The phenotypic and behavioral correlates of mitochondrial variation in three-spined stickleback (*Gasterosteus aculeatus*)

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Abstract

Life-history theory provides a unique framework for understanding the characteristics of an organism that makes them successful in life. Life-history traits such as growth and reproduction are influenced by mitochondrial DNA (mtDNA), making this organelle an interesting study topic within life-history theory. While the effects of mtDNA can be isolated and identified in a laboratory setting through conplastic experiments, identifying what mtDNA influences in the wild is much harder due to the rarity of a naturally-occurring well-mixed nuclear genomic background. This study proposes the three-spined stickleback on North Uist, Scotland to be an ideal wild organism for mitochondrial studies, as their populations exhibit a well-mixed nuclear genome, but two separate mitochondrial haplotypes (trans-Atlantic and European). One-hundred and eighty migratory three-spined stickleback were sampled from six saltwater lochs on North Uist to determine the extent to which mtDNA impacts phenotypic traits like organ size, body size, and ovule volume/clutch size, as well as metabolic traits like citrate synthase enzyme activity. Interestingly, results showed that stickleback haplotype influenced citrate synthase enzyme activity and male gonadosomatic index (GSI), as the European haplotype exhibited both more enzyme activity and a higher male GSI. While potentially a result of one or more factors, differences in citrate synthase levels could indicate the Eu haplotype as being better at biosynthesis or potentially having more mitochondria. Differences in male GSI may be explained by the timing of male spermatogenesis or potentially the need for larger testes in Eu fish. While no other phenotypic trait showed differences between mitochondrial haplotypes, there were many differences that emerged between sexes and between populations such as body size and organ/muscle size. This study provides a holistic look at the relationship between mitochondria, sex, and population in North Uist's migratory three-spined stickleback populations and serves as a good starting point for further research in this topic.

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1 Introduction

1.1 The importance of life-history theory

Studying the life-history traits that define an organism is key to understanding how it evolves, survives, and thrives in a changing environment. Life-history theory can provide a deep look into what makes an organism unique and may provide insight into the wide phenotypic diversity seen in the animal kingdom. Life-history traits like longevity and reproduction have long been known to drive evolution and contribute to the success or failure of a species' ability to survive (Veit et al. 2025). While life-history theory is well understood, the molecular genetic basis of life-history variation has not been studied in depth. Until we understand the specifics of an organism's life-history, we cannot begin to understand what drives its evolution.

1.2 Mitochondria influence life-history variation

Equally important as learning about the life-history variation seen between species is the study of the mechanisms that underlie such variation. Emerging research has begun to understand mitochondria as a major contributor to the life-history and physiological variation seen between species (Kurbalija Novicic et al. 2015, Dowling & Wolff 2023). The mitochondria are the main locations of energy production within an organism and therefore will dictate the ways in which that energy is used. The mitochondria are made up of an inner and outer membrane, which allows for the generation of energy as adenosine triphosphate molecules (ATP). Ninety per cent of energy production within a cell occurs in the mitochondria, with the electron transport chain generating 26-28 molecules of ATP per cycle. The ATP generated in the mitochondria is used for life sustaining organismal processes like macromolecule synthesis, muscle contractions, and thermogenesis (Bonora et al. 2012). These processes all require an input of energy, a demand which is filled by mitochondria and the mitochondrial genome which dictates how that energy is generated. The mitochondrial genome was discovered in the 1960s (Nass & Nass 1963), and contains 22 tRNAs, two rRNAs, and 13 genes that code for essential proteins used in the generation of ATP (Wolstenholme & Clary 1985). Interestingly, mitochondria and their genomes are usually inherited maternally (Sato & Sato 2013), meaning that most organisms regardless of sex contain a mostly identical mitochondrial genome to their mothers. While there are some exceptions to this maternal inheritance (leakage, heteroplasmy,

and recombination) this study will work under the assumption that these exceptions are not at work here. In the past, a maternally inherited genome was seen as selectively neutral (Dowling and Wolff 2023), meaning that it does not influence the evolution of a species. This is not the case, as a wide body of research over the past several decades has found that selection does act on the mitochondrial genome and this genome can accumulate mutations (Wolff et al. 2016), contributing to the life-history and physiological variation seen across species. This selection pressure across species can also be seen within species and populations, with the variation in mitochondrial genomes referred to as mtDNA haplotypes (Brand et al. 2024). The ways in which mtDNA haplotypes can manifest, in combination with the mitochondria's ability to generate ATP influences key life-history traits that define an organism, such as reproduction, growth, and metabolism (Metcalfe et al. 2023, Ballard et al. 2007). This study aims to take a closer look at such variation.

While emerging ideas in this field are promising, there is a lack of research on how mtDNA haplotypes affect life-history and physiological traits across species in the wild. This is mainly because it is usually impossible to control for factors like the nuclear genome, which needs to be virtually identical across all study organisms to draw conclusions about the role of the mitochondria (Barnes et al. 2025). While not in the wild, some studies have attempted to control for the nuclear genome in a laboratory setting to study the effects of mitochondrial variation on life-history and physiology (Latorre-Pellicer et al. 2016, Dowling & Wolff 2023, Rand et al. 2006). These studies introduce a concept called 'conplasticity', where two populations have identical nuclear genomes, but dissimilar mitochondrial genomes. Conplastic studies are an effective way to look at traits that are directly affected by the mitochondrial genome but are unique in that they can control for effects by the nuclear genome.

1.2.1 Laboratory studies for understanding mitochondrial variation

Latorre-Pellicer et al. (2016), examined the consequences of mitochondrial variation for life-history and physiological variations through the lens of a conplastic study in mice. Through selective breeding, they created a conplastic mouse strain using the nuclear genome of the C57BL/6 mice and the mitochondrial genome of the NZB/OlaHsd mice. This conplastic mouse strain was developed over ten generations to ensure true conplasticity and was tested against a natural (non-conplastic) mouse strain with both the nuclear and mitochondrial genomes from the

C57BL/6 strain. Results of this study found that mtDNA does have a significant effect on life-history with the conplastic mouse strain showing a median life-expectancy increase by 16% with telomere length slightly longer at two years. The natural strain showed opposite effects with more signs of ageing at two years, and a higher rate of tumor incidents. These results clearly show that differences in mtDNA have a significant effect on ageing and longevity, a crucial life-history trait needed to understand the general success of populations over time. mtDNA does not just affect longevity either, as Lattore-Pellicer et al. 2016 also found significant differences in metabolism between the two strains. Their conplastic mouse strain showed a higher expression of lipid metabolism, yet a lower carbohydrate metabolism, while the non-conplastic strain exhibited greater weight gain and accelerated deterioration of muscle replaced by fat.

This variation in life-history expression due to mtDNA differences can also be seen in insects, namely *Drosophila sp*. *Drosophila sp.* are model organisms, as they have been used in many laboratory experiments over time (Dowling & Wolff 2023). This makes the various species great for genetic research, such as the conplastic study on mtDNA haplotypes performed by Camus et al. (2017). To study how mtDNA influences thermal tolerance in *Drosophila melanogaster*, Camus et al. (2017) created conplastic strains using a common nuclear background, and two different mitochondrial genomes. A mass bred fly line was created by introgressing mtDNA variants into one standard nuclear background from Puerto Montte (PUE), Chile. Certain individuals from this new nuclear isogenic population were then selected to create their own isofemale lines, given they harbored either the A1 or B1 mtDNA haplotype being studied. Seven generations were then bred, leaving the researchers with the A1 and B1 mtDNA haplotypes, both against the PUE nuclear background. Using these conplastic strains to measure thermal sensitivity, Camus et al. (2017) found that the A1 haplotype exhibited higher heat tolerance compared to the B1 haplotype. The opposite can be said for the cold tolerance study, where the B1 haplotype exhibited higher cold tolerance, opposed to the A1 haplotype. This makes sense, given that the A1 haplotypes are more commonly found in Northern sub-tropical environments that are likely to experience warmer temperatures. B1 haplotypes are more commonly found in southern temperate areas with lower temperature (Camus et al. 2017). These results show the distinct effect that mtDNA can have on *D. melanogaster*, with certain mitochondrial variation linking directly to observable changes in fitness.

While not conplastic, Ballard et al. (2007) also demonstrated how mtDNA can influence life-history traits using wild *Drosophila* populations. The authors of this study used *D. simulans*, and two of their naturally occurring mitochondrial haplotypes, "siII" and "siIII" to determine if mtDNA causes any measurable differences in egg size, fecundity, cold tolerance, and starvation resistance in *D. simulans*. The haplotypes in question vary in their mitochondrial genome, yet the flies harboring each haplotype interbreed freely, indicating a nuclear genome that is blended and unlikely to confound results. In addition to simply observing differences in the above-mentioned traits, authors also predicted that tradeoffs between fecundity/egg size and between cold tolerance/starvation resistance may be seen. This is due to how energy must be partitioned by the mitochondria to ensure survival. The observation of trade-offs provides more evidence that mitochondria have a significant influence on life-history traits. Results of this study showed exactly this, with observable differences in both traits and tradeoffs between the two haplotypes. Differences in traits included egg size, with the siII haplotype showing larger eggs than siIII. siII also exhibited significantly higher fecundity on day three, with fecundity staying consistent throughout lifetime. The fecundity of siII declined significantly with age. Egg size and fecundity were not the only traits that differed between haplotypes, as cold-tolerance and starvation resistance differed completely between the two. siII exhibited better cold tolerance, with 75% of males and 60% of females living more than 48 hours in cold conditions, as opposed to only 62% of siIII males and 46% of siIII females. The opposite effect was shown in relation to starvation resistance with siIII flies being more resistant to starvation than siII flies. Putting these data together, I can see a clear trade-off between flies that have larger eggs, higher fecundity, and better cold tolerance, but cannot survive long periods of starvation (the siII haplotype). The opposite end of this trade off shows the siIII haplotype as being excellent in surviving periods of starvation, but as a result cannot allocate energy to larger eggs, higher fecundity, and cold tolerance. Ballard et al. (2007) clearly demonstrated how mitochondria can influence life-history traits, and that differences in mtDNA do contribute to life-history variation.

These studies on mice and *Drosophila sp.* are invaluable to understanding what the mitochondrial genome can affect within an organism: life-history and physiological traits related to ageing, metabolism, and more. These studies also provide what many wild populations struggle to: an identical nuclear genome acting as a control variable. This aspect of conplastic studies is crucial to be able to make claims about mitochondrial influence, because it can mostly

control for any effects due to the nuclear genome. These laboratory studies are limited, however, as they can only make claims about how the mitochondrial genome affect lab-grown populations. Ecologists have long known that studies in the field can often produce different results from laboratory tests done on the same population in the wild (Turko et al. 2023). Because of this, how can I begin to make claims about mitochondrial effects on the traits of a wild population?

1.2.2 Wild studies for understanding mitochondrial variation

While usually unable to control for the nuclear genome, studies on mitochondrial influence in wild populations have been attempted. Scott & Milsom (2011) studied bar-headed geese (*Anser indicus*), famous for their ability to fly at high altitudes (up to 9,000m) for sustained periods of time. This species is of interest in mitochondrial studies because of their unique metabolic processes that allow them to withstand oxygen deficient altitudes while performing oxygen demanding activity (migration). These metabolic processes all center around the mitochondria, so the authors decided to take a closer look at cytochrome C oxidase (COX) in these populations. Cytochrome c oxidase is an essential component in oxidative phosphorylation (using oxygen to generate ATP), and is likely encoded by three mitochondrial genes, among other nuclear genes. To understand how bar-headed geese mtDNA encode for increased fitness in hypoxic environments, Scott & Milsom (2011) compared this species to pink-footed geese (*Anser brachyrhynchus*) and barnacle geese (*Branta leucopsis)*, two species that do not migrate oxygen deficient altitudes. Their results showed that bar-headed geese had significantly different COX function compared to the low-altitude geese species, with a decreased COX capacity measured in both their cardiac myocytes and entire ventricle mass. This adaptation allows bar-headed geese to better manage the production of harmful reactive oxygen species (ROS) that could cause cellular damage. This is relevant, as Scott & Milsom (2011) found a nonsynonymous mutation in COX3 that likely changed the way in which COX subunits 1 and 3 interact, allowing for the decrease in COX function. These findings show that the mitochondrial genome has a clear effect on metabolism, and that a small change within the mitochondrial genome can lead to greater adaptations. While the results presented by Scott & Milsom (2011) are very useful in understanding the ways in which mitochondrial variation can manifest, it is difficult to assess the pure effects of the mitochondrial genome in this example. Mitochondrial variation may manifest

in a phenotypic way, but what remains unclear from wild studies is how much physiological and life-history variation comes purely from mtDNA, as opposed to mitonuclear interactions.

Studying mitochondrial influence between species in the wild has its uses, but comparing mitochondrial function within the members of one species could prove even more beneficial, as the nuclear genome could potentially become less of a confounding variable. Atlantic killifish (*Fundulus heteroclitus*) might provide such a model, with this species having two distinct haplotypes (mitochondrial genomes differing by five amino acid replacements) found in two climatically different areas along the east coast of North America. The haplotypes in question live either North or South of New Jersey, USA with the northern haplotypes experiencing waters 12° C colder than southern fish (Whitehead 2009, Baris et al. 2016). While all one species, the two haplogroups in question differ dramatically in their ability to survive in varying temperature differences and have differing acute temperature responses. To understand the extent to which mtDNA is driving the variation in geographical adaptations and temperature responses, Baris et al. (2016) looked at oxidative phosphorylation function between the northern and southern haplotypes in an admixed population (to control for nuclear effects). The authors found that acute temperature effects were highly influenced by haplotypes. Specifically, it was found that the southern haplotype exhibited acute temperature effects at both 12° C and 28° C, but the northern haplotype showed no temperature effect at 12° C, and a small temperature effect at 28° C. These results show each haplotype as being well adapted to the thermal region they occupy, specifically with southern fish struggling with more extreme temperatures as opposed to the northern fish. These results show that mitochondrial variation can manifest even within one species, and said variation can influence the success for a population in a certain temperature. While only varying by five amino acid replacements in their mitochondrial genome, it is worth noting that the Atlantic killifish haplotypes also differ in nuclear genes that encode for enzymes and enzyme expression, thus influencing metabolism (Baris et al. 2016). While this is a near ideal model for studying mtDNA influence as the authors strategically chose to sample an admixed population, this study species does still come with likely mitonuclear interactions that effect the expression of temperature responses. In order to draw conclusions about the role of the mitochondrial genome in a wild setting, the nuclear genome must be controlled as much as possible.

1.3 The three-spined stickleback as a model

1.3.1 Origination of three-spined stickleback mitochondrial lineages

This study proposes the three-spined stickleback (*Gasterosteus aculeatus*) as an ideal model for studying the effects of mitochondrial variation on life-history and physiology of an organism in the wild. This is because, in the eastern Atlantic, two divergent mitochondrial lineages segregate against what appears to be a well-mixed nuclear genomic background. This means that these populations have identical or nearly identical nuclear backgrounds while retaining highly diverged mitochondrial genomes (Barnes et al. 2025). The recent glacial period during the Pleistocene saw fish globally needing to find refuge in habitable environments to survive cold periods. During warmer interglacial periods, fish that were limited to southern regions could begin to recolonize northern regions. The three-spined stickleback are among the marine fish that were able to do this following the glacial recession and are unique in that they can colonize both marine and freshwater environments (Mäkinen & Merilä 2007). Certain stickleback populations can successfully colonize freshwater environments partly due to their ability to synthesize docosahexaenoic acid (DHA) (Ishikawa et al. 2019), a crucial nutrient for fish in marine environments that is often lacking in freshwater. Being able to synthesize this crucial nutrient allowed European stickleback populations to thrive and could be a big reason why there is a wide phenotypic diversity within stickleback in Europe. These populations on the east of the Atlantic are made up of various mitochondrial lineages that can be traced back to different populations before or during the Pleistocene. mtDNA analysis identified three separate mitochondrial lineages that diverged from each other in the late Pleistocene. These are European, trans-Atlantic, and Black Sea mitochondrial lineages (Mäkinen & Merilä 2007).

1.3.2 Three-spined stickleback on North Uist, Scotland, and their significance

The three-spined stickleback populations that live in the lochs of North Uist, Scotland show such mitochondrial diversity, with their populations exhibiting the European (Eu) and trans-Atlantic (At) mitochondrial haplotypes. Some stickleback found on N. Uist carry the Eu haplotype which likely originated in freshwater refugia across the western coast of Europe, and with the glacial recession, travelled to locations like N. Uist. Similarly, the At haplotype likely originated in refugia on the east coast of North America, and when the glaciers receded, crossed

the Atlantic Ocean to end up in locations like N. Uist (Dean et al. 2019). In addition to the two haplotypes, N. Uist is also home to two stickleback "ecotypes", a word used to describe stickleback populations that either migrate to and from the island annually (called migratory) or populations of stickleback that do not (called resident). This makes N. Uist interesting for mitochondrial studies, as both the Eu and At haplotypes are represented in both the migratory and resident ecotypes. Eu and At haplotypes on N. Uist are also ideal for mitochondrial studies because they exhibit almost identical nuclear backgrounds (Barnes et al. 2025). As the fish with At and Eu haplotypes on N. Uist have been interbreeding for thousands of years (showing nuclear structuring within each of their respective lochs, see section 4.3.2), this system comes close to mimicking a true conplastic system. It provides a constant nuclear genomic background while retaining divergent mtDNA. Given this unique situation, it is possible to look deeper into how mitochondrial variation influences the life-history and physiology in stickleback in the wild, which can inform ecologists about the effects the mitochondria may be playing on whole organisms. In this study, I aim to understand the relationship between mtDNA haplotypes and metabolism/physiology in populations of migratory three-spined stickleback on N. Uist, Scotland. I will do so by looking at these two categories individually and running tests on various aspects of each to get a holistic understanding of this system.

1.4 Study aims

1.4.1 Metabolic study

Our metabolic studies will aim to understand if liver weight and citrate synthase activity are influenced in any way by mitochondria, similar to work done on white-crowned sparrows (*Zonotrichia leucophrys,* Rhodes et al. 2024). The authors of this study looked at citrate synthase, a way of measuring overall mitochondrial density (and therefore metabolism), and linked it to migration abilities. Given that migration requires a significant energetic output, organisms which migrate have a greater metabolic demand (Rhodes et al. 2024). This implies they may also have a higher density of mitochondria and citrate synthase activity to facilitate increased ATP synthesis. Rhodes et al. (2024) found that their migratory bird population, Gambel's subspecies, did in fact have higher levels of citrate synthase activity compared with their non-migratory population, Nutall's subspecies. I may find similar results between the At and Eu haplotypes living in the lochs of N. Uist, as the migratory histories of these haplotypes

vary significantly (Dean et al. 2019). As mentioned above, the historic At haplotype appears to have crossed the Atlantic after originating in a refugium on the east coast of North America. This haplotype is much commoner in migratory than resident populations on N. Uist (Dean et al. 2019). This is some evidence that the At haplotype may have a greater ability to synthesize ATP which allowed them to be successful in meeting the energetic demands of a trans-Atlantic migration. If this is true, I may see increased citrate synthase activity in N. Uist fish possessing the At haplotypes when compared with the Eu haplotype. The Eu haplotype did not cross a body of water as large as the Atlantic to arrive in N. Uist and therefore should not show as high a level of citrate synthase activity and mitochondrial density.

Interestingly, my results challenge this line of thought. Results showed Eu fish with significantly higher citrate synthase activity in an aquarium reared sample, and a similar (yet insignificant) trend in a wild caught sample. These results indicate that migratory history is not the main factor influencing citrate synthase activity, and that geographical variation or mitochondrial efficiency may be a better explanation. This may be due to the freshwater environments that the Eu fish originated in or perhaps Eu mitochondria that is less efficient at energy production when migrating. These hypotheses will be expanded upon in the discussion (see section 4.1).

1.4.2 Physiological study

Physiological analysis in this study will aim to understand if body size/length, clutch size, ovule volume, pectoral fin size, and muscle/organ mass significantly vary between haplotypes. While the conplastic studies described earlier provide insight into the wide range of traits affected by mitochondrial variation, research on how mtDNA influences life-history traits like clutch or body size is almost non-existent. This study will attempt to bridge this gap by drawing on knowledge obtained by Barnes et al. (2025) that looked at physiological differences between mitochondrial lineages in three-spined stickleback on N. Uist, specifically mitochondrial respiration. While not looking at mitochondrial respiration in this current study, results found by Barnes et al. (2025) still provide a good base line for understanding how mitochondrial variation will manifest phenotypically in migratory stickleback. Authors used high-resolution respirometry (HRR) to measure mitochondrial respiration in heart and brain tissues of stickleback from populations with an equal mix of At and Eu haplotypes. This ensures a well-mixed nuclear

genome. Several respiratory parameters were measured, with significant differences discovered in respiration rate when ET capacity with complex II substrates were tested. It was found that at 6° C, respiratory rates varied significantly between mitochondrial lineages in heart tissue. These results were presented as FCRs (the ratio of oxygen flux in a particular respiratory state compared with the maximum oxygen flux for that state). Barnes et al. (2025) found that Eu haplotype tissue seemed unaffected by the 6° C temperature used, while the FCRs in the At haplotype increased with temperature. Interestingly, using frozen heart tissue, authors also found that this variation is likely a result of mitochondrial complex I, which has subunits encoded by the mitochondrial genome. Results presented here provide clear evidence that the mitochondrial genome plays a real hand in the phenotypic variation seen in the three-spined stickleback. This study aims to further work done by Barnes et al. (2025) in uncovering a wide variety of physiological traits likely effected by mtDNA variation.

2 Methods

2.1 Study site and stickleback collection

2.1.1 North Uist and location of sampled lochs

All three-spined stickleback sampled in this study were collected on the island of North Uist in the Scottish Western Isles (57° 34’ 13.4” N; 7° 16’ 52.3” W). Stickleback were collected from six different lochs (Table 1). These lochs were chosen for sampling due to the prevalence of the migratory stickleback ecotype. All sampling was completed between the 21st of April 2025 and the 22nd of May 2025.

Loch	Code	Coordinates	Sample size (n)
Ardheisker	ARDH	57° 34' 47" N, 7° 24' 51" W	48 females 21 males
Clachan an Luib	LUIB	57° 33' 14" N, 7° 18' 44" W	43 females 20 males
Loch an Duin	DUIN	57° 38' 32" N, 7° 12' 30" W	9 females 6 males
Ob nan Stearnain	OBSE	57° 36' 4" N, 7° 10' 21" W	9 females 4 males
Fairy Knoll	FAIK	57° 38' 7" N, 7° 12′ 54" W	5 females 5 males
Ialadh	IALA	57° 37' 12" N, 7° 12′ 20" W	5 females 5 males

Table 1

2.1.2 Stickleback collection methods

The stickleback used for this study were collected using minnow traps (Gee traps, Dynamic Aqua, Vancouver) placed in various parts of each loch. These traps were thrown into the water, ideally near possible stickleback nesting sites along the shorelines, and were anchored to rocks or vegetation on the shore. Traps were placed in pairs, with no pairs closer than 10m apart. All traps were fully submerged. After 24 to 72 hours, captured stickleback were then placed in large buckets containing loch water for sorting by sex and ecotype. Where possible, the sex of stickleback was identified by their general size (length and depth) or any distinguishing color patterns (males being redder during the breeding season). Ecotype was determined by feeling along the edge of the stickleback to determine the number of bony plates each fish has

(migratory stickleback being fully plated, with resident stickleback being low plated) (Dean et al. 2019).

Aquarium-reared stickleback used for the citrate synthase activity assay were taken from an artificially crossed LUIB tank population. These individuals were hatched in May of 2024 after being artificially crossed from wild-caught members of Clachan an Luib in a temporary field laboratory on N. Uist from the 2024 fieldwork season. These crosses were brought back to the MacColl aquarium at the University of Nottingham and hatched/reared there.

2.1.3 Stickleback husbandry

Once sorted, wild-caught fish needed for further study were placed in insulated, waterproof containers equipped with an air stone for transportation to the temporary field laboratory. Upon arrival, containers were immediately brought inside and supplied with a permanent air pump/stone. Fish were euthanized in a separate room from housed fish and brought to our temporary laboratory for dissection. Any fish that were not used were returned to their lake of origin within 48 hours.

Aquarium conditions were set to mimic the natural light cycles and water temperatures of N. Uist and fish were fed bloodworms daily prior to euthanization. Additionally, biweekly water quality tests were performed, and water was changed as needed for the duration of stickleback rearing.

2.2 Laboratory methods

2.2.1 Euthanasia and dissection methods

All fish used in this study were euthanized with tricaine methane sulphonate (MS-222) followed by destruction of the brain to confirm death, as required by the UK Home Office, under project license PP5421721.

Dissections were carried out immediately following euthanasia. Fish were weighed to the nearest 0.0001g and standard-length measurements were taken from the tip of the snout to the end of the caudal peduncle. Both of the pectoral fins and the caudal fin were removed and stored in 80% ethanol for later DNA extraction. The left pectoral muscles, brain and liver were

dissected out of each fish and weighed. Livers were frozen at -20° C for citrate synthase assay. The sex of fish was established from inspection of the gonads.

2.2.2 Obtaining ovule size data

Ovules were extracted as a whole clutch and were counted, before 10 ovules from each clutch were measured using a microscope and graticule. Ovule length was recorded as the longest axis through the ovule and ovule width as the greatest dimension perpendicular to length. To determine ovule volume, the equation $(4/3)(\pi AB^2)$ was used where A and B are the half-axis values for length and width, respectively.

2.2.3 DNA extractions

Fin clips that had been stored in the refrigerator from fieldwork were prepared for DNA extraction by taking two fins from each sample, cutting them up into smaller pieces, and placing them in desired wells on the 96-well plates provided. DNA extractions were carried out using the DNeasy 96 Blood and Tissue Kit from QIAGEN (DNeasy Blood & Tissue Kits | DNA Purification | QIAGEN). The protocol provided with this kit was followed, with one adjustment made by using only 100µL of buffer AE in the final step instead of 200µL. This was done to create more concentrated DNA samples.

2.2.4 Mitochondrial haplotype assay using PCR and Gel Electrophoresis

When all DNA extractions were successfully performed, haplotypes were determined following an assay designed by Barnes et al. (2025) that used a polymerase chain reaction and restriction enzymes to cut DNA based on mitochondrial haplotype. Specifically, this assay used primers matching the ND4 and ND5 regions of the mitochondrial genome and used HindIII or PstI restriction enzymes to cut DNA based on lineage. In the ND4 region, HindIII cuts a segment of DNA pertaining to the Eu haplotype. In the ND5 region, PstI cuts a segment of DNA pertaining to the At lineage. Amplifying and cutting both the ND4 and ND5 regions of the mitochondrial genome ensures no user error has been made when preparing samples; both regions for each sample should provide the same information.

One PCR reaction required 10µl of Accustart, 7µL of nuclease free water, 1µL of forward primer, and 1µL of reverse primer (being careful to keep the ND4 and ND5 primers separate

from each other. 1µL of DNA was added to each ND4 PCR tube and respective ND5 PCR tube (using 2µL total of DNA per extracted fin clip sample).

All 48 PCR tubes were then loaded into the G-Storm GS1 thermocycler to run for two hours. PCR followed this cycle: initial denaturation at 94° C for three minutes. Then, denaturation at 94° C for 30 seconds, annealing at 60° C for 30 seconds, extension at 72° C for 1 minute, with this whole process repeating 36 times. A final extension then occurred at 72° C for five minutes. A 1.5% agarose gel was created to ensure successful amplification. 6µL of each PCR sample ran through the gel at 100V for 25 minutes, along with 4µL of a 100bp ladder (NEB). DNA was then visualized on an iBright CL750 Imaging System (ThermoFisher Scientific) to confirm amplification

All successfully amplified samples were then mixed with 0.8µL of restriction enzyme and 1.5µL of its associated 10X restriction enzyme buffer. For all ND4 samples, HindIII restriction enzyme and r2.1 10X restriction enzyme buffer were added. For all ND5 samples, PstI restriction enzyme and r3.1 10X restriction enzyme buffer were added. These PCR samples were then put back into the G-Storm GS1 thermocycler for incubation at 37° C for one hour. After incubation, 6µL of each PCR sample was run on a 2% agarose gel at 100V for 25 minutes along with 4µL of a 100bp ladder (NEB). At this point, mitochondrial haplotypes could be visualized using the iBright CL750 Imaging System (ThermoFisher Scientific) (see Figure 1 below).

2.2.5 Citrate synthase assay

I used Abcam's Citrate Synthase Assay Kit (ab239712) to measure citrate synthase activity in frozen liver samples from ARDH, OBSE and LUIB. All liver samples were cut and weighed to be as close to 10mg as possible. These tissues were homogenized in 100µL of Assay Buffer 7 and placed in a centrifuge for 5 minutes at 10,000 *x g*. 10µL of supernatant was collected and pipetted into wells on a 96-well plate. Another 10µL of each sample was added to different wells for background readings. Each sample and sample background well were made up to 50µL by adding 40µL of Assay Buffer 7.

Standard dilutions and positive controls were also required for sample comparisons. GSH Standard dilutions were made up by diluting GSH Standard by adding 90µL of Assay Buffer 7 to 10µL of 20mM GSH Standard. 0, 4, 8, 12, 16, and 20µL of this newly diluted standard was then

added to 50, 46, 42, 38, 34, and 30µL of Assay buffer 7, respectively, each in a different well. This created a 0, 8, 16, 24, 32, and 40 nmol GSH Standard curve. To create the positive control, 990µL of Assay Buffer 7 was added to 10µL of CS Positive Control stock solution. 10µL of this positive control dilution was then added to one well.

2.2.6 Sample and background reaction mixtures

After all samples, their backgrounds, GSH Standard dilutions, and the positive control were added to the 96-well plate, two different reaction mixtures were made. A reaction mixture was made for the sample, standard, and positive control wells, where one reaction required 43µL of Assay Buffer 7, 5µL of DTNB, and 2µL of CS Substrate mix. 50µL of this reaction mix was then pipetted into each sample, standard, and positive control wells. Each background sample reaction included 45µL of Assay Buffer 7 and 5µl of DTNB. 50µL of this mix was pipetted into each background well.

2.2.7 Measuring absorbance in kinetic mode and citrate synthase activity equation

Immediately after adding reaction mixtures into the appropriate wells, the 96-well plate was put into the Epoch Microplate Reader (BioTek Instruments, Inc) for 30 minutes at 25° C and 412nm. Results were captured every minute for 30 minutes and were exported as an excel file by Gen5 Microplate Reader and Imager Software (version 3.10, BioTek Instruments, Inc) for data analysis.

Citrate synthase enzyme activity was determined using the equation:

$$sample\ CS\ activity = \left(\left(\frac{B}{\Delta T\ X\ V}\right) X\ D\right)\frac{nmol}{min}/uL$$

where B is the nanomoles of S-H group from the Standard Curve, △T is the reaction time in minutes, V is the sample volume added to each reaction well in µL, and D is the sample dilution factor.

2.2.8 Determining values for citrate synthase enzyme activity calculations

While V equaled 10µL and D equaled a dilution factor of 10 (10µL for 100µL per well), B and △T needed to be determined mathematically. This data analysis first required subtracting the 0 Standard reading from all readings and then plotting the GSH Standard curve, where x =

time (minutes) and y = optical density of the Standards. This plot provided a line of best fit equation (y = 0.037x) which would be used to determine the citrate synthase activity in each sample later. Next, as all sample background readings showed as significant, each sample background reading was then subtracted from its respective sample reading. After subtracting both the 0 Standard and the sample background from each sample reading, a plot of each sample was created to determine the reaction time (the period at which the optical density/time slope was linear). For all samples, 20 minutes to 30 minutes created a linear plot of the optical densities/time. This gave a $\triangle T$ value of 10 minutes, which was used for citrate synthase activity calculations.

Determining B required each sample reading (minus the 0 Standard and the sample background readings) at 20 minutes to be subtracted from its sample reading at 30 minutes. This new optical density value was then used as the Y value in the line of best fit equation given by the GSH Standard curve (y = 0.037x), where X will give us the B values for each sample. Once B for each sample was determined, all values were plugged into the sample CS activity equation to determine citrate synthase enzyme activity. Lastly, all citrate synthase values were divided by the weight of the original liver tissue used in grams.

2.2.9 Obtaining predation data

Pictures of stickleback used in the study were taken and later analyzed for predation marks. Predation marks include any visible scarring or injuries from putative encounters with predators. This includes “V” shaped marks from bird beaks and significant damage to the body visible from either gashes, holes, or blood (Reimchen 1994). Damaged fins were not considered, as traps can often cause the degradation of fins, unrelated to predation.

2.3 Statistical analysis

2.3.1 Models and tests used

All statistical analyses were conducted using R (version R 4.5.0). Linear models were created using R package “tidyverse” (version 2.0.0, Wickham, 2019). Generalized linear models were created using “stats” (version 4.5.0, R Core Team, 2025). Linear mixed effect models were created using “lme4” (version 1.1-37, Bates et al., 2025). Specific factors used in each model are described below (see results). ANOVA Type 1 sums of squares or ANOVA Type 3 sums of

squares (using Scatterthwaite's method for degrees of freedom) were run on models to determine significance where appropriate.

2.3.2 Fitting data

To run accurate statistical models, the female Gonadosomatic Index (GSI) dataset underwent a log() transformation. One individual in the male GSI dataset was removed as there were unclear results for gonad weight. For clutch size analysis, the average ovule volume for each clutch was used. Two samples were removed from the citrate synthase analysis, as the kinetics machine did not read properly, producing a negative and zero result.

3 Results

3.1 Mitochondrial haplotype proportions

The proportion of the At haplotype in each population can be found in Figure 1.

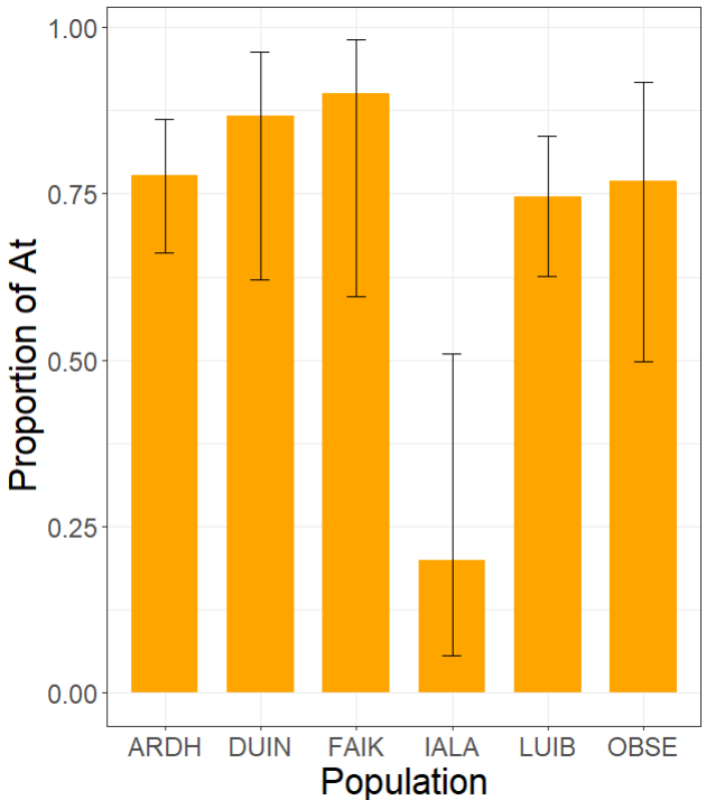

Figure 1. The proportion of the At mitochondrial haplotype present in each population (ARDH, DUIN, FAIK, IALA, LUIB, OBSE). Orange bars highlight the proportion, while black lines represent the 95% binomial confidence intervals.

3.2 Phenotypic data

The locations and numbers of stickleback sampled in this study can be found in Table 1. Data were analyzed with linear models or generalized linear models as appropriate.

3.2.1 Body size data

While there were no significant differences in body size due to mitochondrial haplotype, the stickleback in this study varied in their overall body size between sexes and populations. On average, female stickleback were larger: they were longer in length and weighed more than their

male counterparts. Population variation exists as well, with differences in standard length driven by ARDH fish being small in length, and DUIN and FAIK fish being the longest (Fig. 2, Table 2).

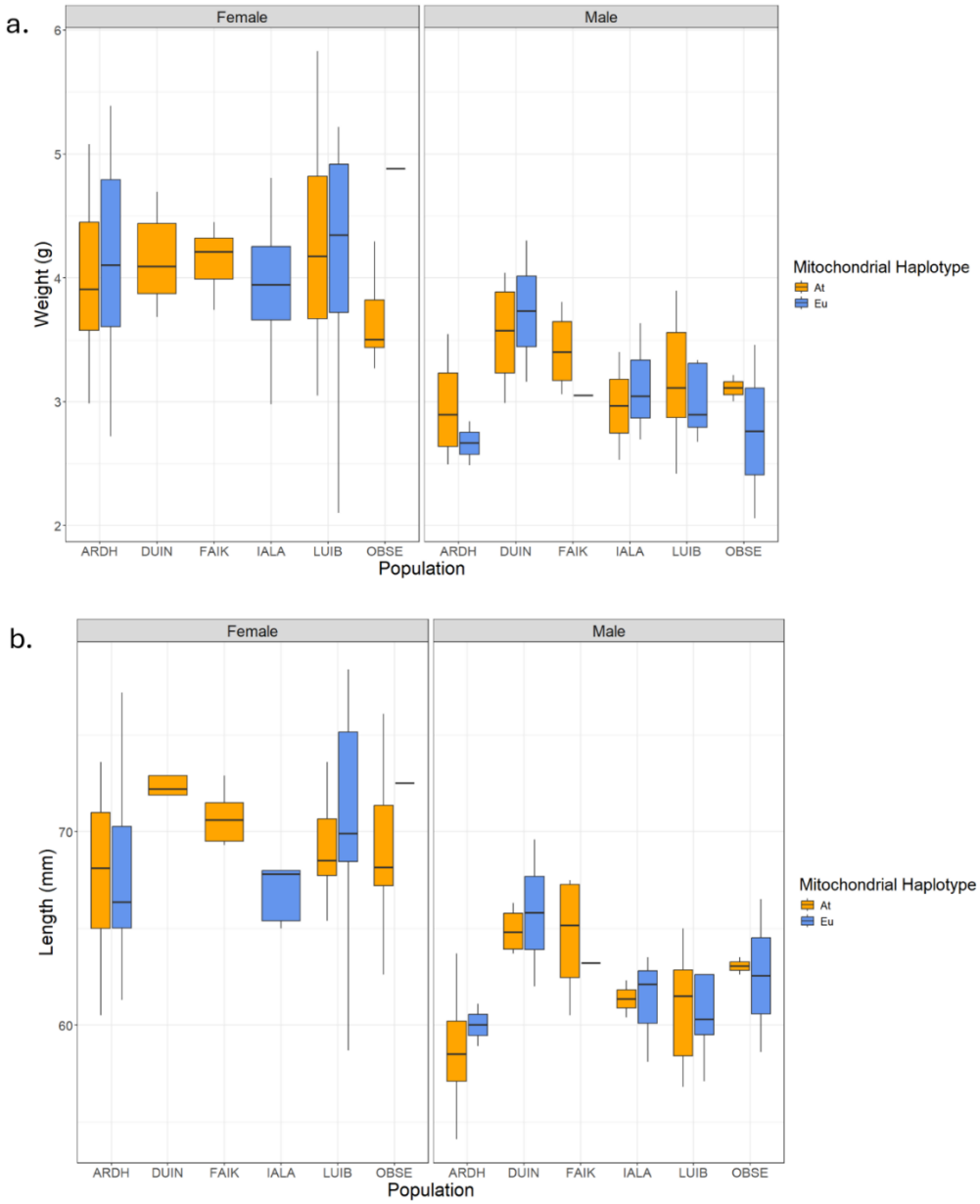

Figure 2. Variation in weight and length of migratory three-spined stickleback from North Uist, between mitochondrial haplotypes, sexes, and populations (ARDH, DUIN, FAIK, IALA, LUIB, OBSE). a) Weight in grams (female, male). b) Length in millimeters (female, male). Mitochondrial haplotypes are designated for each population as orange for At and blue for Eu. 25 and 75 quartile ranges are shown as vertical black lines.

3.2.2 Organ and tissue data

There were no significant differences between mitochondrial haplotypes in almost all the organ and tissue traits measured ($p > 0.05$, Table 2, Figure 3). The only exception to this was male gonadosomatic index (GSI). Male fish carrying the Eu haplotype had a significantly higher GSI compared to the At haplotype ($F_{1,38} = 12.07$, $p = 0.0013$, Table 2, Fig. 5). This pattern was consistent across all populations.

There were also significant differences in the traits measured both between sexes and populations. Females have heavier livers (relative to overall body size) compared to males. Male stickleback, on the other hand, make up for their smaller size by having bigger pectoral muscles and harboring more fat than females ($p < 0.05$, Table 2, Fig. 3).

Interestingly, differences among populations were also observed. HSI, liver weight per gram, fat presence, fat weight, and female GSI all varied amongst populations ($p < 0.05$, Table 2, Fig 3, Fig. 4). FAIK and LUIB are the most different from each other across all liver and fat related traits, with LUIB and IALA specifically harboring the biggest livers. In analyzing this data, I noticed that the longest fish (FAIK) did not have the biggest livers, so I decided to model raw liver weight as a function of fish length, population, and their interaction. The interaction between liver weight and fish length was not significant, implying that liver size/growth scaled similarly between populations ($F_{1, 134}=0.4417$, $p = 0.818717$).

Table 2: Model results[1] for all organ and tissue data collected. Significant p-values have been bolded for emphasis

Trait	Mitochondrial haplotype	Population	Sex[2]	Model used
Hepatosomatic Index (HSI)	P-value: 0.464 F-value: 0.5392 Mean sq: 0.0000598 Sum sq: 0.0000598 Resid. Df: 140 Df: 1	**P-value: 9.369e-5 (***)** F-value: 5.6232 Mean sq: 0.0006237 Sum sq: 0.0031183 Resid. Df: 140 Df: 5	**P-value: <2.2e-16 (***)** F-Value: 237.0654 Mean sq: 0.0262924 Sum sq: 0.0262924 Resid. Df: 140 Df: 1	Linear[3]
Liver weight per gram	P-value: 0.6074442 F-value: 0.2651 Mean sq: 0.0000245 Sum sq: 0.0000245 Resid. Df: 140 Df: 1	**P-value: 0.0001115 (***)** F-value: 5.5305 Mean sq: 0.0005120 Sum sq: 0.0025601 Resid. Df: 140 Df: 5	**P-value: <2.2e-16 (***)** F-value: 174.6949 Mean sq: 0.0161733 Sum sq: 0.0161733 Resid. Df: 140 Df: 1	Linear[3]
Gonadosomatic index (GSI), female	P-value: 0.91185 F-value: 0.012 Mean sq: 0.00203 Sum sq: 0.0020 Resid. Df: 89 Df: 1	**P-value: 0.03131(*)** F-value: 2.5847 Mean sq: 0.42508 Sum sq: 2.1254 Resid. Df: 89 Df: 5	**Date of Collection[2]** P-value: 0.36127 F-value: 0.8421 Mean sq: 0.13849 Sum sq: 0.1385 Resid. Df: 89 Df: 1	Linear[3]
Gonadosomatic index (GSI), male	**P-value: 0.001181 (**)** F-value: 12.0724 Mean sq: 1.1876e-5 Sum sq: 1.1876e-5 Resid. Df: 38 Df: 1	P-value: 0. 270948 F-value: 1.2724 Mean sq: 1.2518e-6 Sum sq: 6.2590e-6 Resid. Df: 38 Df: 5	**Date of Collection[2]** P-value: 0.245129 F-value: 1.3616 Mean sq: 1.3395e-6 Sum sq: 1.3390e-6 Resid. Df: 38 Df: 1	Linear[3]

Trait	Mitochondrial haplotype	Population	Sex[2]	Model used
Pectoral muscle weight per gram	P-value: 0.8002 F-value: 0.0643 Mean sq: 0.00000037 Sum sq: 0.00000037 Resid. Df: 140 Df: 1	P-value: 0.5833 F-value: 0.7556 Mean sq: 0.00000438 Sum sq: 0.00002190 Resid. Df: 140 Df: 5	**P-value: <2.2e-16 (***)** F-value: 120.6506 Mean sq: 0.00069941 Sum sq: 0.00069941 Resid. Df: 140 Df: 1	Linear[3]
Fat presence	P-value: 0.9878192 Deviance: 0.000 Resid. Dev: 151.58 Resid. Df: 140 Df: 1	**P-value: 2.36e-7 (***)** Deviance: 39.014 Resid. Dev: 151.58 Resid. Df: 141 Df: 5	**P-value: 0.0002725 (***)** Deviance: 13.251 Resid. Dev: 190.59 Resid Df: 146 Df: 1	Generalized Linear, family: Binomial[4]
Fat weight (g)	P-value: 0.7993 F-value: 0.0649 Resid. Df: 140 Df: 1	**P-value: 2.793e-6 (***)** F-value: 7.5196 Resid. Df: 140 Df: 5	**P-value: 5.562e-5 (***)** F-value: 17.2909 Resid. Df: 140 Df: 1	Linear[3]
Adiposomatic index (ASI)	P-value: 0.76719 F-value: 0.0880 Mean sq: 0.0275 Sum sq: 0.027 Resid. Df: 139 Df: 1	P-value: 0.09505 F-value: 1.9182 Mean sq: 0.5987 Sum sq: 2.993 Resid. Df: 139 Df: 5	**P-value: 1.671e-7 (***)** F-value: 30.3824 Mean sq: 9.482 Sum sq: 9.483 Resid. Df: 139 Df: 1	Linear[3]

Trait	Mitochondrial haplotype	Population	Sex[2]	Model used
Standard length (mm)	P-value: 0.2023 F-value: 1.6381 Mean sq: 20.24 Sum sq: 20.24 Resid. Df: 172 Df: 1	**P-value: 4.616e-6 (***)** F-value: 7.1008 Mean sq: 87.73 Sum sq: 438.66 Resid. Df: 172 Df: 5	**P-value: <2.2e-16 (***)** F-Value: 204.6032 Mean sq: 2527.89 Sum sq: 2527.89 Resid. Df: 172 Df: 1	Linear[3]
Fish weight (g)	P-value: 0.9951 F-value: 0.0000 Mean sq: 0.000 Sum sq: 0.000 Resid. Df: 142 Df: 1	P-value: 0.2231 F-value: 1.4131 Mean sq: 0.571 Sum sq: 2.857 Resid. Df: 142 Df: 5	**P-value: 1.388e-15 (***)** F-value: 80.8592 Mean sq: 32.695 Sum sq: 32.695 Resid. Df: 142 Df: 1	Linear[3]

[1] The order of factors used in each model is not reflected by the order in which they appear on the table.

[2] For GSI models, sex was not run as a factor as models were split based on sex. Date of collection was considered when modelling GSI as fish were caught on different days of the breeding season depending on the loch sampled.

[3] R package “tidyverse” version 2.0.0, Wickham, 2019

[4] R package “stats” version 4.5.0, R Core Team, 2025

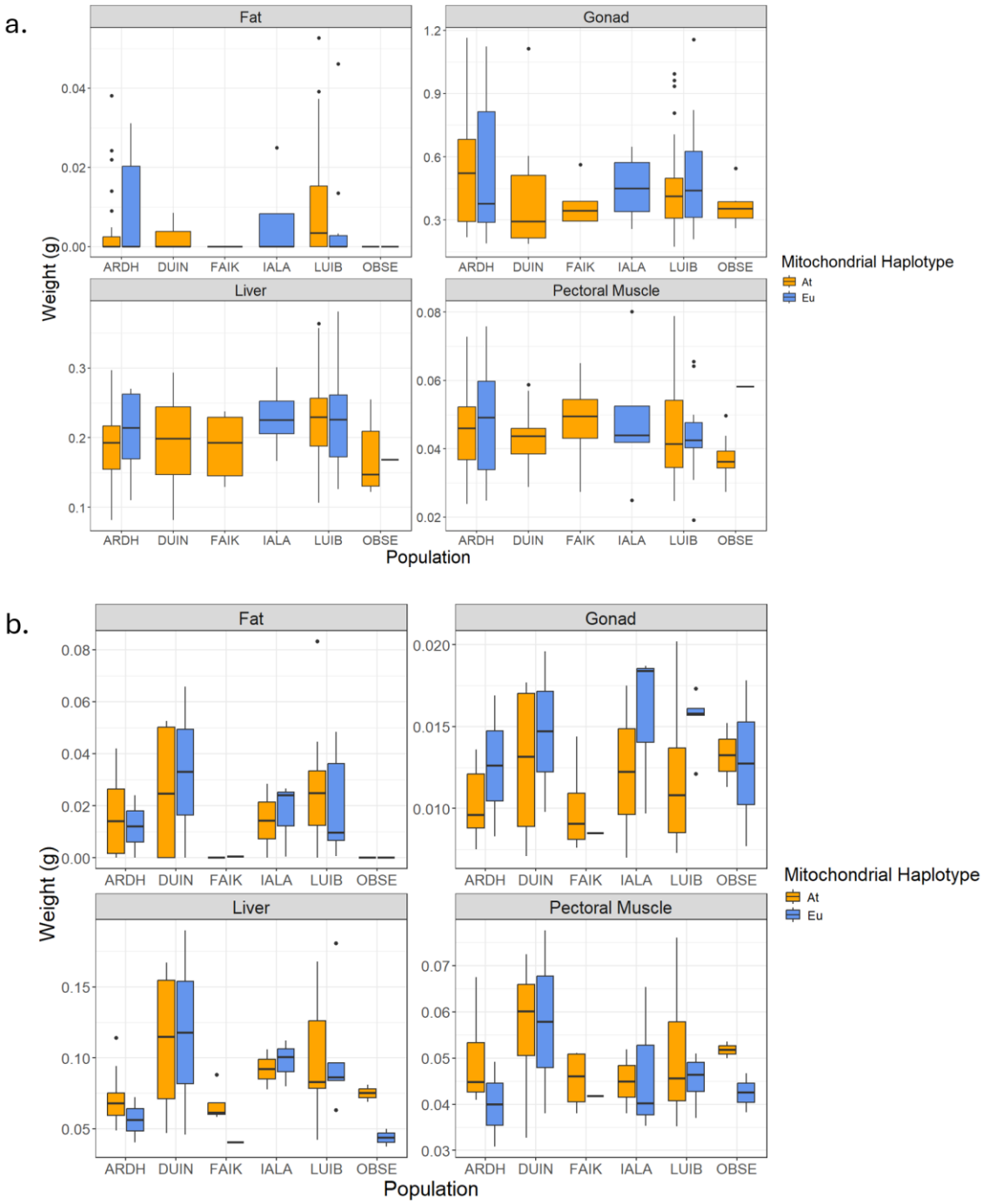

Figure 3. Variation in organ and tissue weights of migratory three-spined stickleback from North Uist, between mitochondrial haplotypes, sexes and populations (ARDH, DUIN, FAIK, IALA, LUIB, OBSE). a) Females (fat, gonad, liver, and pectoral muscle weight in grams). b) Males (fat, gonad, liver, and pectoral muscle weight in grams). Mitochondrial haplotypes are designated for

each population as orange for At and blue for Eu. 25 and 75 quartile ranges are shown as vertical black lines with points representing outliers.

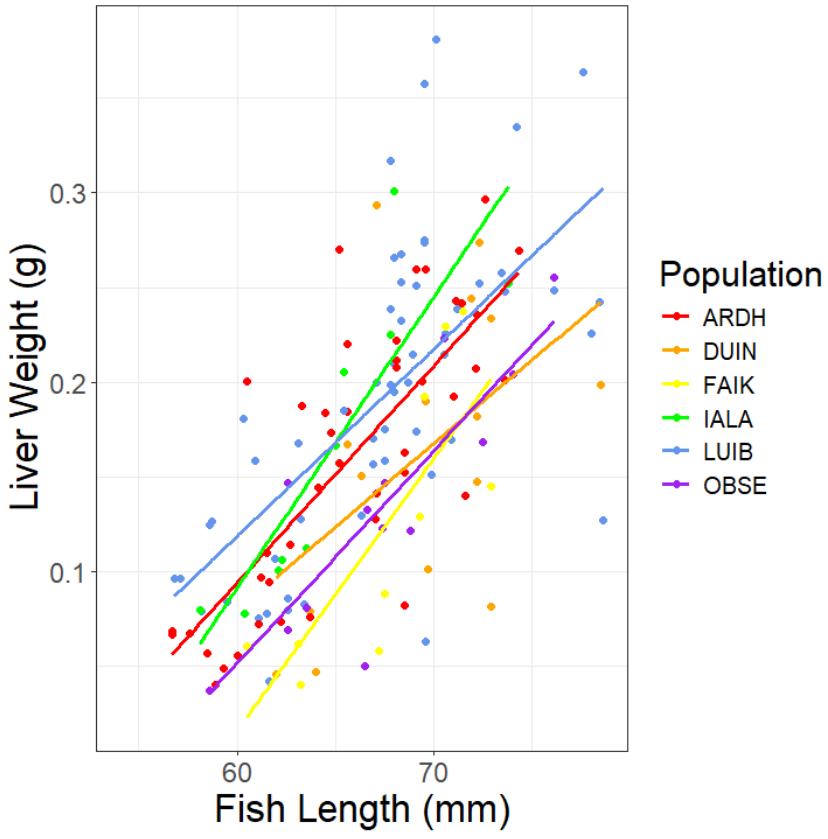

Figure 4. Differences in liver weight in grams between populations plotted against fish length in millimeters. Populations (ARDH, DUIN, FAIK, IALA, LUIB, OBSE) are designated by the various colors. The lines of best fit indicate the change in liver size with increasing fish length, with point showing the individual samples.

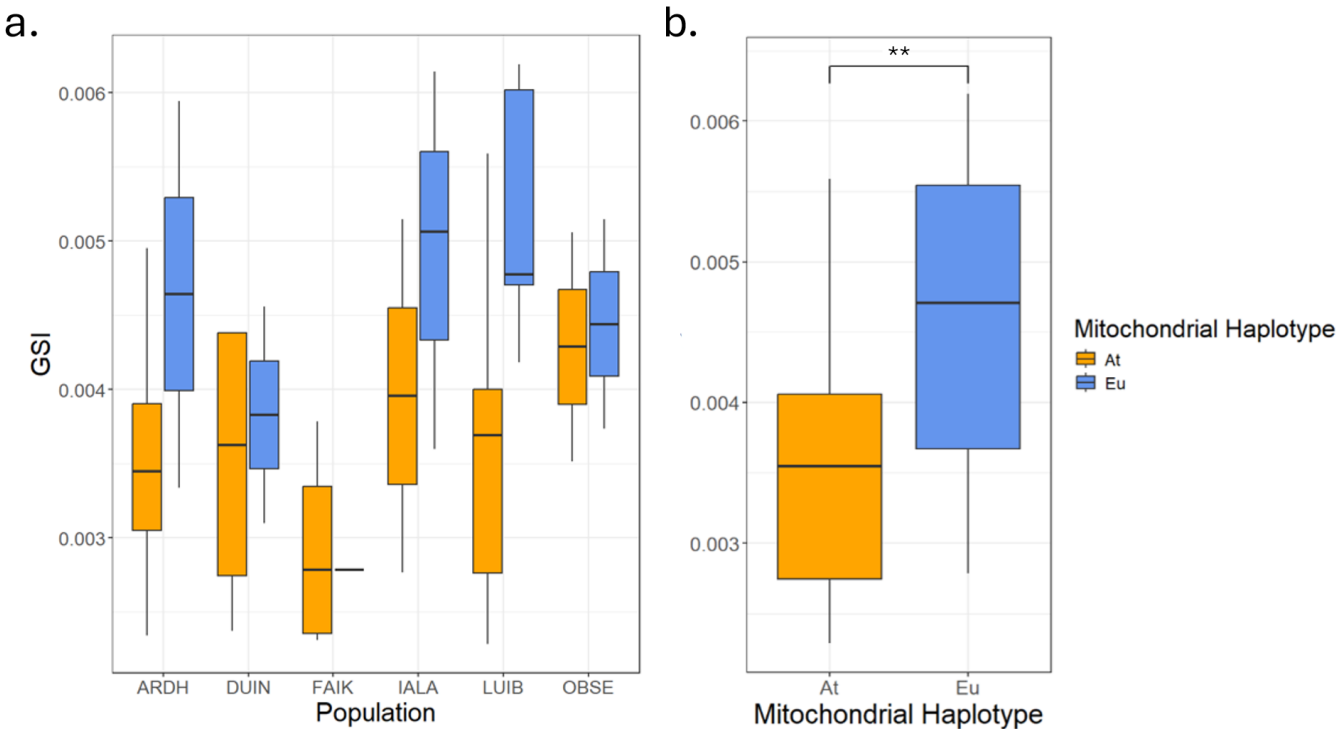

Figure 5. Variation in male GSI measurements. Eu fish had significantly higher GSI compared to At fish. This trend can be visualized across all populations (ARDH, DUIN, FAIK, IALA, LUIB, OBSE). a) GSI differences by population. b) GSI differences between mitochondrial haplotypes. Mitochondrial haplotypes are designated for each population as orange for At and blue for Eu. 25 and 75 quartile ranges are shown as vertical black lines.

3.2.3 Ovule data

Ovules were extracted as a whole clutch from 15 female stickleback (12 from ARDH, one from DUIN, one from IALA, and one from LUIB) and were counted. Clutch size increased with female length but there was no significant difference in clutch size between mitochondrial haplotypes (GLM with Type 1 sums of squares: standard length, $F_{1,12} = 6.94$, $p = 0.022$; mitochondrial haplotype, $F_{1,12} = 0.03$, $p = 0.86$). Ten ovules per stickleback were isolated and used for ovule volume measurements. There was no significant difference in ovule volume between mitochondrial haplotypes or fish length (generalized linear mixed model with normal errors, stickleback ID as the random effect, Type 3 sums of squares: fish length $F_{1,11.998} = 2.62$, $p = 0.13$; mitochondrial haplotype, $F_{1,11.998} = 1.1520$, $p = 0.3042$).

3.3 Metabolic data

3.3.1 Wild caught citrate synthase enzyme activity

Livers from 40 wild caught stickleback (21 from ARDH, 12 from OBSE and seven from LUIB) were used to analyze citrate synthase enzyme activity. Citrate synthase activity did not vary with liver weight or mitochondrial haplotypes although it is worth noting that the means for Eu fish were higher in all populations. There was higher citrate synthase activity in LUIB fish than in OBSE or ARDH (Fig. 6; GLM with Type I sums of squares: liver weight F = 0.11 p = 0.740633; population F = 6.66, p = 0.003528; mitochondrial haplotype F = 0.23, p = 0.637131).

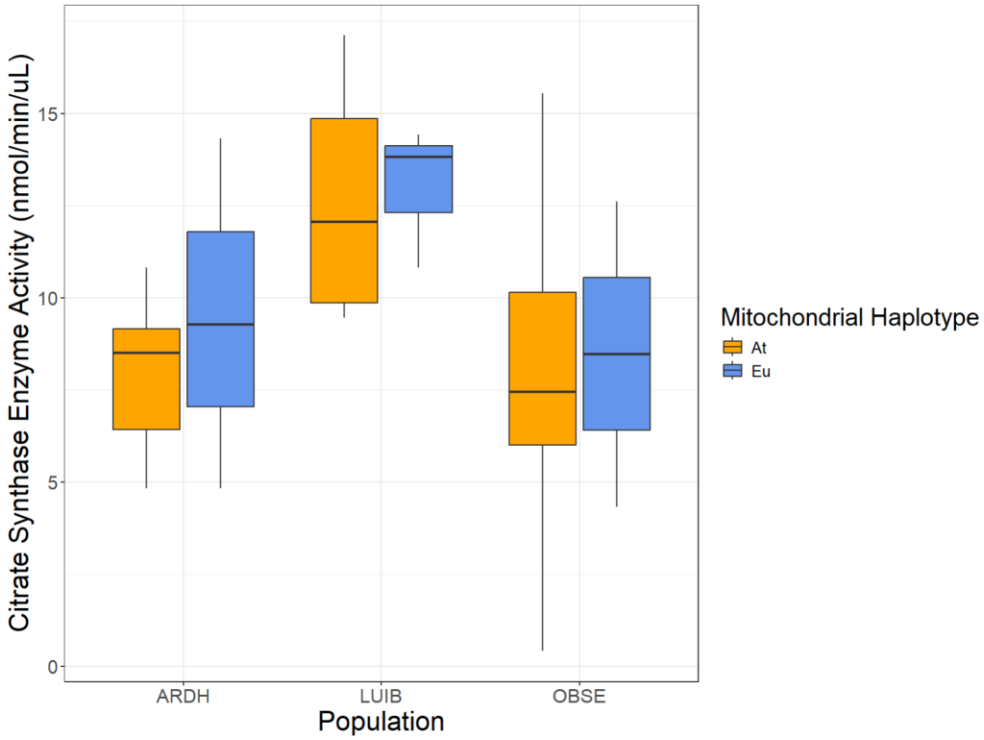

Figure 6. Variation in citrate synthase enzyme activity in migratory three-spined stickleback from North Uist, between mitochondrial haplotypes and populations (ARDH, LUIB, OBSE). Mitochondrial haplotypes are designated for each population as orange for At and blue for Eu. 25 and 75 quartile ranges are shown as vertical black lines.

3.3.2 Aquarium-reared citrate synthase enzyme activity

In 20 freshwater aquarium reared stickleback from 11 families (all LUIB), citrate synthase enzyme activity decreased in fish with larger livers and was significantly higher in fish with the Eu mitochondrial haplotype (Fig. 7; $F_{1,8.6} = 6.56$, $p = 0.0317$).

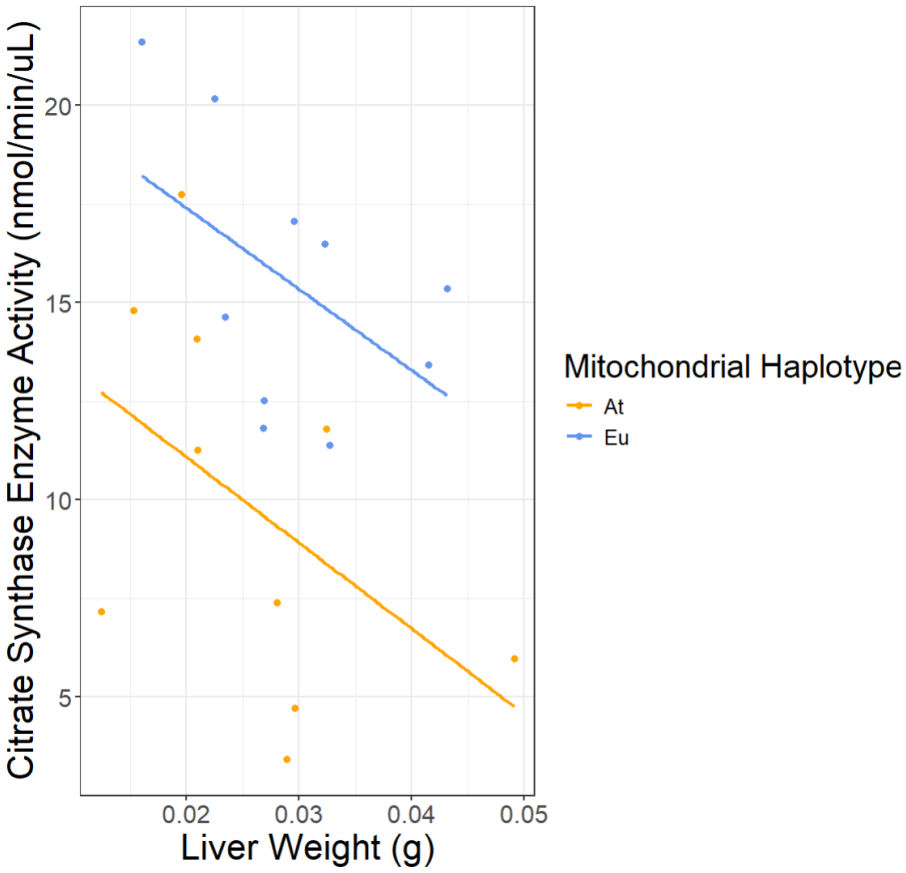

Figure 7. Differences in citrate synthase activity in nmol/min/uL between aquarium reared stickleback plotted against liver weight in grams. At (orange points and line) and Eu (blue points and line) mitochondrial haplotypes are shown.

3.4 Predation Data

Given the differences I observed between populations (see section 3.2), there may be selection acting on each population through the avenue of predation. One hundred and sixty-seven stickleback across my dataset were scanned for predation marks. Twelve stickleback out of 167 showed predation marks, making only 7.2% of the sample appearing to be attacked. I found

no differences between populations, sexes, or lineages ($p > 0.05$). This suggests that if predation does act as a selection agent that varies between populations, I either need a larger sample size to visualize it, or that predation is merely random (Fig. 8).

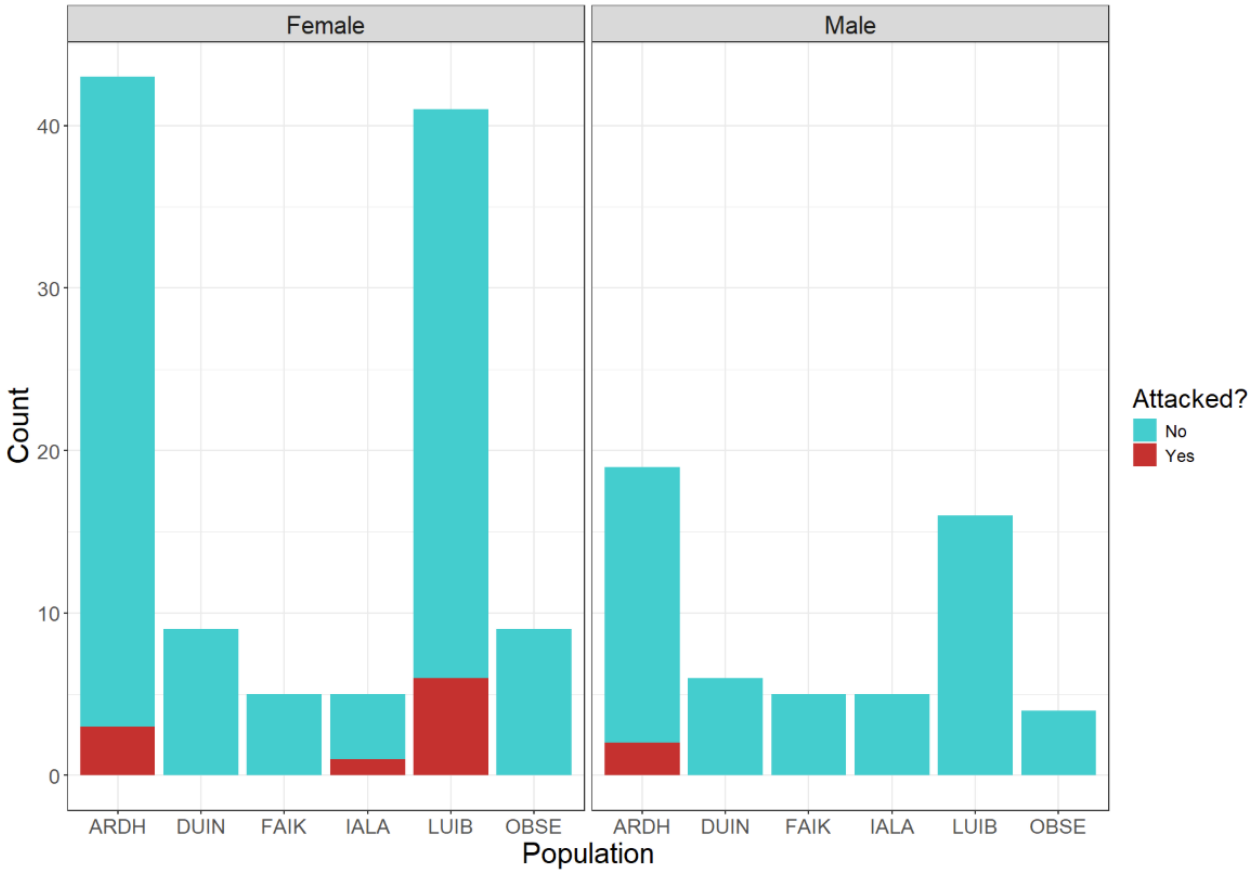

Figure 8. Count of attacked migratory three-spined stickleback from North Uist, between populations (ARDH, DUIN, FAIK, IALA, LUIB, OBSE) and sex (females on left, males on right). Counts of attacked fish are in red, with non-attacked counts in blue.

4 Discussion

Mitochondria have been widely established as organelles responsible for energy production within eukaryotic organisms (Bonora et al. 2012). This energy production is, in part, governed by the mitochondria's own genome, leading us to hypothesize that other physiological and metabolic traits could also be governed by genetic variation in this organelle. My results show that mtDNA impacts citrate synthase enzyme activity levels and male gonadosomatic index, but overall, differences in traits observed were generally between populations or sex levels rather than between haplotypes.

4.1 Citrate synthase enzyme activity

I observed significant differences in the citrate synthase levels of my aquarium reared fish, with the Eu haplotype showing more enzyme activity compared to the At haplotype. I originally hypothesized that the At haplotype would exhibit increased citrate synthase levels due to its association with a migratory haplotype that originated on the other side of the Atlantic (Dean et al. 2019). My results show this is not the case and could be due to a necessity for the Eu haplotype to perform biosynthesis (Lewis et al. *in preparation*, Ishikawa et al. 2019). The Eu haplotype originated in refugia along the west coasts of Europe, probably with continuous access to freshwater-only environments (Dean et al. 2019). Freshwater does not usually contain prey species high in long-chain polyunsaturated fatty acids, a crucial macromolecule for fish, perhaps requiring freshwater fish like the original Eu stickleback, to use other methods of fatty acid acquisition (Xie et al. 2021, Ishikawa et al. 2019). Freshwater fish living in fatty acid depleted areas can synthesize their own fatty acids through a process called endogenous production, or biosynthesis. During biosynthesis, fish convert C18 polyunsaturated fatty acids, α-linolenic acid, and linoleic acid into a biologically active form, like docosahexaenoic acid (DHA) (Xie et al. 2021). It is possible that these biosynthesis pathways use citrate synthase enzymes as a key player in macromolecule production. Citrate synthase enzymes are a major part of the citric acid cycle as they catalyze the first step which turns oxaloacetate and acetyl-coenzyme A into citrate and coenzyme A (Aleksandrov et al. 2014). When not used for those purposes, citrate synthase enzymes can be used by the cell for biosynthesis by providing necessary carbon for fatty-acids and membrane lipids (Peng et al. 2022).

It is possible that the biosynthesis pathways that use citrate synthase are more active in the N. Uist Eu fish and this could be why they have increased citrate synthase enzyme activity. As the Eu haplotype likely originated in freshwater environments, fatty acid acquisition through biosynthesis would have been necessary, and is a process that was likely passed down to the modern Eu stickleback in this study. Interestingly, my hypothesis that freshwater-originating stickleback partake in biosynthesis has been tested elsewhere. In Japan, three-spined stickleback that can colonise freshwater have duplications of a gene, Fads2, that is active in biosynthesis pathways allowing for the production of DHA. In contrast, Japan Sea stickleback (*G. nipponicus*) lack this duplication and are unable to colonize freshwater (Ishikawa et al. 2019). Given this pattern of increased DHA biosynthesis in Japanese stickleback populations, it is possible that similar compensatory phenomena occur in the stickleback populations of N. Uist. Recent research performed by Lewis et al. (*in preparation*) provides evidence for the Eu mitochondrial haplotype as having a higher copy number of the Fads2 gene, and therefore a higher expression of the biosynthesis pathway involved in producing DHA. This is consistent with the theory that freshwater-originating fish have evolved to better synthesize fatty acids and could be why our Eu fish show upregulated citrate synthase in this study.

Although not statistically significant, I found a similar citrate synthase enzyme activity trend in the wild caught stickleback. It is possible that the saltwater environments these fish live in provide sufficient dietary long-chain fatty acids, like DHA, to 'override', to some extent, the need for upregulated Fads2 and biosynthesis, therefore lowering the need for citrate synthase activity within a cell.

Another possibility that may explain the pattern I observed here is that fish with the Eu haplotype simply have more mitochondria, resulting in a higher citrate synthase enzyme activity. This might be because Eu fish require a higher number of mitochondria to meet the energetic demands of a migratory lifestyle because they harbor mitochondria that are less efficient than the At haplotype. As the Eu lineage has dispersed a shorter distance to reach N. Uist, they may not have evolved mitochondria as efficient at energy production as the At haplotype. At fish could have evolved to be more efficient at ATP synthesis, given the energetic demands required to cross the Atlantic. While more laboratory tests are needed to understand what is truly happening,

preliminary results show intriguing patterns on how mtDNA may affect metabolism in N. Uist three-spined stickleback.

4.2 Male GSI

Male GSI was also found to be statistically different between mitochondrial haplotypes, with Eu fish having a higher GSI. GSI is a measure of gonad weight when taking the total body weight (minus the liver) into account. Normalizing gonad weight in this way allows for accurate assessment of gonad size comparative to body composition. In aquatic organisms, GSI has been used to determine the fertility or reproductive condition of individuals in a species. Traditionally, it is assumed that GSI is highest during breeding seasons. In females, this correlates to enlargement of the ovules and is when ovules are considered ripe for laying. In males, this looks a bit different, as the highest GSI is an indication of the onset of spermiogenesis, with a lower GSI correlating with the completion of this process and elimination of the germ cell cytoplasm. Because of this, a male fish may be considered at peak reproduction when GSI measurements are lower comparative to other gonadal development stages (Stoumboudi et al. 1997). While I cannot say that a higher GSI in male fish correlates exactly to peak reproduction (like in females), I could use my GSI data to assess differences in the timing of peak reproduction between mitochondrial haplotypes. As I am seeing differences in GSI between the At and Eu fish in my study, it could be possible that mtDNA may influence the timing of migration in from the sea or the timing/onset of spermiogenesis. As migratory stickleback spend most of the year out at sea, the time at which stickleback may return to saltwater lochs could be influenced by mtDNA (such as enlargement of ovules for female stickleback). Interestingly, the mitochondria play a major role in male reproductive processes like spermatogenesis (in which spermiogenesis is included) and may influence when these stickleback choose to migrate away from the sea. Throughout spermatogenesis, mitochondria are frequently fragmented and re-built, ultimately ending up in a “sheath” surrounding the axoneme during spermiogenesis (Varuzhanyan et al. 2021). This mitochondrial sheath is crucial in sperm mobility, and it is the onset of this process when male GSI is highest in fish (Stoumboudi et al. 1997). Additionally, studies have found that mitochondrial energetics influence sperm fertilization capacity and quality, and dysfunctional mitochondria can lead to reproductive problems (Ulloa-Rodríguez et al. 2017).

Given the complex nature of spermatogenesis and the role of mitochondria in spermiogenesis, it is possible that the underlying mitochondrial genome may affect the timing of these processes. It could be that Eu and At fish exhibit slightly different timescales for mitochondrial dependent processes like mitochondrial fragmentation and sheath formation, therefore affecting the timing of higher or lower GSI measurements. My models showed that the date of collection did not have a significant effect on GSI measurements in male fish, allowing us to draw conclusions about why GSI is higher in the Eu haplotype. A higher GSI measurement for Eu fish could indicate that this haplotype is either farther along in spermatogenesis (having already completed spermiogenesis) or that Eu fish are still in the initial meiotic stages of spermatogenesis compared to At fish. To say for certain, future studies will need to assess other markers of reproduction that better determine timing of peak fertility in male fish.

Another possibility is that the testes of the fish with the Eu haplotype are simply larger, therefore giving us a higher GSI in Eu fish. In vertebrates, testis size is likely a result of selection, whereby the larger the organ, the higher likelihood of successful reproduction that organism has. Larger testes are associated with a higher sperm count or potentially bigger sperm cells (Stockley et al. 1996), something that is likely selected based on the environments they are found in, or the morphology of the eggs to be fertilized. On N. Uist, the Eu haplotype is more commonly found in freshwater lochs, as opposed to the saltwater lochs that this study focused on. Hill (2023) took a deeper look at stickleback reproduction within the freshwater lochs on N. Uist and discovered differences in the egg morphologies of various freshwater lochs, potentially impacting reproduction. His results showed that fish from alkaline lochs had a difficult time hatching in acidic waters. The reason for this is that the alkaline eggs appeared to be more "flexible" and therefore harder for small fish fry to break through in acidic conditions, resulting in death. While these results deal with the effect of pH in freshwater environments, they clearly show the impact a specific loch and its abiotic conditions can have on stickleback reproductive success. A similar abiotic phenomenon could be occurring in my saltwater lochs, causing Eu fish testes to increase in size. It may be that Eu fish sperm are comparatively weaker or less efficient than At fish sperm in saltwater. This would require Eu stickleback to produce more sperm cells to successfully reproduce, resulting in a higher GSI. This idea also fits in with the fact that a higher percentage of Eu fish are found in freshwater versus saltwater: Eu fish may be less reproductively successful in saltwater than At fish. A study similar to Hill et al. (2023) is needed

to understand the true nature of egg morphology and its relationship with GSI in Eu stickleback. Further studies could expereiment with different pairing

4.3 Body size

4.3.1 Sex differences

Apart from those traits that differed between the haplotypes, I also saw many traits that varied either among populations or between sexes. Body size was one of these traits, with female stickleback exhibiting significantly longer and heavier bodies than male stickleback. Sexual dimorphism, a phenomenon where sexes in the same species appear physically different, occurs in many species globally and is well documented in fish. Reasons for sexual dimorphism within fish species vary, but often have to do with mating patterns, parental care, and environmental factors (Ronco et al. 2019, Horne et al. 2019, Qiao et al. 2016). Like other fish species, three-spined stickleback populations throughout Japan, North America, and Europe have also been documented as exhibiting sexual dimorphism (Leionen et al. 2010, Kitano et al. 2012), consistent with my N. Uist findings. My body size data in this study show females as both larger and longer, and this may be an adaptation that influences reproductive success. Body size in females is often associated with fecundity; the larger the female, the more reproductively successful they will be. This is different for male fish, as body size is more a result of male-male competition and sexual selection (Parker 1992). In stickleback, other factors often influence male reproductive success, such as coloration (redder males tending to be more successful in mating), so size is not the only factor driving sexual selection (Bakker and Mundwiler 1994). From a reproductive standpoint, the sole job of a female stickleback is to grow ovules in preparation for external fertilization, thus driving larger size in females.

4.3.2 Population differences

Population differences in body size also provide a look into the three-spined stickleback of N. Uist. ARDH fish were significantly smaller than other populations, driving length results that lead to DUIN and FAIK individuals being significantly longer. Interestingly, DUIN and FAIK are the two lochs in this study that are geographically closest to one another, both found in the northeast of the island. ARDH, on the other hand, is found on the opposite, southwestern side of the island. It could be that differences in location result in abiotically or biotically different

environments that affect stickleback body size. Geographic location has been found to affect morphology in stickleback in other parts of the world. Morris et al. (2018) studied a similar system to N. Uist, where several marine stickleback populations are found across Alaska, British Columbia, and northwestern US states (Washington, Oregon, and California). Their results showed high variation in body shape across locations, with northern British Columbian stickleback showing "narrow, streamlined bodies" and southern Californian stickleback showing "squatter, less streamlined bodies" (Morris et al. 2018). These results provide evidence that local adaptation occurs in (marine) stickleback populations globally and may be able to explain the body size differences I see in my migratory populations on N. Uist. Local adaptations arise within a population due to the differing environments certain populations find themselves in. The environments found within and around both DUIN and FAIK may be similar enough to select for longer bodies. This includes the environments that these migratory fish swim into such as Loch Maddy (57° 35' 58" N, 7° 09' 31" W) or the Minch (58° 01' 51" N, 5° 59' 36" W). This contrasts with the environments found within and around ARDH (including the Atlantic Ocean), as those fish have shorter lengths. These local adaptations have arisen for a reason and may have to do with factors such as nutrient availability, predation patterns, or intra-population competition. It may be that lochs located in similar geographic ranges exhibit similar conditions in these areas and therefore contribute to the body size patterns I see.

Additionally, body size may be affected by the early-life growth rate and behaviors of migratory stickleback in each individual loch. It could be that stickleback growth is greatly influenced by their early, pre-adult life, similar to other marine species (Jonsson and Jonsson 2014). The growth of migratory stickleback after hatching depends on a variety of factors: many abiotic components like pH or nutrient availability, but also the behavior and presence of other competitors or predators. For example, freshwater stickleback on North Uist exhibit "dwarfed" body sizes in nutrient poor conditions when certain small species like brown trout or the nine-spined stickleback (*Pungitius pungitius*) are not present (MacColl et al. 2013). Interestingly, further studies show that the three-spined stickleback has the opposite effect on body size of the nine-spine stickleback, with their absence allowing the nine-spine stickleback to grow very large (Herczeg et al. 2009). While this evidence provides a strong example of biotic factors impacting growth, I must also consider the behavior of my migratory fish themselves. It is hypothesized that migratory stickleback have a "homing" ability that allows them to return to their loch of

hatch each year. If migratory stickleback did not have the ability to return to their loch of origin, then I would likely see more consistent body size across the migratory lochs in this study. Unfortunately, outside of speculation, I am unable to say for certain if N. Uist migratory stickleback are in fact returning to their own lochs, and future telemetry studies would need to be employed.

4.4 Organ sizes and indexes

4.4.1 Sex differences

Outside of body size measurements like length and weight, the stickleback in my study showed differences in their internal makeup between sexes. In general, female stickleback have larger livers than males, as my results showed both HSI and liver weight per gram increased in females. Sexual dimorphism in fish liver is well documented, with Qiao et al. (2016) reporting female Japanese meduka (*Oryzias latipes*) hepatocytes (functional liver cells) as containing large, isolated reserve vesicles where male liver cells show smaller versions. The liver is an essential organ when it comes to the reproductive processes of female fish, as it plays a major role in oocyte production; the process by which an immature ovarian cell is produced. Additionally, in female hepatocytes, granular endoplasmic reticulum, mitochondria, and peroxisomes are reported as increased, providing further evidence for larger liver size in female fish (Qiao et al. 2016).

While they do harbor smaller livers, the male stickleback in my study exhibit a tradeoff, whereby they have much larger pectoral muscles and greater fat than female stickleback. The pectoral muscles are a particular interesting muscle group in male stickleback, as they play a direct role in male parental care strategies. It is well established that male stickleback provide parental care through the continuous “fanning” of oxygenated water over fertilized eggs in their nest (Lachance and Fitzgeralds 1992). Male stickleback will continue this fanning behavior throughout the duration of egg development until hatching begins (Bakker et al. 2006). The pectoral muscle is the main muscle used for this “fanning” motion and is likely why the male stickleback in this study show larger pectoral muscles compared to females. As females invest little to no parental care in their eggs, needing simply to lay their eggs in a male’s nest (Bakker et al. 2006), female pectoral muscles would not be as well developed. Male stickleback also exhibit

greater fat reserves. This is likely an adaptation required for the energetic and aerobic demand of male parental care. Given the need for male stickleback to maintain constant watch over their eggs, and only a small, defended territory around them, it is likely they do not have the opportunity to hunt for food or gain many nutrients while providing care. If this is the case, it is likely that they draw on the nutrients stored in their fat reserves for survival during egg development.

4.4.2 Population differences

Similar to the population differences in body size, my results also show population differences in liver and fat related traits. I found significant differences between FAIK and LUIB, indicating that these two populations are most different from each other compared to other populations. Interestingly, while FAIK was identified as some of the longest fish in the dataset, they also have some of the smallest livers and fat content. This is different from LUIB fish, that did not stand out for their length but do stand out for their large liver measurements. Given that I have FAIK fish with long bodies and small internal organs and LUIB fish with average sized bodies but above average sized organs, this could imply that body size and organ size may not be correlated completely. There are likely other patterns going on that could account for the discrepancies in organ sizes. If geographical variation is influencing this system, this would have to imply that IALA (being near FAIK) would also show smaller organ sizes, but this is not the case. Fish from IALA, which is found on the opposite side of the island also had larger livers, like LUIB. A more likely explanation for the differences in organ size could be nutrient availability (and associated abiotic factors) and reproductive timing.

Nutrient availability could be a likely explanation due to the ways in which specific food items and diet effect biomass gain. Some nutrients may digest differently in the body and therefore affect the energy that an organism can produce (Talbot 1993). It could be that each loch contains slightly different food stuff, causing sticklebacks in each to show varying organ sizes. Additionally, the availability of nutrients also depends on various abiotic factors like dissolved oxygen levels and salinity (Talbot 1993), therefore influencing the diets of each population. Reproductive timing may also be a likely explanation for variation in organ sizes, as fish are known to greatly differ in size depending on the time of year and breeding season. In fact, reproductive cycles, in combination with available nutrients, are known to affect body protein

and lipid depletion and retention (Talbot 1993). It is possible that each population follows their own slightly different reproductive cycles and that is therefore why I see slightly different fat and liver sizes (refer to sections 4.2 and 4.4.1 for further information on reproductive cycles and organ sizes). This idea that populations may be on differing reproductive cycles fits well with the fact that body size differs amongst populations and the theory that stickleback return to mate with individuals from their own population (see section 4.3.2).

4.5 Ovule volume and clutch size

While showing no differences between mitochondrial haplotypes, my ovule results continue a theme seen throughout fish reproductive literature: with increasing female length, clutch size also increases (Takatsu et al. 2023, Karino and Arai 2006). This makes sense, as a physically larger body size will be able to accommodate more ovules. Unfortunately, due to my small sample size I am unable to determine any significance on the mitochondrial haplotype or population level. Further studies on this interaction should focus more on obtaining a larger, more even sample size from several populations.

4.6 Predation

To achieve a holistic understanding of the migratory populations in this sample, I looked at marks of predation across the six populations. There were no differences in frequency of predation on the mitochondrial haplotypes, across populations, or between sexes. While this is statistically the case, graphical results visually show a slight trend: female fish appear more frequently attacked than male. While the total number of stickleback (male or female) attacked in this study was low, more populations had attacked female stickleback (LUIB, IALA, ARDH) compared to male (only ARDH). Given what I know about female body size relative to male body size, this may lend some insight into the preferences of stickleback natural predators: the bigger the fish, the better. Three-spined stickleback predators on N. Uist often include bird species like terns (Arctic, Common, and Little) heron, shag, and cormorants, and larger marine species like trout and eel. Additionally, this sample only represents stickleback that were able to evade death by predation and subsequently caught by my traps and does not give a truly accurate representation of predation patterns in each population. It could be that some populations have evolved to evade predation better than others or that the majority of predation occurs in parts of a

loch that I did not sample at (like the middle/deepest areas). Additionally, a behavioral component to this must be assessed as well. It may be that stickleback previously attacked by a predator will be more wary of unfamiliar objects in their environment, like a trap, and therefore stay away from them. While my study does provide a good start to understanding predation patterns on N. Uist, to discover the causes and consequences of predation between populations, a larger and more focused study must be designed.

4.7 Conclusion

North Uist provides an ideal environment for mitochondrial studies. It houses three-spined stickleback from two mitochondrial lineages that originated on different sides of the Atlantic, while providing an environment for them to interbreed within their own lochs. While not providing true conplasticity, this environment resembled conplastic experiments and allowed me to design a study that tested what mitochondrial DNA affects in a well-mixed nuclear background, specifically in the wild. This is not often found in a wild setting and is why N. Uist is an ecosystem with such value to mitochondrial research. Given the importance of mitochondria in growth and reproduction, migratory abilities, and even organ function, it is crucial that research focuses on understanding what mitochondria influence, and how variation in this organelle can lead to differing phenotypic traits. This study aimed to do exactly that, with a wide range of phenotypic traits measured.

4.7.1 Citrate synthase enzyme activity

While I found that mitochondrial variation influences only a couple of phenotypic traits (citrate synthase enzyme activity and male GSI), these traits are potentially very crucial to the success and survival of migratory stickleback populations. My citrate synthase study showed interesting differences between enzyme activity levels. Eu stickleback have increased enzyme activity, perhaps a result of their ancestral need for biosynthesis. If freshwater fish, like the ancestral Eu stickleback did not develop the ability to synthesize their own fatty acids, we may not see the Eu haplotype represented in the migratory populations of N. Uist. While more in-depth research should be performed to fully understand this relationship, this study has provided an insight into the complexity of mitochondrial function and its implications for freshwater fish survival.

4.7.2 Male GSI

Male stickleback GSI was also identified as being different between mitochondrial haplotypes. As GSI is a measure of gonad weight relative to body weight, it gives us a good estimate on the ways in which testes physically differ between haplotypes. Results found Eu stickleback as having a higher GSI. As I have identified GSI as being increased during the onset of spermiogenesis (Stoumboudi et al. 1997), we can infer one of two possibilities. Either the reproductive cycles of the Eu and At fish are slightly offset, or Eu fish simply have larger testes. If the former is the case, we caught our stickleback at a time when Eu fish were theoretically closer to their spermiogenesis phase, and the At fish were slightly ahead or behind it. If the latter is the case, Eu male fish may need either more sperm, or physically bigger sperm to deal with the abiotic conditions that a saltwater loch presents. Understanding why this is the case would need further research, but this study begins a conversation around how mitochondria influence reproductive timing or the strength/resilience of adult sperm cells.

4.7.3 Sex differences

While the primary focus of this study was to identify differences between mitochondrial haplotypes, I decided to also take a deeper look at traits that differ between sexes and populations. This is in an effort to achieve a holistic understanding of the environments that both haplotypes of stickleback reside in on N. Uist. The results I found when comparing male and female fish paint an interesting picture on the role that each sex plays in reproduction. Female stickleback are longer and less fatty, with bigger livers; this is likely so that the females can produce many, healthy eggs. Males on the other hand are smaller but have larger pectoral muscles and contain more fat. This is likely an adaptation to ensure parental care success, as male fish spend a long time physically fanning oxygenated water over their eggs, leaving little time to feed (Lachance and Fitzgeralds 1992, Bakker et al. 2006). These body size and organ adaptations are crucial to the successful reproduction, and it would be curious to see if similar patterns occur in the freshwater stickleback populations on N. Uist.

4.7.4 Population differences

The six populations sampled also showed much diversity in body and organ size, likely due to geographical variation and differences in abiotic conditions. My population results

identify unique patterns in FAIK and LUIB fish, as statistical analysis often pulled these two populations out as very different. FAIK fish are long but have very small organs relative to their body size. This is the opposite case for LUIB fish, as they have an average body size, but very large organs relative to body size. This pattern indicates that body and organ sizes are not positively correlated and are likely influenced by different abiotic or geographical factors. Body size can often be influenced by early life-history and migratory patterns (Jonsson and Jonsson 2014). Organ size can also be linked to these traits, but potentially even more so to nutrient availability and pH (things that are often influenced by geographical variation) (Talbot 1993).

4.7.5 Ovules and predation

My ovule and predation data, while lacking in sample size, showed a few patterns. My ovule data proved existing knowledge about the positive correlation between body length and clutch size (Takatsu et al. 2023, Karino and Arai 2006). In order to make any claims about differences in haplotypes, populations, or sexes, a larger and more diverse sample is needed.

My predation data, while not significant, did show a slight trend of females being more frequently attacked. This could be due to the large female size, and the preference of predators for larger prey. Beyond this explanation, there were no differences between populations or sex, and the sample did not well represent the true rate of predation, as it does not account for fish that have been eaten or ones that stay wary of unfamiliar objects, like traps.

4.7.6 Final thoughts

It is my hope that this thesis provides a holistic look into the migratory stickleback populations on the mitochondrial level, and between populations and sexes. I believe that future studies should use N. Uist for its immense potential in mitochondrial work and understanding of life-history theory.

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