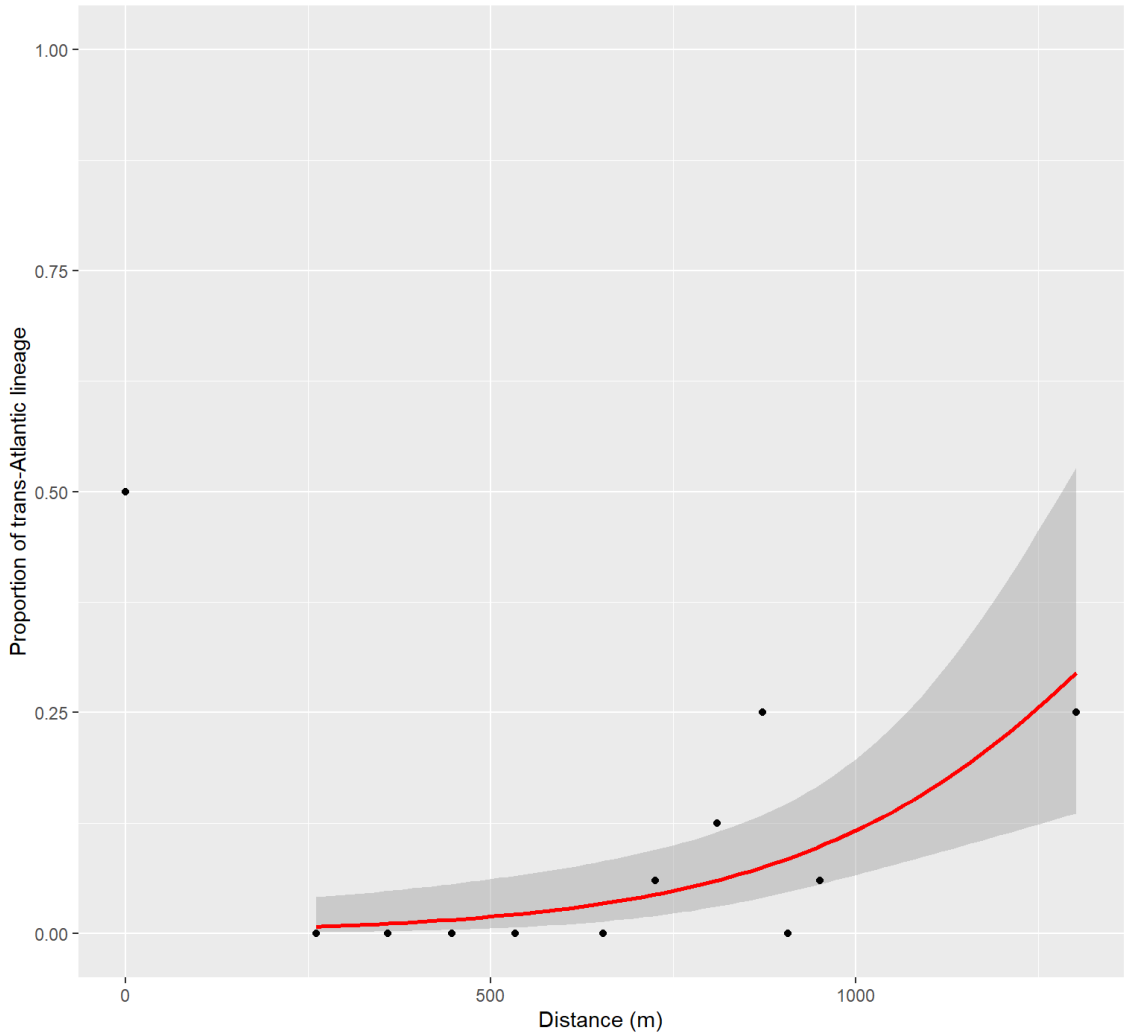


Figure 14. The proportion of fish carrying the trans-Atlantic mitochondrial haplotype across the Hosta hybrid zone with 95% confidence interval in 176 three-spined stickleback (sample A). The data point for Grogarry (0m, marine) was not included in the model.

6.3.2 Eda genotype, armour plate phenotype and mitochondrial lineage

Eda genotype did not predict plate phenotype consistently in Hosta stream and loch. The discrepancy between Eda genotype and plate phenotype is significant ($t_{180} = 9.5$, $P < 0.001$). There were 23 mismatches out of 181 fish. The majority of mismatches were completely plated fish that were Eda CL homozygous (Fig. 15). No low plated fish were Eda CC homozygous, and similarly no completely plated fish were Eda LL homozygous. Mitochondrial lineage was not significantly different between armour plate phenotypes (ANOVA, $F_{2, 178} = 1.364$, $P = 0.258$), whereas the proportion of phenotype-genotype mismatching between the three armour plate phenotypes was significant (ANOVA, $F_{2, 178} = 36.64$, $P < 0.001$).

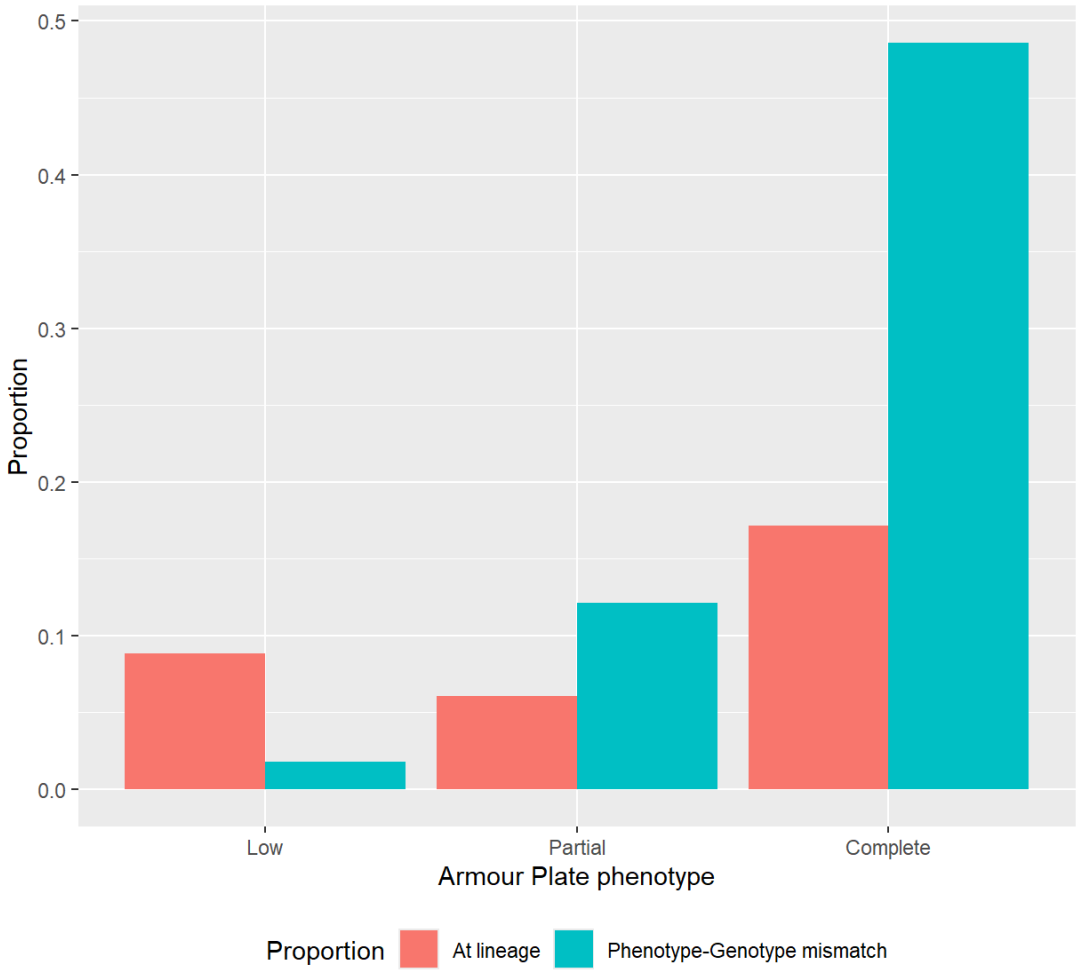


Figure 15. The proportion of trans-Atlantic lineage and the proportion of armour plate phenotype – Eda genotype mismatch within the three armour plate morphs: low plated, partially plated and completely plated three-spined stickleback in 181 sample A stickleback: Hosta stream, loch and Grog (marine).

6.3.3 Chromosomal inversions

The frequency of the 'marine' allele at the chromosome I inversion increased significantly with distance from the sea (Fig. 16; r = 0.61, t_9 = 2.33, *P* = 0.045). Contrastingly, there was no significant correlation between the frequency of chromosome XI marine alleles and the distance from the sea within Hosta hybrid zone (Fig. 17; r = 0.50, t_9 = 1.75, *P* = 0.115).

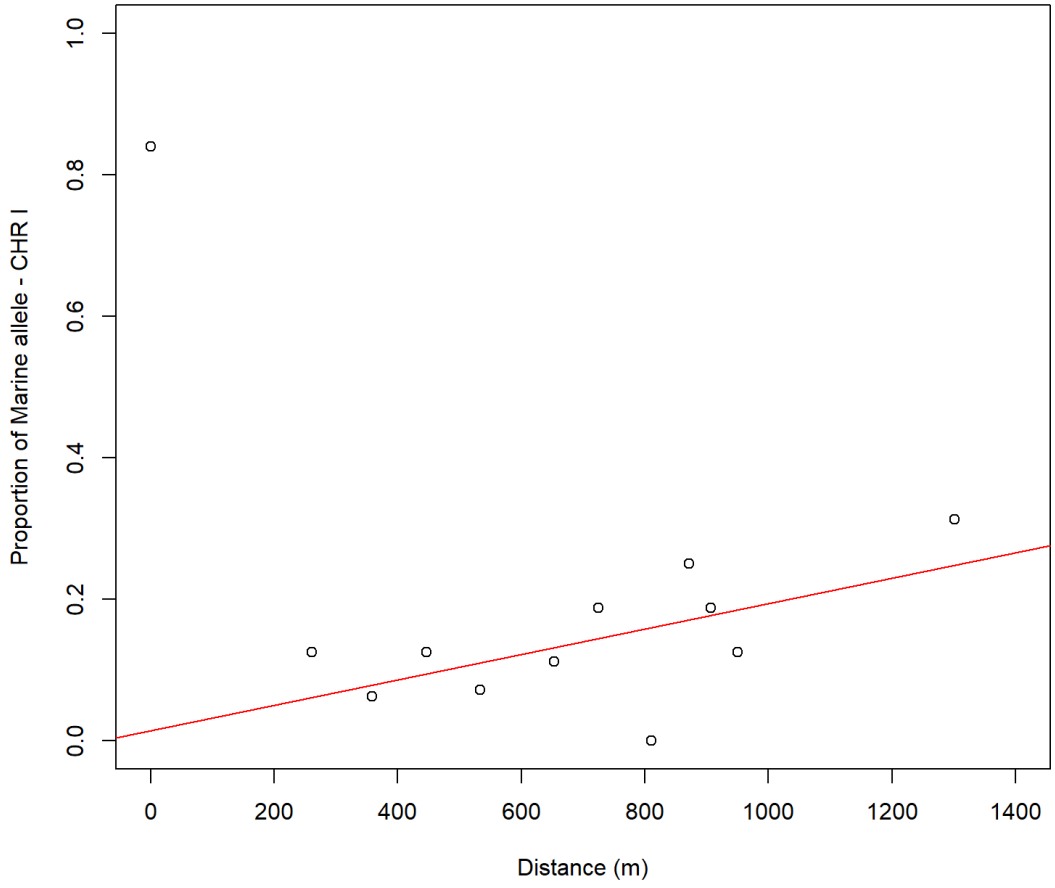


Figure 16. The proportion of marine alleles at the chromosome I inversion in 192 three-spined stickleback across Hosta stream, loch and grog (sample A). Red line indicates significant positive correlation. Data point for Grogarry (0m) not included in linear model.

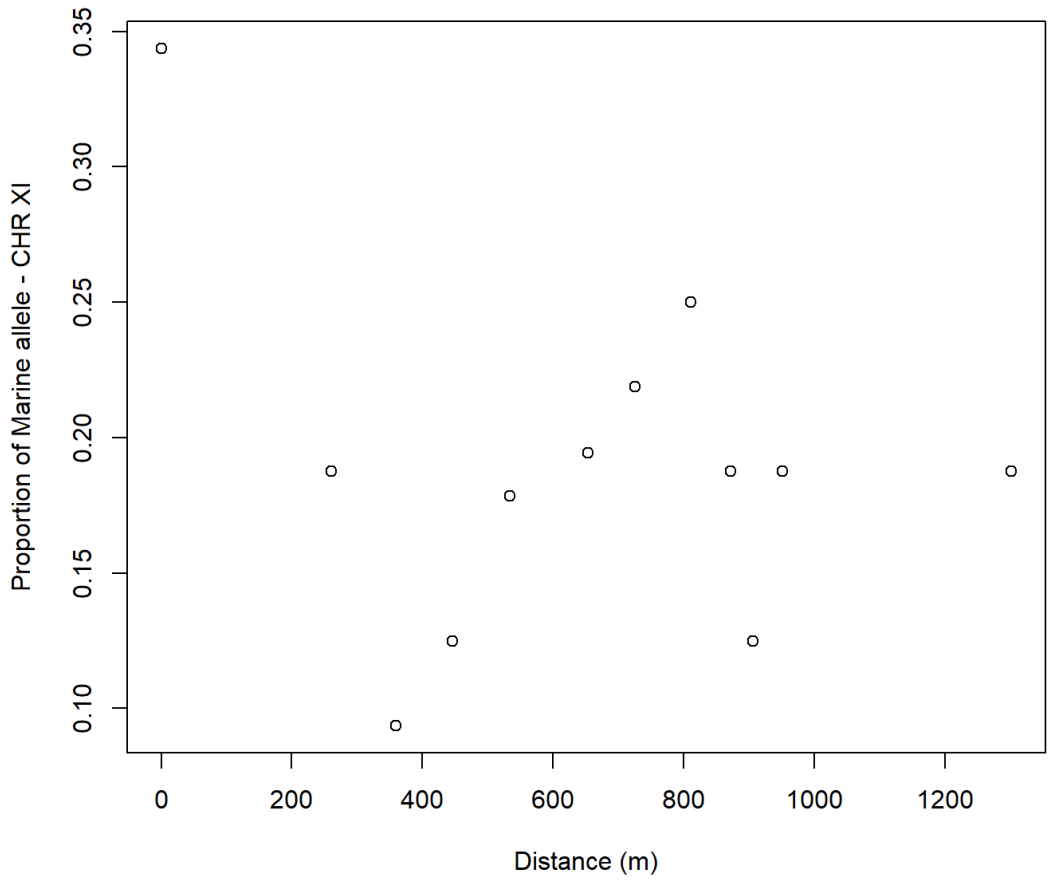


Figure 17. The proportion of marine alleles on the chromosome XI inversion in 192 three-spined stickleback across Hosta hybrid zone (sample A). Data point for Grogarry (0m, marine) not included in statistical testing.

Across 17 freshwater lochs there was a strong positive association between the marine chromosome XI inversion alleles and the pH of the water (Fig. 18; r = 0.831, $t_{15} = 5.79$, $P < 0.0001$). The lochs range in pH from 6.1 to 8.8. Loch a'Bharpa, Loch Sgadabhagh, Trosavat stream, a'Bharpa stream and Loch a'Chadha Ruaidh were all completely of the freshwater chromosome XI inversion orientation. Whereas Loch Scolpaig was the only loch showing completely marine chromosome XI inversion orientation fish, followed by loch Sann at 0.94 marine orientation proportion.

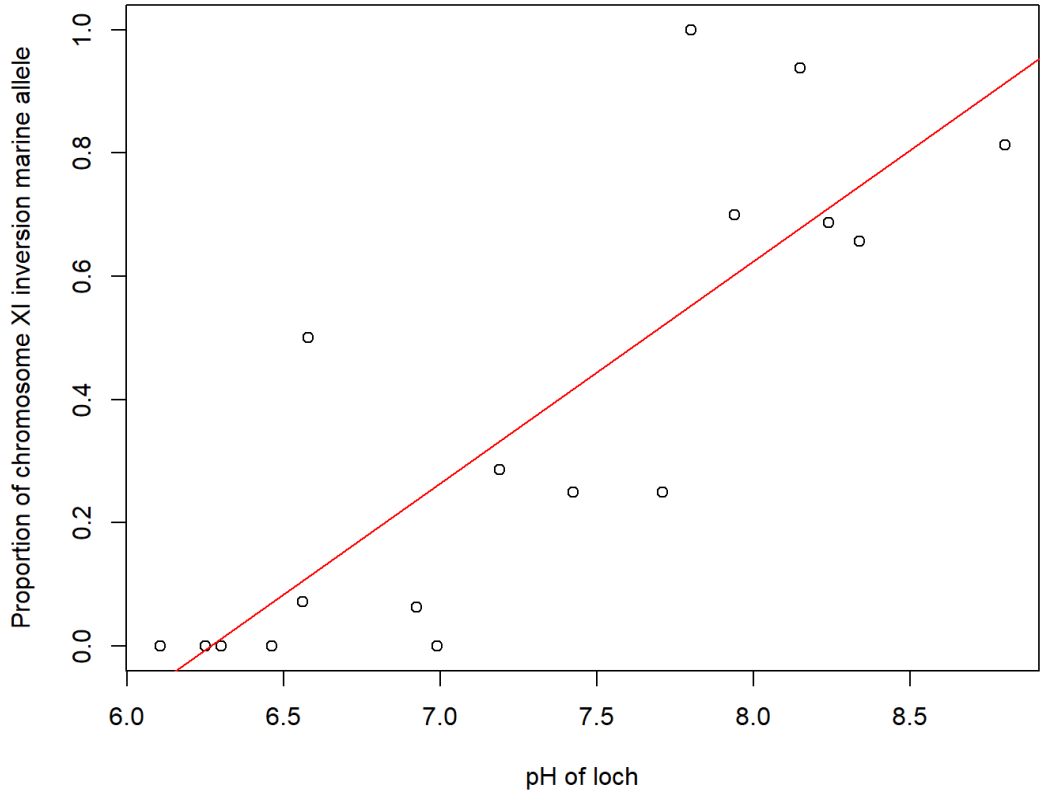


Figure 18. The frequency of chromosome XI inversion marine alleles in 159 three-spined stickleback across lochs of varying pH (sample D). Red line indicates significant positive correlation.

7 Discussion

Hosta hybrid zone exhibits a remarkable amount of morphological and genetic variation within a small distance, including large variations in size, lateral plating, shape and adaptive allele frequencies. There appears to be little structure in morphology throughout the hybrid zone, with length, measures of armour (including plate count) and shape remaining relatively constant, suggesting a panmictic population where migration and gene flow overcome any selection that might be associated with the parental populations inhabiting contrasting environments (freshwater loch, open sea) at either end. However, in sharp contrast, the genotyping results suggest some strong structuring, with substantial variation in allele frequencies of putatively adaptive alleles such as mitochondrial lineage, Eda genotype and the chromosome I inversion alleles. More detailed examinations of the associations and geographical patterns in these alleles suggest that Eda is not associated with variation in relative liver weight, and that the chromosome XI inversion is likely to contribute to adaptation to variation in pH or its consequences.

7.1 Morphological variation within Hosta hybrid zone

There is little pattern in the morphological variation within Hosta hybrid zone. Neither size nor shape, nor elements of armour, with the exception of lateral plate count showed any significant variation through the hybrid zone, and the variation in plate counts was small. A similar study involving observations of shape variation across sea, river and lake habitats showed contrasting results, where significant changes in body depth and length were shown between lake and river environments (Ravinet et al., 2013). Benthic-limnetic divergence is

common in freshwater three-spined stickleback systems (Dean et al., 2022, Ravinet et al., 2013, Aguirre, 2009), but the adaptation towards these niches is not apparent in Hosta stream as body shape did not seem to change between loch and stream environments. The lack of an anadromous-freshwater gradient in morphological characteristics across the hybrid zone suggests that Hosta hybrid zone is comprised of one rather homogeneous population that is infrequently permeated by anadromous three-spined stickleback during their migration. However, shape analysis including the anadromous Grogarry population did show significant differences to the Hosta loch sample, suggesting that there is some barrier to gene flow from Hosta stream to the loch.

7.1.1 Reproductive Isolation

This barrier to gene flow between Hosta stream and Hosta Loch is somewhat suggested in the lateral plate count result, as there is a lack of partial and completely plated phenotypes in the loch, but no other morphological data supports this. The homogeneity of the results and the lack of a cline in anadromous morphological features with distance from the sea suggests that the migration of anadromous stickleback into Hosta hybrid zone is rare and/or that any selection on morphological variation is limited. Alternatively, changes in shape between the sites may be due to phenotypic plasticity to environmental conditions, and not divergent selection (Svanbäck and Schluter, 2012), but genetic analysis on body shape is required to test this. Nevertheless, the frequencies of completely plated and partially plated fish in the stream strongly suggest that the population there has been subject to admixture from the sea in the past or continues to be.

The factors that affect the distance anadromous stickleback will travel within a freshwater stream before reproducing are not well documented but can play an important role in reproductive isolation between the anadromous and freshwater three-spined stickleback in Hosta stream. Previous hybrid zone studies find a steady gradient in characteristics between the two divergent populations (Vines et al., 2016, Barton and Hewitt, 1985), thus we might expect a steady decrease of standard length and lateral armour plate count as well as spine length, lateral plate size and pelvic plate size with an increase in distance from the sea. However, this is not the case in Hosta stream, as we observe a decrease in lateral armour plate count with distance from the sea but no consistent trend in standard length or in dorsal spine length, lateral plate size or pelvic plate size. This might suggest that there are no environmental or biotic cues that cause anadromous three-spined stickleback to choose to breed or inhabit specific sections of the stream and therefore, no gradient of anadromous length or plating characteristics is visible across the hybrid zone.

We did not observe any definitely anadromous fish in any samples from Hosta stream and loch (personal observation), which suggests that anadromous migration into Hosta stream is infrequent and rare. This may have left time since any anadromous immigration for the mixing and movement of fish within Hosta stream to blur any distinct trends in armour plating or other anadromous characteristics across the hybrid zone, which is an explanation for the lack of morphological structure or gradients in traits across the hybrid zone. However, lateral armour plate count decreased significantly within Loch Hosta, indicating a lack of complete and partially plated three-spined stickleback, suggesting some

morphological structure at the extremes. The rarity of completely plated phenotypes in Hosta loch in the face of the easy gene flow within Hosta stream suggests that direct dispersal is rare, but also that there may be strongly structured selection against the Eda^{C} allele in the loch. One explanation for this is that there may be habitat preference associated with the Eda alleles between the fast-flowing stream water and the slow-moving loch water, as well as the various water chemistry and flora differences. Previous studies show that stickleback can be selective of their habitat as lake and stream stickleback differ significantly in their preference for nesting habitat (Hendry et al., 2002, Bolnick et al., 2009), however how/if this translates to freshwater and marine stickleback and the Eda allele is unclear. Similarly, the Eda gene has been shown to affect schooling behaviour (Greenwood et al., 2013) which might also affect the distribution of plate phenotypes within Hosta stream and loch.

7.1.2 Heterozygote Deficiency

Three-spined stickleback have repeatedly adapted reduced lateral armour plating in freshwater systems. Hosta hybrid zone appears to be no exception, with vastly more low plated fish than completely and partially plated fish. It is unexpected that partially plated fish had a smaller standard length on average than low and completely plated phenotypes, which might suggest specific selection pressures for heterozygous fish. For this reason, we investigated heterozygote deficiency in Hosta stream. Hardy Weinberg tests on armour plate phenotype revealed a significant lack of partially plated fish within the Hosta stream population, consistent with the theory that selection is acting against the partially plated phenotype. This result is supported by the absence of partially plated fish

larger than 50mm across Hosta hybrid zone, and further by the partially plated fish falling within the Hardy Weinberg equilibrium in younger fish (<35mm), both of which indicate that the reduced heterozygote count in the stream might be due to heterozygote fish experiencing a reduced fitness rather than assortative mating. This result is similar to a previous study where Eda CL heterozygous fish were transplanted into a natural lake system and monitored for changes to Eda allele frequency, ultimately resulting in a deficit of heterozygotes overall and within embryos, suggesting disruptive natural selection via the selection against heterozygotes (Schluter et al., 2010). However, the lack of any departure from Hardy Weinberg equilibrium for the Eda genotypes in this study does not support the existence of heterozygote disadvantage, as has been documented elsewhere previously (Marchinko et al., 2014). Instead, there may simply be incomplete penetrance of the Eda allele in determining plating phenotype, and/or some pattern of selection on modifier alleles. The plating phenotype is known to have three modifier alleles that normally explain only a small proportion of the variation in plate phenotype (Colosimo et al., 2004, O'Brown et al., 2015), but that might be enough to explain the discrepancy between phenotypic and genotypic data.

7.1.3 Hepatosomatic index

Previous research shows mixed evidence for heterozygote deficiency, suggesting a heavy dependency on environmental factors within each system (Jones et al., 2006). Following the discovery of a deficit of partially plated fish in Hosta hybrid zone, we used the hepatosomatic index as a proxy for fitness to test one hypothesis for what might drive selection (pleiotropic metabolic effects acting through the BAFF gene that is physically linked to Eda (Chan et al., 2021, Colosimo et al., 2005)). However, the results indicated that liver weight variation and hence lipid stores were not affected by Eda genotype, which suggests that Eda genotype did not have a significant effect on that measure of fish fitness.

7.2 Genetic variation and patterns of selection across environmental gradients

7.2.1 Eda

The frequency of Eda^{C} alleles decreases steadily with distance from the sea within Hosta stream and loch. This suggests a pattern of selection against the Eda^{C} allele in freshwater systems, which is exactly as we would expect from previous studies (Colosimo et al., 2005, Magalhaes et al., 2016) and follows explicitly the pattern described by the hypothesis that reduced armour plating was repeatedly adapted in freshwater systems from the ancestral marine population during freshwater colonisation (Colosimo et al., 2004). It is hypothesised that freshwater systems select for reduced armour plating in stickleback for a number of reasons. Firstly, a reduction in armour plating facilitates increased body flexibility and therefore manoeuvrability (Taylor and McPhail, 1986), which

means that freshwater fish will have a predator avoidance advantage to completely plated phenotypes. Secondly, there is evidence for the lack of calcium ions in some freshwater systems causing a reduced armour plate count (Giles, 1983), although this explanation is aimed at plate deficient stickleback found in acidic lakes where calcium ions are absent, and as such is not applicable to Hosta stream. Thirdly, armour plating and spines were originally evolved in ancestral marine populations as defences; lateral armour plates against the puncturing of toothed predators and spines against swallowing mechanisms, leading to increased escape chances (Reimchen, 2000). In freshwater systems the reduction of predation pressures may have caused the reduction in armour plates, allowing resources and energy to be allocated towards faster maturation and reproduction (Schluter et al., 2010). Nevertheless, the pattern of selection against the Eda^{C} allele across Hosta hybrid zone is prominent and will cause divergence between the Hosta freshwater population and the anadromous population.

7.2.2 Eda genotype and plate phenotype mismatch

As Eda genotyping revealed no heterozygote deficit within Hosta hybrid zone, the mismatch between the armour plate phenotype results and Eda genotype results supports the possibility that other genes responsible for armour plating are experiencing the selection pressures shown. The majority of research supports the idea that Eda genotype accurately predicts lateral plate phenotype (Colosimo et al., 2004, Leinonen et al., 2012, Archambeault et al., 2020), however there is evidence of mismatching in some wild populations (Ravinet et al., 2015, Lucek et al., 2012), for which the Eda receptor gene (Laurentino et al., 2022) or Eda modifier genes (Colosimo et al., 2004) may be responsible. In this

study, the completely plated phenotype exhibited a higher proportion of phenotype-genotype mismatching than both low plated and partially plated phenotypes combined. This means that Eda CL genotypes expressed completely plated phenotypes more often than other forms of mismatching.

A finding by Lucek et al. (2012) showed unexpected diversions from the plate phenotype expected by Eda genotyping in a freshwater stickleback population, which they concluded to be a result of divergent selection on the Eda modifier genes or a restriction to the expression and formation of lateral plates due to environmental constraints (Lucek et al., 2012). Furthermore, a study by Rennison et al. (2015) showed that selection pressures on Eda genotype and armour plate phenotype differ in freshwater systems, and specifically that the reduced armour plating in freshwater systems is a result of selection on plate count as well as selection on the Eda gene and the unmeasured traits affected by Eda (Rennison et al., 2015), which may also explain the discrepancy between plate phenotype and Eda genotype in this study. Lastly, a study on a western Irish stickleback hybrid zone found a similar genotype-phenotype mismatch, and attributed this to plate morphology being greatly affected by a gene other than Eda (Ravinet et al., 2015), as well as hypothesising that modifier genes may also be responsible. The conclusions of this study cannot be directly applied to Hosta hybrid zone as the Irish Eda CL heterozygous fish averaged 10.5 lateral plates, vastly different from other Eda-determined systems with 29+ plates (Berner et al., 2014), nonetheless, the evidence given in this study does suggest similarities. Overall, this study and previous research suggests modifier genes or other

variables play a greater role in determining plate count in three-spined stickleback than previously thought and that Eda is not the sole determinant of plate morphology.

7.2.3 Mitochondrial lineage

Hosta stream and loch are inhabited by stickleback of two distinct mitochondrial lineages, the trans-Atlantic lineage and the European lineage. It is thought that after the end of the last glacial period 15,000 years ago, the European lineage migrated north from an Iberian refugium, while the trans-Atlantic lineage migrated north and east from a refugium on the east coast of America, meeting and mixing around the British Isles and Iceland (M. Barnes and A.D.C MacColl, unpublished data).

Previous research has suggested that the European lineage has a greater affinity for freshwater adaptation than the trans-Atlantic lineage (Dean et al., 2019). The pattern we found was therefore unusual. At and Eu lineages occurred at approximately equal frequencies in anadromous (Grogarry) fish, as expected (Dean et al., 2019), but the frequency of the At lineage was also high in Loch Hosta, relative to other Uist freshwater lochs (M. Barnes, unpublished data). This may be the chance result of colonisation by the two lineages or a consequence of the environmental conditions in Loch Hosta, which are better for fish than most other Uist freshwater lochs. Given the haplotype frequencies in the parental populations at either end of the Hosta stream, it therefore seems remarkable that the At lineage was missing from a large proportion of the stream. Given our sample sizes, this is unlikely to be chance, but might result from some adaptive ad-

vantage of the Eu haplotype in the stream e.g. because of nutritional or other environmental conditions, for example, the water oxygen concentration, pH (from 9.32 within the stream to 8.34 within the loch), temperatures (from 13.4C within the stream to 11.3C within the loch), salinity (from 0.47 ppt within the stream to 0.18 ppt within the loch) or nutrient availability are all expected to vary between a loch and a stream due to the geographical differences, which will affect the three-spined stickleback drastically. Previous studies support these results very closely, despite the predictions which may be drawn based on European lineages adaptational bias towards freshwater (Mäkinen and Merilä, 2008). This pattern is unlikely to be the result of morphological differences, as previous studies show no difference in shape or size between At and Eu lineages (Ravinet et al., 2013), but may arise from behavioural differences between the lineages, as it is possible that one lineage prefers to nest in slow moving loch water than in turbulent stream waters, however previous research would suggest that this is also not a plausible explanation (Begum M., 2021. unpublished). It is possible that this pattern is an indirect result of linkage disequilibrium with another gene which is under selection due to the variation in environmental conditions, but this would require further investigation.

7.2.4 Chromosomal Inversions I and XI

The chromosome I inversion is known to be divergent between anadromous and freshwater three-spined stickleback (Jones et al., 2012). However, the adaptive significance of the inversion is under-researched and little is known of the genes within and what they contribute to freshwater adaptation apart from ATP1a1, a gene coding for a sodium/potassium transport channel. ATP1a1 is key for fish osmoregulatory function (Kaplan, 2002) and is expected to be affected by salinity (Hohenlohe et al., 2010, Magalhaes et al., 2021), thus we expect a negative trend in the frequency of marine alleles within the inversion with distance from the sea. Interestingly, within Hosta stream and loch the opposite is observed; the frequency of marine alleles within the chromosome I inversion was positively correlated with distance from the sea. This result is unexpected, as it suggests that the marine alleles within the chromosome I inversion are best suited to the top of the stream and the loch, furthest from the sea. This suggests that the ATP1a1 gene is not as affected by salinity as expected, that there are other genes within the inversion that are more important for freshwater adaptation and are taking priority in being selected for. Further investigation into the contents of the chromosome I inversion will be able to reveal more about the result shown here, but this is beyond the scope of this thesis.

On the other hand, the chromosome XI inversion showed no significant trends across Hosta hybrid zone. However, by investigating the genes contained within the inversion we may predict the environmental variables that would produce a trend. The chromosome XI inversion contains the KCNH4 gene, which codes for a voltage-gated potassium channel. Being located within the inversion suggests

the possibility of two isoforms specific to marine and freshwater chromosome XI inversion orientations (Jones et al., 2012). The function of the KCNH4 gene and its marine-freshwater isoforms is not known. But we may predict that voltage-gated channels will be affected heavily by changes in water pH. Here we find a strong association between the chromosome XI inversion and the water pH across 17 lochs. This aligns with similar findings by Haenel et al. (2019), which showed that the chromosome XI inversion differed between acid and alkaline lochs (Haenel et al., 2019). As water acidity is an important environmental condition that often changes drastically between marine and freshwater environments, we can hypothesise that the chromosomal inversion is an adaptation to accommodate this change in pH which developed during the freshwater colonisation by ancestral marine populations. The lack of any pattern in the Hosta hybrid zone is consistent with the fact that the pH in the stream remains high all the way to the loch: higher than in most freshwater populations on Uist. This result is the first step in determining the function of the chromosome XI inversion in freshwater adaptation but more research is required if we are to fully understand the extent of divergence between marine and freshwater three-spined stickleback.

7.3 Conclusion

Standard length of fish, dorsal spine length, lateral plate length, pelvic plate length, and fish shape showed no significant patterns across the hybrid zone. Lateral armour plate count showed some structure, but this could mainly be explained by the lack of high plate counts within Hosta loch. Population composition in terms of lateral plate armour and the ratios of the armour plate morphs was quite consistent with previous studies and the lack of partially plated fish has been documented previously (Schluter et al., 2010). These results suggest no barriers to gene flow and that there has been sufficient time since the last anadromous immigration for the population to be diverse and well mixed, with little pattern in morphological variation across distance from the sea.

The pattern of mitochondrial variation aligns with a previous study in this system (Begum M., 2021, unpublished), with an increase in trans-Atlantic lineage fish with distance from the sea. Similarly, the decrease in Eda^{C} allele frequency with distance from the sea aligns with previous studies on armour plate reduction in three-spined stickleback (Schluter et al., 2010). The increase in chromosome XI inversion 'marine' alleles with water pH is also supported by a previous study (Haenel et al., 2019), and explained by the KCNH4 gene which is divergent between freshwater and marine populations (Jones et al., 2012) and predicted to be affected by changes in pH (Taugbøl et al., 2014). However, the chromosome I inversion 'marine' alleles showing a positive pattern with distance from the sea was unexpected, and the explanation for this result is unclear. Genetic variation is highly structured with distance from the sea, suggesting selection is

acting on genes within Hosta hybrid zone and loch. This selection promotes divergence between freshwater and anadromous populations.

In summary, morphology was highly variable within Hosta stream, however was of little structure across the distance of the hybrid zone. Which, independent of genetic analysis, would suggest that the three-spined stickleback within were of one, rather homogeneous population. Contrastingly, genetic variation was strongly structured, with Eda allele frequency, mitochondrial haplotype, and chromosome I inversion 'marine' allele frequency all strongly associated with distance from the sea. This suggests that selection is primarily acting on the genetic differences within the hybrid zone, indicating that there is more selection within Hosta hybrid zone than immediately visible, but also that there is divergent selection between the freshwater and marine populations occurring. It is also possible that the selection within the hybrid zone is acting on underlying physiological traits rather than the 'classical' morphological traits targeted in this study, which would not be detected.

7.3.1 Further research

Further research into the morphological variation in three-spined stickleback would benefit from an investigation into the modifier genes that influence armour plating and the extent to which they do so. If modifier genes or otherwise do indeed have the ability to alter the plating of a stickleback sufficiently from the prediction of Eda so that it may be misidentified using the classic method (Bell, 1981), the full determinants of armour plate phenotype should be uncovered. The mismatch between plate phenotype and Eda genotype suggested in

this thesis and others (Lucek et al., 2012, Ravinet et al., 2015) hint at this, but these results alone cannot conclude with certainty that this is the case.

Chromosomal inversions have recently been suggested to be key to divergence in some cases (Dean et al., 2019, Lowry and Willis, 2010), and as such they are important to the understanding of evolutionary ecology. In this thesis, the association between the chromosome XI inversion and water pH is clear, however the explanation for the association between the frequency of chromosome I inversion 'marine' alleles and distance from the sea remains obscure. Further research into the genes within the chromosome I inversion in three-spined stickleback and the observation of allele frequency over other environmental gradients will allow a disentangling of the factors affecting it and lead to a greater understanding of the impact of the chromosome I inversion on freshwater colonisation and the freshwater-anadromous divergence.

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