Fatty acid variation in three-spined stickleback (*Gasterosteus aculeatus*)

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Abstract

Omega-3/omega-6 long-chain polyunsaturated fatty acids, particularly EPA and DHA, are key biological molecules necessary for growth, survival and reproduction. Environmental heterogeneity in the dietary availability of these compounds across aquatic environments has resulted in differential evolution of traits associated with DHA biosynthesis among populations of the same species exploiting divergent resources. Such disparities in DHA biosynthetic capacities have resulted in population-level differences in omega-3/omega-6 long-chain polyunsaturated fatty acid phenotypes. Here, fatty acid profiles of three-spined stickleback ecotypes inhabiting or originating from lochs on North Uist drastically differing in environmental parameters, notably pH and nutrient conditions, were measured to understand how EPA, DHA and total fatty acid concentrations differ between distinct environments. To support any conclusions drawn from corresponding results, a summary analysis on algal EPA and DHA contents was performed to showcase fatty acid resource heterogeneity between aquatic habitats. Moreover, fatty acids of plankton sampled from an acidic and alkaline loch were profiled to understand how the dietary availability of polyunsaturated fatty acids varies between analysed freshwater environments. Algae EPA and DHA contents were significantly influenced by habitat and taxonomy. Furthermore, significant differences were discovered between loch plankton in concentrations of all analysed polyunsaturated fatty acids, suggesting divergent selection pressures may act on corresponding freshwater stickleback populations. Finally, EPA, DHA and total fatty acid concentrations significantly varied between lochs, ecotypes and sexes. Results were used to predict divergent metabolic evolution between three-spined stickleback populations.

Chapter 1: General Introduction

The colonisation of unoccupied niches can result in species diversification and adaptive radiations, defined by rapid local adaptation to varying environments prompting divergent phenotypic change (Magalhaes *et al*., 2016). These events are notably evidenced in Darwin's finches (Grant and Grant, 2002), East African cichlid fish (Brawand *et al*., 2014) and the numerous accounts of postglacial invasions of northern hemisphere freshwater environments by marine fish (e.g., Campbell, 1985; Schluter, 1993; Kitano *et al*., 2010; Magalhaes *et al*., 2016). However, the ability to colonise novel environments can vary amongst lineages, including between stickleback lineages, which exhibit differential capacities to establish in freshwater habitats (Ishikawa *et al*., 2019). Determinants influencing successful movements have seldom been identified (Ishikawa *et al*., 2019).

Food webs differ between ecosystems (Twining *et al*., 2021), therefore, ecological transitions between them typically entail dietary shifts for corresponding organisms. Within ecosystems, ontogenetic development is regularly associated with dietary alterations, as well as changes in habitat and trophic interactions between the ecological community (Nonaka and Kuparinen, 2021). These life-history stage dependent diets result in highly composite, multidimensional ecological systems (Nonaka and Kuparinen, 2021). Research regarding evolutionary adaptations required to cope with dynamic nutritional changes commonly investigate behavioural and morphological traits (Twining *et al*., 2021). For example, the evolution of trophic polymorphisms in pharyngeal jaw and head morphology in the cichlid fish *Cichlasoma citrinellum*, which change during ontogenetic development, are hypothesised to reduce intraspecific competition through divergent niche usage (Meyer, 1990). The cephalic differences

influence feeding mode, enabling the optimal exploitation of different dietary resources by each morph, subsequently permitting population growth (Meyer, 1990).

Metabolism governs cellular-, organismal- and ecosystem-level interactions, acting as a constraint in trophic ecology and numerous other systems in nature (Braakman and Smith, 2013; Twining *et al*., 2021). The evolution of metabolic traits influencing key biosynthetic (anabolic) and degradation (catabolic) processes likely regulates ecological opportunities to invade novel adaptive zones, species diversification and the maintenance of resultant biodiversity (Braakman and Smith, 2012; Twining *et al*., 2021). This is because nutrient deficiencies, for example the paucity of docosahexaenoic acid in freshwater ecosystems, limit organismal fitness (Ishikawa *et al*., 2021). Therefore, habitat- and ecosystem-level disparities in resource availability and quality should impose divergent selection pressures on indigenous populations and colonising species to optimize levels of resource intake and nutrient biosynthesis (Twining *et* al., 2018; Nishio *et al*., 2020; Ishikawa *et al*., 2021; Twining *et al*., 2021). This may contribute to natural selection driving both speciation and radiations (Twining *et al*., 2021). Until recently, studies have failed to identify the genetically encoded metabolic alterations required to subsist across nutritional gradients between environments during ecological transitions.

Environmental resource availability of inorganic elements and organic compounds rarely aligns with the physiological demands of species, a limiting factor for population viability (Twining *et al*., 2018; Twining *et al*., 2021). For example, the nutrient contents of consumers in arthropod communities are correlated with the nutrient compositions of prey, themselves comprised of increasing protein and decreasing lipid quantities at higher trophic levels (Wilder *et al*., 2013).

This decreasing lipid availability is hypothesised to place an upper limit on the size of food chains and overall webs, as energy production through amino acid catabolism cannot compensate for lipid reductions, potentially resulting in the decreased fitness of high-level predators (Wilder *et al*., 2013). However, nutrient quality is increasingly recognised as of equal or greater importance than the sheer quantity of resources distributed across ecosystems (Twining *et al*., 2018). For example, stoichiometric food quality is typically low for herbivores inhabiting freshwater and terrestrial environments, with increasing C:N and C:P rations resulting in gross growth efficiency reductions in grazers (Elser *et al*., 2000). Under stoichiometric food quality models, feeding on primary producers with average carbon-nutrient rations is predicted to yield corresponding primary consumers gross growth efficiencies of between 10%-30% (Elser et al., 2000). Moreover, the consumption of low-quality (highly indigestible) prey by insectivorous avian chicks with small guts may limit fitness due to reduced nutrient uptake capabilities (Reeves *et al*., 2021). For example, the growth and survival of Northern bobwhite (*Colinus virginianus*) chicks, which lack the capacity to effectively digest chitinous compounds, could be affected by consuming arthropods with large exoskeletons (Reeves *et al*., 2021). Furthermore, diet trials performed on Japan Sea stickleback (*Gasterosteus nipponicus*) revealed significant increases in survival when individuals were fed high quality (docosahexaenoic acid (DHA)-rich) compared to low quality (DHA-free) foodstuffs, suggesting nutritional quality does affect survival (Ishikawa *et al*., 2019). Inorganic elements are solely acquired through dietary intake (Twining *et al*., 2021), therefore, nutritional constraints in environments cannot be overcome by evolving compensatory metabolic adaptations. In contrast, nutritional shortages in organic compounds, such as DHA, can be subsisted if organisms have evolved metabolic capacities to synthesise required molecules from precursors (Twining *et al*., 2021).

Fatty acids – digestion and storage

Metabolic, particularly lipid phenotypes, are significant contributing sources of fitness variation between individuals (Twining *et al*., 2021). This is especially apparent in aquatic organisms, which evolved metabolic adaptations for lipid dependency as a main energy source within environments scarce in simple carbohydrates (Turchini *et al*., 2021). Phenotypic flexibility in physiological traits associated with energy metabolism permits the maximisation of growth rates under nutritional constraints, likely becoming a factor governing species viability under anthropogenic environmental alterations (Auer *et al*., 2015).

Lipids are a significant class of hydrophobic biological molecules required for cellular structure, signalling, metabolism and transport (Muro *et al*., 2014; Turchini *et al*., 2021). Fatty acids, a component of lipids, are carboxylic acids comprising a terminal methyl group and an aliphatic chain varying in length (Turchini *et al*., 2021). The properties and functions of fatty acids are determined by their chemical structure (Turchini *et al*., 2021). The production of ATP through mitochondrial beta-oxidation is a key role of fatty acids (Sargent *et al*., 2002). Ecologically, the presence and relative concentrations of certain fatty acids within organisms can be used as markers of trophic relationships and ecosystem dynamics (Dalsgaard *et al*., 2003; Rudy *et al*., 2016; De Carvalho and Caramujo, 2018).

Fatty acids take numerous biochemical forms, often being esterified to associated molecules, to construct products classified by either polarity or complexity (Turchini *et al*., 2021). For example, triacylglycerols are common and effective storage molecules, defined by three identical

or varying fatty acids esterified to a glycerol molecule, resulting in many compounds comprised of numerous possible combinations of precursors (Turchini *et al*., 2021). Long-chain polyunsaturated fatty acids (LC-PUFAs) have a regiospecific preference to binding at the *sn-2* position of these molecules, as showcased by the predominant binding of DHA, an omega-3 long-chain polyunsaturated fatty acid, at this location in triglycerides of Atlantic salmon (*Salmo salar*) (Ruiz-Lopez *et al*., 2015; Turchini *et al*., 2021). Moreover, preferential regiospecificity of DHA to the *sn-2* position of phosphatidylcholine molecules was observed in the mud crab (*Scylla paramamosain*) (Wang *et al*., 2022). Phosphatidylcholine is the commonest phospholipid, a significant molecule within the lipid bilayer of cells with a basic structure similar to triacylglycerols, consisting of a glycerol molecule with fatty acids esterified at the *sn-1* and *sn-2* positions, but diverging from them by the incorporation of a phosphate group at *sn-3* position, (Turchini *et al*., 2021). Phospholipids are often more complex in structure and chemistry, as other molecules can be integrated via binding to the phosphate head, such as in phosphatidylcholine, which comprises choline adjoined to this group (Turchini *et al*., 2021). Molecules have greater stability when bound at *sn-2* compared to both *sn-1* and *sn-3* which may explain such LC-PUFA positional preferences (Wang *et al*., 2022). Wax esters are another common lipid storage compound, particularly throughout trophic levels of marine environments, comprising a variable fatty acid esterified to a fatty alcohol (Turchini *et al*., 2021). Fatty acids define several other lipid classes (Turchini *et al*., 2021).

In animals, after lipid digestion, free fatty acids are assimilated into gut enterocytes, a process dependent on evolutionary conserved transport proteins, including fatty acid transport proteins, which prevent leakage of such molecules from the cell (Turchini *et al*., 2021). Once absorbed,

fatty acids are esterified to assemble lipid compounds, including triacylglycerols and phospholipids, through various synthesis pathways (Turchini *et al*., 2021). In fish, the liver, muscle and adipose tissue are key lipid deposition organs, generally possessing higher relative lipid contents compared to the whole body (He *et al*., 2015; Turchini *et al*., 2021). Although the lipid contents of each organ differ between species, individual variation can result from sex, age and multiple environmental factors including nutrient availability (Turchini *et al*., 2021).

Omega-3/omega-6 long-chain polyunsaturated fatty acids

Omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs), particularly alpha-linolenic acid (ALA; C18:3n-3), eicosapentaenoic acid (EPA; C20:5n-3) and DHA (C22:6n-3), are essential structural components of the brain and nervous system, present in and affecting the properties of neuronal membranes and in turn, cellular function (Dyall and Michael-Titus, 2008). Omega-3 LC-PUFAs acquired dietetically are assimilated into the cell membrane (Surette, 2008). Studies suggest these molecules increase mitochondrial biogenesis in certain tissues (Albracht-Schulte *et al*., 2018). Moreover, they are used as precursors for lipid messenger synthesis, and act as ligands, activating peroxisome proliferator-activated receptors (PPARs), which regulate the expression of many genes, such as the acyl-CoA oxidase gene, and subsequent biological processes (Keller *et al*., 1993; Schoonjans *et al*., 1996; Hihi *et al*., 2002; Dyall and Michael-Titus, 2008; Kersten, 2014). For example, PPARα, which is primarily expressed in tissues characterised by high fatty acid degradation, governs genes necessary for lipid metabolism and metabolic homeostasis (Yoon, 2009; Kersten, 2014). Investigations utilising *Xenopus laevis* PPARα showed greater activation of the receptor in the presence of

PUFAs compared to shorter chain fatty acids (Keller *et al*., 1993). As well as neurogenesis (Innis, 2008) and visual development (Dyall and Michael-Titus, 2008), these molecules, particularly DHA and EPA, affect key processes including immune function, through influencing the anti-inflammatory process and providing immune-modulating effects (Mori *et al*., 2004; Calder, 2010) in addition to being necessary for growth, reproduction and survival (Twining *et al*., 2018; Twining *et al*., 2021; Turchini *et al*., 2021). The evolutionary conservation of DHA as a vital constituent of neuronal membranes showcases its biological importance (Crawford *et al*., 2013). Omega-6 long chain polyunsaturated fatty acids (n-6 LC-PUFAs) are similar molecules necessary for survival (Hudson *et al*., 2022). They often act synergistically with n-3 LC-PUFAs, with imbalances in optimal n-6/n-3 ratios correlating with increased incidence of various diseases, including ulcerative colitis, in which individuals housing higher relative concentrations of arachidonic acid (ARA; C20:4n-6) in adipose tissue have a significantly higher risk of developing the disease (de Silva *et al*., 2010; Scaioli *et al*., 2017; Turchini *et al*., 2021; Hudson *et al*., 2022). Oxygenated products of C_{20} n-x LC-PUFAs (eicosanoids), produced using COX and LOX enzymes, function as ligands for nuclear receptors and bind to G-protein coupled receptors (Calder, 2020; Turchini *et al*., 2021). Eicosanoids regulate many networks, including the female reproductive system (Calder, 2020).

Omega-3/omega-6 LC-PUFA biosynthesis

The ability to synthesise and modify n-3/n-6 LC-PUFAs, including DHA, varies greatly across the tree of life and between closely related species (Ishikawa *et al*., 2019; Twining *et al*., 2021). Simplified, the n-3/n-6 LC-PUFA biosynthetic pathway (**Fig. 1**) comprises several distinct stages which utilise combinations of alternating desaturases and elongases. These introduce kink

inducing double bonds and increase aliphatic chain length respectively (Twining *et al*., 2021; Turchini *et al*., 2021). Firstly, nonlipid precursors (carbon sources) are degraded into citrate and subsequently transported from the mitochondria to the cytosol, which, through multiple *de novo* synthesis reactions involving a carboxylase, lyase and fatty acid synthase, is converted into palmitic acid (C16:0) (Twining *et al*., 2021; Turchini *et al*., 2021). This can be further elongated via saturated fatty acid (SFA) elongase to stearic acid (C18:0) (Henderson, 1996; Twining *et al*., 2021; Turchini *et al*., 2021). These events are similar among mammals and fish (Turchini *et al*., 2021). Stearoyl-CoA desaturase, exhibiting Δ9 activity, converts stearic to oleic acid (C18:1n-9c), a monounsaturated fatty acid (MUFA), defined by the presence of one double bond at the ninth carbon from the terminal methyl end (Twining *et al*., 2021). The desaturation of palmitic acid to palmitoleic acid (C16:1n-7) may also occur (Turchini *et al*., 2021). Further Δ12 desaturation transmutes oleic acid into linoleic acid (LIN; C18:2n-6c), an omega-6 PUFA necessary for survival (Twining *et al*., 2021; Hudson *et al*., 2022). ALA is produced through the conversion of LIN via Δ15 desaturase activity (Twining *et al*., 2021). Unlike, for example, marine microalgae as well as some copepods and invertebrate lineages, including Mollusca (Kabeya *et al*., 2018; Twining *et al*., 2021), vertebrates cannot de novo synthesise ALA or LIN from MUFA precursors due to an absence of Δ12 and Δ15 desaturases, therefore, the dietary acquirement of these PUFAs is essential (Xie *et al*., 2021).

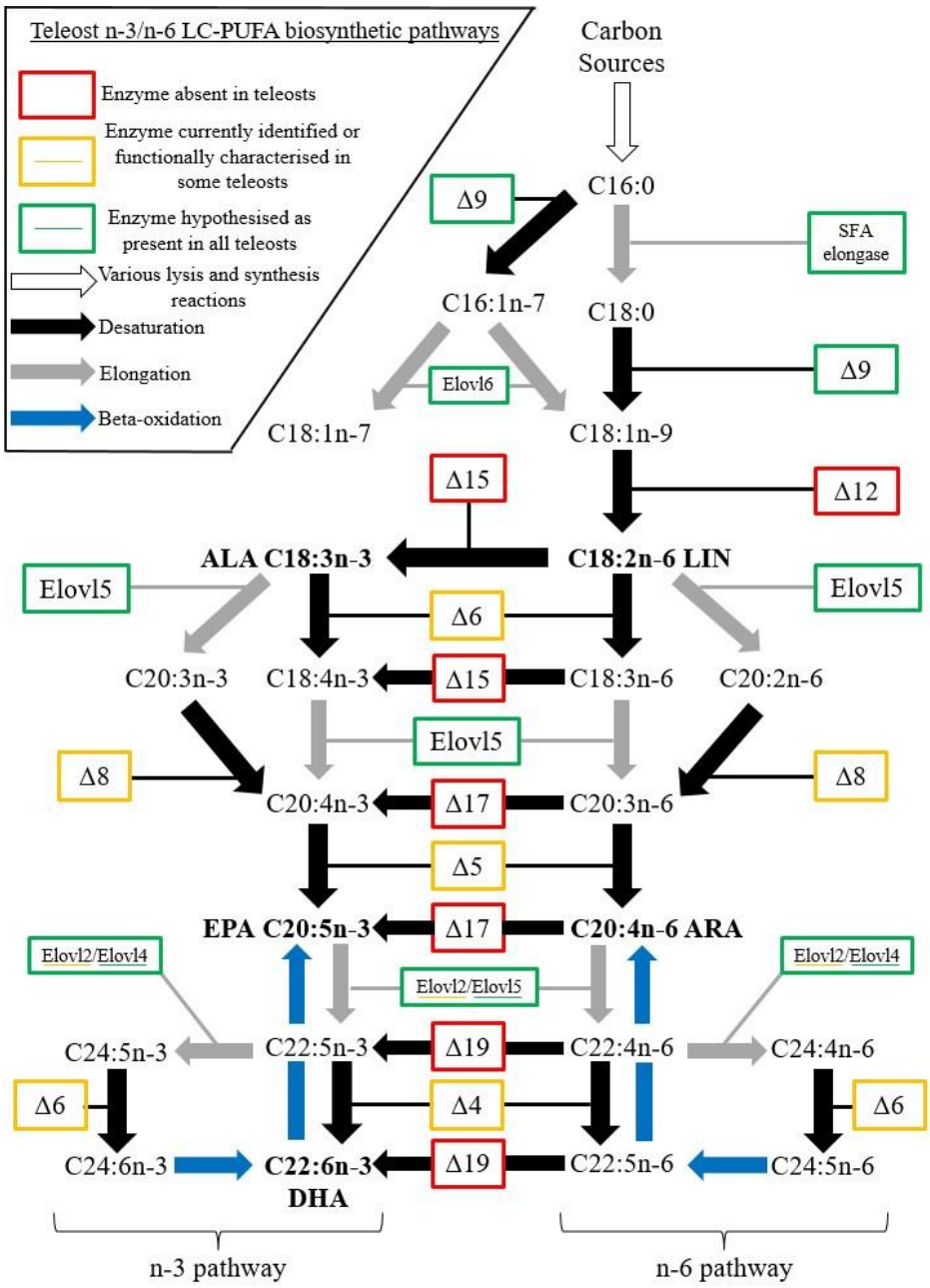

Figure 1. The n-3/n-6 LC-PUFA biosynthetic pathways of teleosts. Key molecules are emboldened. Desaturase and elongases are highlighted based on their presence within the lineage. The "Sprecher pathway" and the elongation of EPA and ARA to C22:5n-3 and C22:4n-6 can be performed by Elovl2, only apparent in some teleosts. However, these reactions can also be conducted utilising either Elovl4 or Elovl5.

Both LIN and ALA, through a subset of front-end desaturases and PUFA elongases, can be synthesised into C_{20} and subsequently C_{22} n-6 and n-3 LC-PUFAs respectively, including ARA, EPA and DHA (Oboh *et al*., 2017 Twining *et al*., 2021). The production of n-3 LC-PUFAs from n-6 precursors can arise, although, such mechanisms require methyl-end desaturases with Δ15, Δ17 and Δ19 activity (Twining *et al*., 2021). Δ17 and Δ19 desaturases have been identified in fungi, oomycetes and invertebrates (Xue *et al*., 2013; Kabeya *et al*., 2018), however, they are absent in vertebrates, resulting in an inability to synthesise n-3s, particularly EPA and DHA, through these pathways. Single enzymes can possess multiple desaturation activities, as showcased in the two n-x (where x is 3 or 6) invertebrate desaturases present across corresponding metazoan lineages (Kabeya *et al*., 2018). A vertebrate's ability to synthesise EPA and DHA from ALA is conditional on the presence of genes encoding such fatty acyl desaturase (Fads) enzymes and elongation of very long chain fatty acid (Elovl) proteins (Surm *et al*., 2018; Turchini *et al*., 2021). Mammals possess a Δ5 and Δ6 fatty acyl desaturase, encoded by the fatty acyl desaturase 1 (*Fads1*) and the fatty acyl desaturase 2 (*Fads2*) genes respectively, as well as two elongase genes, being *Elovl5* and *Elovl2*, encoding enzymes of the same name, all of which are involved in the synthesis of C_{20} and C_{22} n-3 and n-6 PUFAs (Monroig *et al*., 2016). Competition for enzymes arises between both biosynthesis systems (Turchini *et al*., 2021). To interconvert EPA to DHA, mammals, as well as fish, utilise the "Sprecher pathway", consisting of sequential elongation from 22:5n-3 to 24:5n-3, with subsequent Δ6 desaturation to 24:6n-3 and peroxisomal beta oxidation (retroconversion) to DHA (Leonard *et al*., 2002; Oboh *et al*., 2017). Many mammals possess incomplete biosynthesis pathways due to an absence of these enzymes (Turchini *et al*., 2021).

Desaturases and elongases in teleosts

Although some fish possess functional orthologues of both *Fads1* and *Fads2* genes, their evolution preceding gnathostome radiation has resulted in an absence of *Fads1* in teleosts, although the resultant loss of Δ5 desaturase activity has been recovered in a subset of corresponding lineages through the mutation, potential duplications and functional diversification of teleost *Fads2* (Castro *et al*., 2012; Garrido *et al*., 2019). For example, Atlantic salmon, through multi-functionalisation and neofunctionalization, have a bifunctional Δ5Δ6 Fads2 (Oboh *et al*., 2017). Although teleost Fads2 has a greater diversity in regioselectivity and specificity compared to the mammalian equivalent, it predominantly occurs as a Δ6 desaturase in most species (Monroig *et al*., 2011). Oboh *et al*. (2017) confirmed teleost Fads2 can showcase Δ6 activity towards C_{24} n-3 LC-PUFAs, permitting DHA synthesis through the "Sprecher pathway". Moreover, they identified 11 Acanthopterygii species possessing sequences for a Fads2 with Δ4 desaturation, enabled by the YXXN amino acid domain within the translated protein. Two of these species (*Oryzias latipes* and *Oreochromis niloticus*) had their Fads2 function as a Δ4 desaturase confirmed. Garrido *et al*. (2019) further identified Δ4 activity in Fads2 of an additional two teleost species (*Pegusa lascaris* and *Atherina presbyter*) which contained this amino acid residue. A subset of teleosts possess Fads2 containing a YXXQ domain, another residue sequence allowing Δ4 desaturase activity (Kuah *et al*., 2015; Garrido *et al*., 2019). Δ4 desaturation capabilities permit DHA interconversion through a second alternative pathway (Oboh *et al*., (2017). The "Δ4 pathway" comprises a one-step procedure characterised by such desaturation (Oboh *et al*., 2017; Twining *et al*., 2021). An organism can possess and

synthesise DHA through both the "Δ4" and "Sprecher pathway" (Oboh *et al*., 2017). Although the Δ4 Fads2 of *P. lascaris* and *A. presbyter* investigated did not exhibit any Δ5 activity, many Δ4 Fads2 in fish have expressed such activity (Oboh *et al*., 2017; Garrido *et al*., 2019). Furthermore, Garrido *et al*. (2019) suggested their results were inconclusive, due to a lack of investigation for *Fads2* copy number variants in respective genomes which could have neo-functional or multifunctional Fads2 expressing divergent specificities. Therefore, these species may possess Fads2 with Δ5 activity. Ancient teleost lineages lack Δ4 Fads2 due to a seemingly recent evolution within the phylogeny (Oboh *et al*., 2017).

Teleost Fads2 can also provide Δ8 activity, which enables an alternative pathway to the Δ6 equivalent for the interconversion of ALA to 20:4n-3 through desaturation of 20:3n-3 (Monroig *et al*., 2011; Twining *et al*., 2021). For example, Garrido *et al.* (2019) identified two teleost species (*Sarpa salpa* and *Chelon labrosus*) possessing bifunctional Δ6Δ8 Fads2. These species contained a FXXQ domain at the region determining regioselectivity, suggesting these residues are required for Δ8 activity, although, this sequence is present in previously characterised teleost *Fads2* expressing bifunctional Δ5Δ6 phenotypes, such as in the freshwater carnivore *Channa striata*, reducing its effectiveness as a determinant sequence (Kuah *et al*., 2016; Garrido *et al*., 2019). The amino acid sequence determining bifunctional Δ5Δ6 desaturases is unknown (Garrido *et al*., 2019). Δ8 activity has been lost in many, but not all, species possessing Δ4 Fads2, with selection pressures, or physiological incompatibilities resulting in them being currently undetermined (Garrido *et al*., 2019). Trophic level and phylogeny, due to their effect on Fads2 functional diversification, influence the capacity of teleosts to synthesise n-3 and n-6 LC-PUFAs, although, the later factor was found to be more important (Garrido *et al*., 2019). Species-level

variation exists between fish in the presence and efficiency of Δ8 desaturation activities (Monroig *et al*., 2011). Variation in Fads2 specificities and DHA synthesis capabilities between closely related species (e.g., Ishikawa et al., 2019; Garrido et al., 2019) support hypotheses regarding the independent evolution of Fads2 enzymatic activities, such as Δ5 regioselectivity, across the teleost phylogeny (Castro *et al*., 2012). Fads2 possessing Δ4, Δ5, Δ6 and Δ8 specificities have been identified in a variety of freshwater, marine and diadromous fish (Monroig *et al*., 2011; Xie *et al*., 2021). Numerous studies have investigated or reviewed findings on methyl-end and front-end desaturases of other taxonomic groups (e.g., Monroig and Kabeya, 2018).

Research regarding elongases required for LC-PUFA biosynthesis, and the genes encoding them, is lacking in comparison to investigations into Fads proteins (Monroig *et al*., 2016). Eight Elovl proteins, defined by the required HXXHH motif, have been identified and functionally characterised in teleosts, each of which are involved in the fatty acid network resulting in DHA or n-6 DPA (Ferraz *et al*., 2022). Teleosts are similar to mammals in the functional utilisation of Elovl2 and Elovl5 in PUFA elongation throughout the n-3/n-6 LC-PUFA synthesis pathway, suggesting conservation of these enzymes throughout the vertebrate phylogeny (Ferraz *et al*., 2022). Although Elovl2, functionally characterised in a small number of teleosts (e.g., Xu *et al*., 2020), has an affinity to C_{18} and more so C_{20} substrates, it possesses a high specificity towards C_{22} PUFAs and therefore is important for efficient DHA synthesis through the "Sprecher pathway" (Xu *et al*., 2020; Turchini *et al*., 2021; Ferraz *et al*., 2022). Opposingly, Elovl5, hypothesised as present in all teleosts and showing greater adaptivity compared to mammalian orthologues, has an affinity to C_{18} and C_{20} PUFAs yet a low specificity to C_{22} substrates (Castro

et al., 2016; Xu *et al*., 2020; Turchini *et al*., 2021; Ferraz *et al*., 2022). Gene loss events have resulted in the removal of *Elovl2* in some teleosts (Xu *et al*., 2020). Two isoforms of Elovl4, Elovl4a and Elovl4b, were first isolated and functionally defined in zebrafish, display abilities to elongate numerous FA substrates, particularly very long-chain PUFAs ($>C_{20}$) with carbon chain lengths of up to C_{36} (Castro *et al*., 2016). Elovl4b is known to be involved in DHA biosynthesis (Turchini *et al*., 2021). Functional evolution of Elovl4 in some marine teleosts is hypothesised to have occurred as an adaptive response to losing *Elovl2* and in turn C_{22} elongation capabilities (Castro *et al*., 2016; Monroig and Kabeya, 2018). Moreover, a strongly supported hypothesis suggests teleosts all possess *Elovl4a* and *Elovl4b* genes varying in copy number (Castro *et al*., 2016). Xue *et al*. (2014) also discovered two paralogs, *elovl4c-1* and *elovl4c-2*, present in Atlantic cod, which may also be involved in DHA biosynthesis, although no functional studies have been performed subsequently to confirm this. *Elovl4a* and *Elovl4b* are predominantly expressed in tissues characterised by functional requirements for very long-chain fatty acids, contrary to the more general use of Elovl2 and Elovl5 (Turchini *et al*., 2021).

As well as the whole genome duplication which occurred around the vertebrate radiation, a teleost-specific genome duplication arose within the phylogeny (Castro *et al*., 2016). Such instances affect gene copy number, subsequently resulting in gene loss, sub-functionalisation and neofunctionalization events (Castro *et al*., 2016). These processes influence organismal physiological capabilities by, for example, altering the number and function of genes encoding necessary desaturases and elongases (Castro *et al*., 2016). Therefore, these events may explain repertoire differences and the functional divergence of the enzymatic cascade associated with the

n-3/n-6 LC-PUFA pathways amongst the vertebrates, as teleosts experienced a second genome duplication, unlike other taxonomic groups (Castro *et al*., 2016).

Environmental n-3 LC-PUFA resource heterogeneity and corresponding adaptive strategies

The capacity of organisms to biosynthesise n-3/n-6 LC-PUFAs is likely determined by the environmental availability of such resources, resulting in species diversification through disparities in evolutionary allocation trade-offs between dietary intake and associated physiological capabilities (Twining *et al*., 2021). Behavioural approaches to fulfil nutritional requirements include dietary discrimination, as observed in juvenile rainbow trout (*Oncorhynchus mykiss*), which were able to differentiate between diets composed of varying levels of EPA and DHA (Roy *et al*., 2020). Levels of n-3 LC-PUFAs predominantly impacted feeding behaviour, with the juvenile rainbow trout at all tested ages preferring and wasting less of the high n-3 diets compared to both the low n-3 and medium n-3 feeds they were compared against (Roy *et al*., 2020). For example, in one rich versus medium n-3 diet test, a ~76% preference for the higher quality feed was reported (Roy *et al*., 2020). The fitness related costs of biosynthesis to individuals and species may influence the required n-3 dietary intake (Twining *et al*., 2021).

The n-3 LC-PUFA content of primary producers and in turn, the dietary availability of such molecules, varies substantially between ecosystems (Twining *et al*., 2021). Between aquatic ecosystems, marine primary producers often exhibit higher amounts of EPA and DHA compared to freshwater phytoplankton communities, which exhibit large amounts of ALA yet little to no

EPA and DHA, so much so that DHA deficiencies in freshwater food webs are a determinant obstructing freshwater colonisation by marine fish lineages lacking necessary metabolic adaptations (Ishikawa *et al*., 2019; Twining *et al*., 2021).

The cell membrane of plants, composed in part of a lipid bilayer largely responsible for its conformation, is reliant on LC-PUFAs, with the degree of unsaturation influencing its structure, fluidity and permeability (Jiang and Chen, 1999; Lundbæk *et al*., 2010; Sui *et al*., 2010). For example, investigations into the length and shape of DHA, which is often a component of phospholipids in cell membranes, suggest its short, compact and helical-like structure which varies in conformity influences lipid bilayer fluidity (Huber *et al*., 2002; Valentine *et al*., 2004). Sui *et al*. (2010) observed an increasing degree of unsaturated fatty acids in the lipid bilayer with increasing salinity, suggesting its effectiveness for protecting photosynthetic machinery in saline environments, potentially by increasing membrane fluidity and in turn permitting related membrane protein activity. In turn, it is hypothesised the osmoregulatory requirement for high amounts of EPA and DHA in saline environments explains the observed differences between marine and freshwater ecosystems in aquatic primary producer n-3 compositions (Twining *et al*., 2021). Moreover, strategies adopted by migratory consumers, such as anadromous fish which move from marine to brackish or freshwater environments to spawn, may include exploiting n-3 rich environments and subsequently rationing preserved EPA and DHA stores in n-3 poor environments (Twining *et al*., 2021). For example, adult salmon rely on lipid stores during saltwater to freshwater migrations (Miller *et al*., 2009).

Variation in the n-3 composition of phytoplankton further exists within marine, freshwater and brackish ecosystems, as many environmental and phylogenetic factors can result in spatiotemporal differences between species (discussed in Chapter 2) (Twining *et al*., 2021). Further refined, apparent disparities in the nutritional availability of n-3s between spatially proximate habitats has driven metabolic evolution, as showcased in lake-stream three-spined stickleback (*Gasterosteus aculeatus*) species pairs (Ishikawa *et al*., 2021; Twining *et al*., 2021).

Metabolic adaptations to dietary n-3 LC-PUFA deficiencies

Numerous metabolic strategies are available for species to overcome nutrient (particularly DHA) deficiencies in home or colonised environments, many of which involve changes to the regulation of genes. For example, in the liver of the Nile tilapia (*Oreochromis niloticus*), during periods of low dietary lipid availability compared to medium and high fat diets, there is significantly higher expression of both the sterol regulatory element binding protein 1 (*SREBP1*) gene and *ApoE*, a gene encoding a very-low density lipoprotein, suggesting strategies to increase lipogenesis when nutrient attainability is poor (He *et al*., 2015). SREBP1 is a transcription factor regulating the expression of numerous key lipogenic and fatty acid transport genes, all of which were either upregulated or shared similar expression to other tested diets, which resulted in efficient triacylglycerol synthesis from esterified precursors (He *et al*., 2015). Moreover, upregulation of ApoE lipoprotein is hypothesised to increase transport efficiency of triacylglycerols to other tissues and in turn, maintain lipid homeostasis within the liver (He *et al*., 2015). He *et al*. (2015) also discovered greater expression of fatty acid binding protein 4 gene, another important protein for fatty acid synthesis. Low lipid intake further resulted in higher

expression of some glycolysis related enzymes, which would result in a greater abundance of acetyl-CoA, a coenzyme which can be converted via proteins regulated by SREBP1 to malonyl-CoA, another non-protein molecule directly involved in *de novo* saturated fatty acid synthesis from non-lipid carbon sources (He *et al*., 2015). All of these results suggested higher fatty acid metabolism is associated with low lipid intake in the Nile tilapia (He *et al*., 2015). However, the metabolic strategy varied when dietary lipid attainability was high, with increased free fatty acid uptake into adipocytes with simultaneous increased fatty acid and glycerol synthesis resulting in greater triacylglycerol synthesis (He *et al*., 2015). Additionally, upregulation of lipolysis to maintain lipid homeostasis results in excess triacylglycerols being broken down into free fatty acids (He *et al*., 2015). Free fatty acids act as ligands, with PUFAs activating and upregulating PPARgamma (Hihi *et al*., 2002; He *et al*., 2015). A combination of high dietary intake of free fatty acids, as well as high triacylglycerol hydrolysis to produce these molecules, will cause high expression of PPARgamma, a main function of which is adipogenesis, resulting in greater lipid storage capabilities (Hihi *et al*., 2002; He *et al*., 2015).

Adaptations directly influencing EPA and DHA synthesis capabilities are often associated with regulatory, coding and structural variation in genes encoding elongases and desaturases utilised within this pathway. Selection pressures permitting metabolic adaptive evolution are normally correlated with environmental- and habitat- level disparities in n-3 LC-PUFA availability (Twining *et al*., 2021). For example, the independent evolution (through sub- and neo-functionalisation events) of Fads2 and Elovl5 enzymes utilised in the n-3 LC-PUFA pathway was associated with the successful colonisation of freshwater environments by three Archiridae

species with catadromous or obligate freshwater lifestyles by enhancing DHA synthesis capabilities in nutrient poor environments (Matsushita *et al*., 2020).

Atlantic salmon have greater numbers of respective *Elovl* and *Fads* genes and in turn greater LC-PUFA biosynthetic capabilities compared to other non-salmonid fish due to a recent genome tetraploidisation within the lineage (Leong *et al*., 2010; Carmona-Antoñanzas *et al*., 2013). As stated above, these events contribute to mechanisms culminating in adaptation and species diversification through the non-, neo- and sub-functionalisation of newly duplicated genes and resultant proteins (Leong *et al*., 2010; Carmona-Antoñanzas *et al*., 2013; Twining *et al*., 2021). Salmonids have experienced four genome duplications, whereas teleosts have only endured three (Carmona-Antoñanzas *et al*., 2013). Comparisons against the Northern pike (*Esox lucius*), the closest non-polyploid taxa to salmonids with a divergence time predating the whole genome duplication in the lineage, showed that Atlantic salmon possess homologous, symmetrically evolving and selectively retained *Elovl5* paralogs (Leong *et al*., 2010; Carmona-Antoñanzas *et al*., 2013). Life history and dietary variation exists between the two species, with Northern pike having a predominantly piscivorous diet, which is characterised by high EPA and DHA contents within prey tissues, compared to Atlantic salmon, which are dependent on terrestrial detritus and invertebrates often containing negligible amounts of n-3 LC-PUFAs, particularly DHA (Carmona-Antoñanzas *et al*., 2013). Therefore, the selective retention of the *Elovl5* paralogs is theorised to be an adaptation to improve their n-3 biosynthetic capacity, permitting the colonisation of nutrient poor freshwater environments (Carmona-Antoñanzas *et al*., 2013). Moreover, the Atlantic salmon *Elovl5* paralogs were predominantly expressed in the liver and intestines, the premier organs for fatty acid biosynthesis, in contrast to primary *Elovl5* expression

in the brain of Northern pike, a similar expression profile to other carnivorous fish which rely on dietary LC-PUFA intake, further supporting the hypothesis regarding the metabolic adaptive strategy of Atlantic salmon in freshwater environments (Carmona-Antoñanzas *et al*., 2013). Similarly, some three-spined stickleback populations have adapted to freshwater environments, characterised by low dietary availability of EPA and DHA (Twining *et al*., 2021) by evolving compensatory capacities for increased DHA biosynthesis through increased *Fads2* copy number (Ishikawa *et al*., 2019).

Recent research has argued phylogenetic position, not disparities in dietary n-3 LC-PUFA availability between marine and freshwater ecosystems, is the predominant driver influencing the evolution of differential enzymatic capacities for DHA biosynthesis between teleosts (Xu *et al*., 2020). However, recent studies have also found disparities in DHA biosynthetic capabilities between closely related species. Ishikawa *et al*. (2019) not only demonstrated DHA as a limiting factor for freshwater niche colonisation by marine fishes, but also discovered copy number variation in the *Fads2* gene within and between Pacific three-spined stickleback and closely related Japan Sea stickleback (*G. nipponicus*), which diverged between ~0.68 and 1 million years ago (Ravinet *et al*., 2018). Marine and freshwater ecotypes of the Pacific three-spined stickleback, which have successfully colonised and radiated freshwater environments, had significantly higher *Fads2* copy number compared to the Japan Sea stickleback, which have failed to perform this transition (Ishikawa *et al*., 2019). Functionally, the *Fads2* copy number variation resulted in Pacific three-spined stickleback having lower mortality compared to Japan Sea stickleback when fed DHA-deficient diets during diet trials, regardless of salinity (Ishikawa *et al*., 2019). Moreover, transgenic Japan Sea stickleback upregulating *Fads2* had significantly

greater survival compared to control transgenics with no *Fads2* overexpression when fed DHA-free diets (Ishikawa *et al*., 2019). Therefore, although phylogenetic position may influence general patterns between higher taxa, the largest determinant of species- and individual-level variation in enzymatic capacity and in turn biosynthetic capabilities is habitat.

The majority of studies regarding metabolic evolution in fish have investigated differences in the enzymatic network between marine, migratory and freshwater species. Rarely investigated are the differences in the fatty acid content, metabolome and therefore divergent metabolic adaptive evolution of populations of the same species occupying freshwater environments varying in environmental parameters and by direct association, nutrient availability. This is surprising, given the known disparities in food chains and in turn dietary fatty acid availability between them (Ishikawa *et al*., 2021; Twining *et al*., 2021). A meta-analysis on three-spined stickleback prey items demonstrated disparities between marine, brackish and freshwater environments in DHA content, whilst also showcasing variation within marine and freshwater habitats in the DHA content of benthic and pelagic prey items (Ishikawa *et al*., 2019). Prey items in marine benthic and pelagic habitats have greater DHA content compared to their freshwater counterparts (Ishikawa *et al*., 2019). DHA-poor benthos are the predominant prey items of shallower freshwater habitats including streams, in contrast with limnetic (pelagic) prey items, including DHA-rich copepods, which are more abundant in deep water bodies (Ishikawa *et al*., 2021). Ishikawa *et al*. (2021) showed three-spined stickleback residing in lakes have significantly lower *Fads2* copy number compared to stream conspecifics. This suggests divergence in dietary DHA availability between freshwater habitats can prompt differential selection pressures for the evolution of greater *Fads2* copy numbers and in turn, complete or more efficient DHA synthesis

pathways (Ishikawa *et al*., 2021). Moreover, significant correlations were observed between some foraging traits and *Fads2* copy number, with planktivore-like three-spined stickleback populations possessing lower *Fads2* copy number compared to populations expressing more benthic-like phenotypes, indicating morphological adaptations may directly influence metabolic evolution (Ishikawa *et al*., 2021).

Variation in *Fads2* copy number has also been unveiled between European three-spined stickleback lineages which invaded freshwater at time points interspaced by the last ice age, with the earlier colonising lineage (Lake Geneva population) possessing a greater relative *Fads2* copy number (Ishikawa *et al*., 2019; Hudson *et al*., 2022). These results support findings from Ishikawa *et al*. (2021), as the Lake Geneva population predominantly feed on DHA-poor benthos whereas the primary prey item of Lake Constance populations is DHA-rich zooplankton, suggesting divergent selection pressures for compensatory biosynthetic capacities (Hudson *et al*., 2022). In turn, Hudson *et al*. (2022) discovered significant differences in the concentrations of $C_{18\text{-}22}$ n-x LC-PUFAs, including DHA, between wild Geneva and Constance lake and stream three-spined stickleback populations. This included variation between Geneva and Constance lake populations as well as between recently diverged lake and stream ecotypes (Hudson *et al*., 2022). Disparities between lake and stream populations in EPA and DHA are hypothesised to be determined by variation in prey item quality (Ishikawa *et al*., 2021; Hudson *et al*., 2022). Surprisingly, in all bar the Constance lake population, wild males had higher concentrations of EPA and DHA compared to females, even though *Fads2* is ancestrally an X-linked gene and no X-inactivation mechanisms are present in fish, meaning females should express *Fads2* more than male conspecifics (Hudson *et al*., 2022). Overall, LC-PUFA profiles of wild fish were

predominantly determined by ecotype with n-3 concentrations being further influenced by sex and lineage (Hudson *et al*., 2022). During rearing trials, when fed n-3-poor diets, Geneva lake and stream stickleback had considerably greater DHA and less EPA and ALA compared to Lake Constance conspecifics, with lineage significantly influencing concentrations of almost all tested fatty acids (Hudson *et al*., 2022). These results are consistent with findings from Ishikawa *et al*. (2019) by showcasing populations with higher relative *Fads2* copy number comprise greater abundances of DHA under nutritional restrictions, suggesting more efficient or complete metabolic pathways compared to Constance lake and stream populations. Geneva populations possessed greater DHA concentrations compared to both Constance populations when reared under common garden conditions (Hudson *et al*., 2022). Moreover, wild Geneva lake and stream females possessed greater concentrations of DHA than Constance stream equivalents (Hudson *et al*., 2022). Disparities in DHA concentrations during n-3-poor diet trials were predominantly influenced by lineage (Hudson *et al*., 2022). Results from Hudson *et al*. (2022) show quantitative differences in the concentrations of n-3/n-6 LC-PUFAs, particularly DHA, arise between freshwater populations varying in *Fads2* copy number, as previously presented by Ishikawa *et al*. (2019). Therefore, knowledge of the n-3/n-6 LC-PUFA profiles of both prey items and divergent fish populations is beneficial for generating hypotheses regarding the potential presence of associated metabolic adaptations in three-spined stickleback. Large disparities were displayed between lab-reared and wild-caught fish. For example, a more than ten-fold increase in DHA concentrations was found in Constance lake wild-caught males compared to those reared in the lab (Hudson *et al*., 2022). As Constance lake fish predominantly feed on zooplankton, and results indicate effective foraging capabilities, it can be hypothesised selection for planktivory and corresponding morphological adaptations may relax selection pressures for increased

productivity of DHA biosynthesis, in line with previous hypotheses regarding selection for benthivory (Ishikawa *et al*., 2021; Hudson *et al*., 2022).

Although studies have investigated genomic variation and resultant fatty acid phenotypes of divergent freshwater three-spined stickleback populations, few have presented ecological-based reports investigating how dietary disparities in lakes varying in environmental parameters influence fish fatty acid contents. Deep water bodies often have greater abundances of DHA relative to other freshwater environments (Ishikawa *et al*., 2021). However, among such habitats, environmental conditions, such as total phosphorus concentrations, can reduce the quantity and quality of n-3-rich phytoplankton (Taipale *et al*., 2019). These reductions directly influence the EPA and DHA contents of fish prey items and therefore their dietary availability (Taipale *et al*., 2019), a selection pressure which has resulted in adaptive metabolic evolution of three-spined stickleback (e.g., Ishikawa *et al*., 2019).

Study overview

In this study, the fatty acid, specifically n-3 LC-PUFA profiles of three-spined stickleback populations occupying fresh- and saltwater lochs varying in environmental parameters, particularly pH and nutritional conditions, were investigated to identify the presence of divergent fatty acid phenotypes among and between ecotypes. Moreover, fatty acid analyses were performed on plankton samples collected from an acidic and alkaline freshwater loch to test hypotheses regarding dietary disparities between lochs and draw conclusions regarding differences in corresponding three-spined stickleback populations. Finally, a summary-analysis

was performed to understand how the EPA and DHA contents of algae vary between aquatic habitats and taxa. Understanding the variation in concentrations of EPA and DHA between three-spined stickleback populations, as well as the differences in the dietary availability of n-3/n-6 LC-PUFAs between aquatic habitats and among freshwater lochs, will permit the generation of evidence-based hypotheses regarding potential divergent metabolic adaptive evolution between these populations.

Chapter 2: Fatty acid variation in algae

Introduction

Zooplankton are a critical and usually high-quality prey item in marine and freshwater (particularly lake) environments, often containing large amounts of essential n-3/n-6 LC-PUFAs, including EPA and DHA, compared to aquatic benthic macroinvertebrates (Burns *et al*., 2011; Ishikawa *et al*., 2021; Twining *et al*., 2021; Hudson *et al*., 2022). Essential LC-PUFAs are required for peak performance in aquatic consumers, of which DHA is the most important in copepods and numerous fish species (Taipale *et al*., 2019). However, the fatty acid composition of freshwater zooplankton is determined by taxonomy (phylogenetic position) as well as diet (the fatty acid composition of phytoplankton) (Ravet *et al*., 2010; Burns *et al*., 2011). Moreover, zooplankton fitness and population dynamics are influenced by the dietary intake of both fatty acids and phosphorus (Gulati and Demott, 1997; Taipale *et al*., 2019; Taipale *et al*., 2020). Other environmental factors impact the digestibility and assimilation of zooplankton foodstuffs (Gulati and Demott, 1997). The complex shape and large size of some phytoplankton taxa, additionally to the morphological and chemical defences deployed by a subset of primary producers, on top of consumer physiological constraints, affect herbivorous zooplankton by limiting the quantity of phytoplankton consumed (Gulati and Demott, 1997; Taipale *et al*., 2019; Taipale *et al*., 2020). The n-3 LC-PUFA content of algae and in turn the dietary availability of these molecules is affected by phylogeny as well as major chemical and physical attributes of environments, a subset of which are reviewed below. Moreover, I use a simple summary analysis of EPA and

DHA content of algae species in an attempt to understand the variation in dietary n-3 LC-PUFA availability within and between taxa and aquatic habitats predominantly varying in salinity.

Salinity

Evidence suggests unsaturated fatty acids enhance resistance of plants to salt stress through effects on lipid bilayer characteristics. In turn, salinity has been suggested a significant determinant explaining the higher amounts of EPA and DHA observed in marine algae communities compared to freshwater alternatives (Sui *et al*., 2010; Ishikawa *et al*., 2019; Twining *et al*., 2021). However, between saltwater environments, salinity levels influence n-3 LC-PUFA composition of algae. For example, *Dunaliella sp*. sampled from a hypersaline lake exhibited decreases in n-3 PUFA composition with increasing salinities (Xu and Beardall, 1997). The fatty acid composition of *Dunaliella sp*. was ~57% n-3 PUFA when growth medium comprised low molar NaCl concentrations compared to ~48% when cultures had a high molar NaCl concentration (Xu and Beardall, 1997). Moreover, *Pinguiococcus pyrenoidosus*, a marine unicellular alga, exhibited increased growth rates with increasing culture salinity (Sang *et al*., 2012). However, growth rate suppression occurred at the highest salinities (Sang *et al*., 2012). Percentage EPA and DHA in samples also drastically decreased at higher salt concentrations (Sang *et al*., 2012). Analysing three strains of *Crypthecodinium cohnii*, Jiang and Chen (1999) showed growth rates as well as DHA contents were highest at either 5 or 9 g L^{-1} NaCl. DHA compositions and growths rates decreased as salinity was further increased to 23 and 35 g L^{-1} NaCl respectively (Jiang and Chen, 1999). These studies suggest there are optimal salinities for algae growth, lipid compositions as well as n-3 LC-PUFA production and contents, with

increases and decreases in salinity from such optima decreasing rates or compositions. Such trends may be due to desaturation of enzymes involved in n-3 LC-PUFA biosynthesis. Sui *et al.* (2018) showed fatty acid desaturase activity and in turn n-3 PUFA content decreased in *Arachis hypogaea* L. under salt stress but subsequently increased in standard conditions. Correlations between salinity and n-3/n-6 LC-PUFA content vary between phytoplankton groups commonly found in saltwater environments, suggesting a phylogenetic influence (Galloway and Winder, 2015).

pH

Similarly to salinity, although pH can influence algal cellular growth rates and impact PUFA production, optimum pHs are species specific (Gutierrez, 2009; Sang *et al*., 2012). For example, the lowest cell densities of *P. pyrenoidosus* occurred when cultured at acidic pHs (pH 5 and 6) (Sang *et al*., 2012). Growth rates increased with culture pH, with higher cell densities at the most alkaline pHs tested (pH 7.7, 8 and 9) (Sang *et al*., 2012). The highest cell density occurred at pH 8. Opposingly, the highest EPA and DHA production occurred when *P. pyrenoidosus* was grown at pH 6, although, the lowest contents were observed at pH 5 (Sang *et al*., 2012). Gutierrez (2009) showed the highest dry weight (growth rate) of a mixed algae sample occurred at pH 8, with little growth at pH 5 and 9. Moreover, although *Ankistrodesmus* had optimum growth at pH 6, the majority of algae grew best at more alkaline pHs (Gutierrez, 2009). The highest triacylglycerol and neutral lipid contents occurred at pH 7, 8 and 9, with low content being observed at acidic pHs (Gutierrez, 2009).

Ocean acidification, defined by the decrease in ocean pH resulting from anthropogenic CO_2 emission, has resulted in decreasing PUFA contents in zooplankton (Rossoll *et al*., 2012). For example, feeding *Acartia tonsa*, a copepod, with low pCO_2 treated phytoplankton and zooplankton resulted in high PUFA content (Rossoll *et al*., 2012). However, when *A. tonsa* was fed a diet consisting of high pCO_2 treated zooplankton and either high or low pCO_2 treated phytoplankton, PUFA composition was ~30% lower (Rossoll *et al*., 2012), suggesting decreasing dietary availability of PUFAs with decreasing pH in marine environments. Opposingly, although a decrease from pH 8.1 to 7.7 (acidified conditions) resulted in a percentage decrease in total fatty acids of the algae *Ulva compressa*, a percentage increase in PUFAs was observed (Vinuganesh *et al*., 2022). Similarly to salinity, trends in pH may be due to desaturation of enzymes associated with fatty acid biosynthesis. Overall, variations in pH among freshwater and marine environments can affect the EPA and DHA contents of plankton.

Temperature

Algae are primary producers, therefore, the growth rate and in turn amounts of n-3 LC-PUFAs are dependent on the rate of photosynthesis (Singh and Singh, 2015). Temperature, as well as pH, light intensity and season, are hypothesised as critical factors influencing the productivity of photosynthesis and in turn, growth (Singh and Singh, 2015). Evidence suggests the highest cell density of *P. pyrenoidosus* occurred when grown at the highest tested temperature (28^{o}C) (Sang *et al*., 2012). However, EPA and DHA compositions significantly decreased with increasing temperature, with highest contents occurring at the lowest tested temperature (20^{o}C) (Sang *et al*., 2012). Moreover, Renaud *et al*. (1995) found three species of marine algae, one of which was

sampled from two locations, all exhibited slow or no growth, as well as low PUFA contents at high temperatures. *Nitzschia closterium* and *N. paleacea* produced higher amounts of EPA and the Tahitian *Isochrysis sp.* strain produced higher amounts of DHA at low temperatures (Renaud *et al*., 1995). Negative correlations between phytoplankton PUFA content and temperature have been found in other studies (Hartwich *et al*., 2013). Hypothesis regarding algal n-3 LC-PUFA variation with temperature suggest a correlation exists between increasing temperatures and increasing enzymatic reaction rates and therefore, greater EPA and DHA degradation (Twining *et* al., 2021). Moreover, Twining *et al*. (2021) suggest homeoviscous adaptations to retain bilayer fluidity at low temperatures results in increased amounts of membrane lipids possessing double bond-containing fatty acids, such as EPA and DHA. Overall, closely related algae species, as well as individuals of the same species, can vary in PUFA content when occupying environments differing in temperature.

Temperature further affects algal growth and n-3 LC-PUFA composition through its influence on the effects of light intensity (Markou *et al*., 2012). Low temperatures increase the threshold light intensity level at which photoinhibition occurs, defined by damage to photosynthetic machinery resulting from too much light (Markou *et al*., 2012). As algal growth rates increase with light intensity, low temperatures may result in greater growth rates difficult to achieve in high temperature environments due to the algae's ability to withstand higher light intensities (Markou *et al*., 2012). However, increasing light intensities seemingly reduce n-3 LC-PUFA compositions in some algae (e.g., Sang *et al*., 2012).

Light intensity

Disparities in light intensity between aquatic environments influence EPA and DHA production in algae (Twining *et al*., 2021). For example, DHA production in *P. pyrenoidosus* was highest when cultures were at the lowest light intensity tested (50 µmol photons $m^{-2}s^{-1}$) and decreased thereafter with increasing light intensity, becoming non-discoverable at 150 and 200 µmol photons $m^{-2}s^{-1}$ (Sang *et al*., 2012). EPA production was highest at 100 µmol photons $m^{-2}s^{-1}$ and decreased with increased light intensity (Sang *et al*., 2012). Negative correlations between PUFA content and light intensity are apparent in additional studies (Hartwich *et al*., 2013; Twining *et al*., 2021). Growth rates did not significantly differ with light intensity (Sang *et al*., 2012). PUFA contents further differ between light spectra, with the highest EPA production in *Tisochrysis lutea* batch cultures occurring under white and green light in the exponential and stationary growth phase respectively (del Pilar Sánchez-Saavedra *et al*., 2016). Similarly, DHA production was highest under red and green light depending on the growth phase of cultures (del Pilar Sánchez-Saavedra *et al*., 2016).

Season

Seasonality is suggested to be another critical factor affecting primary producer growth and lipid composition, for reasoning including changes in day length altering environmental parameters including light intensity and temperature (Singh and Singh, 2015). Ravet *et al*. (2010) showed differences in n-3 PUFA content in phytoplankton, from 8% in summer to 30% in spring. These changes influenced freshwater zooplankton fatty acid composition, suggesting seasonality has strong effects on consumer n-3 PUFA contents (Ravet *et al*., 2010). Hartwich *et al*. (2013) found

seasonal variation of DHA in the largest analysed size fraction, with late summer and autumn periods having the highest accumulation of this molecule. Such seasonal patterns were hypothesised to result from an increased proportion of anticipatory DHA storing copepods in the fraction (Hartwich *et al*., 2013). In contrast, Lau *et al*. (2012) argued seasonality has little influence on zooplankton (and benthic macroinvertebrate) fatty acid compositions, which are instead strongly influenced by taxonomy. Different zooplankton taxa have different strategies for PUFA accumulation (Hartwich *et al*., 2013), therefore, seasonality may affect the dietary availability of n-3 LC-PUFAs in freshwater environments differently depending on zooplankton community compositions between such.

Phylogeny

Although environmental factors likely account for variation between lower taxonomic ranks, including between strains of the same species, phylogeny is hypothesised to account for fatty acid variation at the level of phyla and class (Lang *et al*., 2011). Results from Cañavate (2018) supported previous conclusions, suggesting phylogeny exerted the strongest influence on algae fatty acid variation, with habitat only accounting for a small amount. In freshwater environments, only some phytoplankton taxa are hypothesised to possess capacities for EPA and DHA biosynthesis (Taipale *et al*., 2019). Therefore, between freshwater environments, variance in algae community composition itself can result in differential dietetic n-3 LC-PUFA availability for higher trophic levels, including fish.

Phosphorus

As phylogeny is hypothesised to explain n-3 LC-PUFA variation between algal groups, factors affecting the community composition of algae may be important factors influencing the dietary availability of ALA, EPA and DHA within and between freshwater, brackish and marine ecosystems. Phosphorus enrichment has resulted in anthropogenic eutrophication, characterised by increased phytoplankton and cyanobacterial growth (Taipale *et al*., 2019). Taipale *et al.* (2019) showed high phosphorus concentrations in lakes may enhance the growth of n-3 LC-PUFA-deficient cyanobacteria. Ultra-oligotrophic, lower-eutrophic and eutrophic lakes (based on total phosphorus concentrations) had the lowest sestonic concentrations of EPA and DHA compared to oligotrophic and mesotrophic alternatives (Taipale *et al*., 2019), although, other unmeasured factors could contribute to or explain the observed variation. Therefore, differential phosphorus input or concentrations may result in divergent dietary n-3 LC-PUFA availability between marine, brackish and freshwater environments.

Growth phase

Other than abiotic and phylogenetic factors, variation in algal PUFA compositions can also result from changes to their growth phase (Liu *et al*., 2016). Boelen *et al.* (2017) showed four marine microalgae species had increased EPA contents during stationary compared to exponential growth phase, although no change in levels of DHA occurred. Fidalgo *et al.* (1998) found EPA and DHA production in *I. galbana* was highest during either early or late stationary compared to exponential growth phase when cultured under every tested nitrogen source. Moreover, an overall significant increase in total fatty acids from exponential to stationary growth phase

occurred in an investigation of 19 brackish and marine algae (Schwenk *et al*., 2013). These studies suggest PUFA variation within and between species can result from differences in current growth phase, as algae normally have greater EPA and DHA contents during stationary growth.

Hypotheses

A summary analysis was performed to understand the variation in percentage EPA and DHA contents of algae between aquatic habitats and taxa. Based on existing literature, it was hypothesised the percentage EPA and DHA contents of algae were significantly influenced by habitat. Saltwater (marine and brackish) algae were hypothesised to contain greater mean EPA and DHA contents compared to freshwater alternatives. Moreover, it was hypothesised these n-3 LC-PUFA contents differed between phyla. Results will provide support for or reject previous hypotheses regarding algal fatty acid variation between environments and taxonomic groups.

Methods

Search string creation

Data regarding the percentage EPA and DHA contents of freshwater, brackish and marine algae were obtained from papers extracted from Web of Science. Three search strings were used during literature searches, varying in the keywords necessary or required absent in the topic field search (which scans the title, abstract, author keywords and Keywords Plus of papers). The search strings were: '(Phytoplankton OR *algae) AND ("fatty acid composition" OR "fatty acid profile*") AND marine NOT zooplankton NOT freshwater NOT larvae NOT fish', '(Phytoplankton OR *algae) AND ("fatty acid composition" OR "fatty acid profile*") AND freshwater NOT zooplankton NOT marine NOT larvae NOT fish' and '(Phytoplankton OR *algae) AND ("fatty acid composition" OR "fatty acid profile*") AND freshwater AND marine NOT zooplankton NOT larvae NOT fish'. Search strings were composed to return papers investigating the fatty acid profiles of algae originating from specific habitats. The final search string was used to find papers investigating both marine and freshwater algae which were excluded from previous searches. Zooplankton, fish and larvae were excluded in each search string to avoid investigations regarding the aquaculture of such organisms.

Data collection

Data regarding the percentage EPA and DHA content of algae, as well as their habitat of origin (freshwater, brackish, marine), strain (if applicable), cell count at harvest (if available), amount

of EPA and DHA (if available), number of replicates, growth stage and measure (percentage fatty acid, percentage fatty acid methyl ester (FAME) or percentage weight) were extracted from returned papers (see references). The references and citations of papers returned in the original scans were also searched to find data. Studies investigating the effect of environmental variables, such as light intensity, on the fatty acid contents of algae were excluded from the meta-analysis. Where amounts of fatty acid were provided, percentage EPA and DHA content were calculated. Many studies analysed the same algae species isolated from different environments or cultured in different conditions. Therefore, multiple data points exist for certain species as means could not be calculated from values obtained in separate studies. Numerous papers failed to clearly define the type of habitat that samples originated from. In these situations, two methods were used. If geographic locations were noted, google earth and internet searches were performed to identify habitat type. Secondly, if strain number was provided, samples were cross-referenced with corresponding algae databases to identify habitat isolation source. This approach was predominantly used for data from Lang *et al*. (2011) and Mitani *et al*. (2017), which profiled algae strains held in the SAG and NIES culture collections respectively. As Lang *et al*. (2011) and Mitani *et al*. (2017) are not recently published, some algal strains originally analysed have been reclassified as different species. Databases provided details of previous names if strains were reclassified. Therefore, during the cross-referencing process of these two papers, out-of-date species names were updated using information from corresponding database pages, to permit more accurate analyses regarding n-3 LC-PUFA variation between phyla. In each case, a name change was noted in the master spreadsheet. Data points with unidentifiable habitats of origin were excluded from all analyses. EPA and DHA are described as non-discoverable (n.d.), occurring in trace amounts (tr.) or represented by a hyphen (-) to signify their undetectability in

numerous studies. In each of these cases, a percentage content of zero was noted. Once all data had been extracted, values were checked by a further review of used papers. Many papers measured EPA and DHA as percentage of FAMEs. A difference of ~14 exists between the molecular weight of fatty acids and their associated FAMEs. Therefore, percentage FAME values for EPA and DHA will be overinflated by 4.4% and 4.1% respectively compared to percentage fatty acid values for these molecules, resulting in inaccurate results. Moreover, studies quantifying EPA and DHA as percentage weight did not define the measure as either percentage weight FAME or fatty acid. Therefore, only papers which either measured or could be used to calculate EPA and DHA as a percentage of total fatty acids were included in the analysis.

Taxonomic data collection

Once data had been extracted from papers and organised, AlgaeBase (<https://www.algaebase.org>) and the Integrated Taxonomic Information System (ITIS) (<https://www.itis.gov>) were used to obtain taxonomic information regarding each species. ITIS was used to determine cyanobacteria genera and AlgaeBase was used to identify the phylum, class, order and family of each analysed species. Eleven observations had unidentifiable, out-of-date or no genera in original papers. Although they could be defined as cyanobacteria or non-cyanobacteria, other taxonomic information could not be collected. Therefore, these observations were excluded from phyla analyses.

Statistical Analysis

A summary of data collected is provided (**Table 1**). In total of 1844 observations were collected in this meta-analysis. After excluding observations in which EPA and DHA were measured by percentage FAME or percentage weight, 1751 observations measuring by percentage of total fatty acids remained for statistical analysis. 1695 and 1748 of these observations possessed percentage DHA and EPA values respectively. Using R ver. 4.1.2, the variance in percentage EPA and DHA of algae between original habitats and taxonomic groups was tested through Tweedie GLMs with gamma response distributions, which permit the incorporation of zero-value data. Wilcoxon tests were used to test for significant differences between cyanobacteria and non-cyanobacteria taxa of specific habitats in relative amounts of EPA and DHA. No analyses on other reviewed environmental variables were performed.

Results

Habitat

The EPA and DHA contents of algae were significantly influenced by habitat (**Table S1-S2, Fig. 2-3**). In terms of non-cyanobacteria, the median EPA content was lowest in freshwater environments and highest in brackish environments, although median contents were similar between saltwater habitats (**Fig. 2**). The median DHA contents of non-cyanobacteria followed similar trends, however, was highest in marine environments (**Fig. 3**). Although freshwater non-cyanobacteria have a low average EPA and DHA content, some species are noted as being highly composed of these fatty acids (**Fig. 2-3**). Almost all cyanobacteria possessed negligible EPA and DHA contents regardless of habitat (**Fig. 2-3**).

Cyanobacteria

The EPA and DHA contents of algae were also influenced by taxa (cyanobacteria versus non-cyanobacteria) (**Table S1-S2, Fig. 2-3**). Within each habitat, EPA contents significantly varied between corresponding cyanobacteria and non-cyanobacteria. DHA also varied significantly between taxa sampled from freshwater and marine environments (**Table S3, Fig. 2-3**).

Table 1. A summary of data collected and used for the summary analysis.

Number of Studies	*Number of EPA Measurements*	*Number of DHA Measurements*	*Number of Observations Classified by Phyla*	*Number of Observations Classified by Class*
35	1748	1695	1740	1740

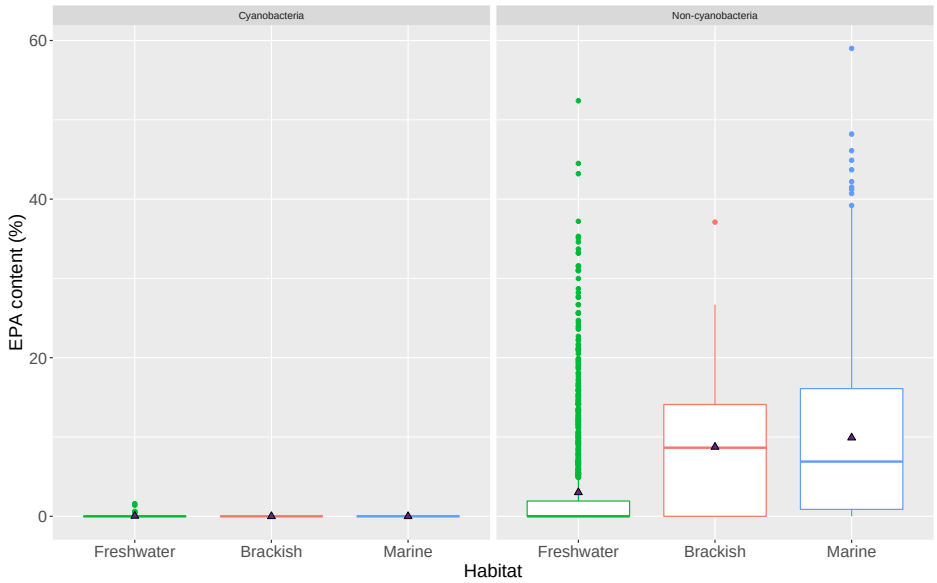

Figure 2. The EPA contents on algae isolated from freshwater (n=1205), brackish (n=57) and marine environments (n=486), separated by taxa (cyanobacteria and non-cyanobacteria). Triangular points represent the mean EPA contents of each group.

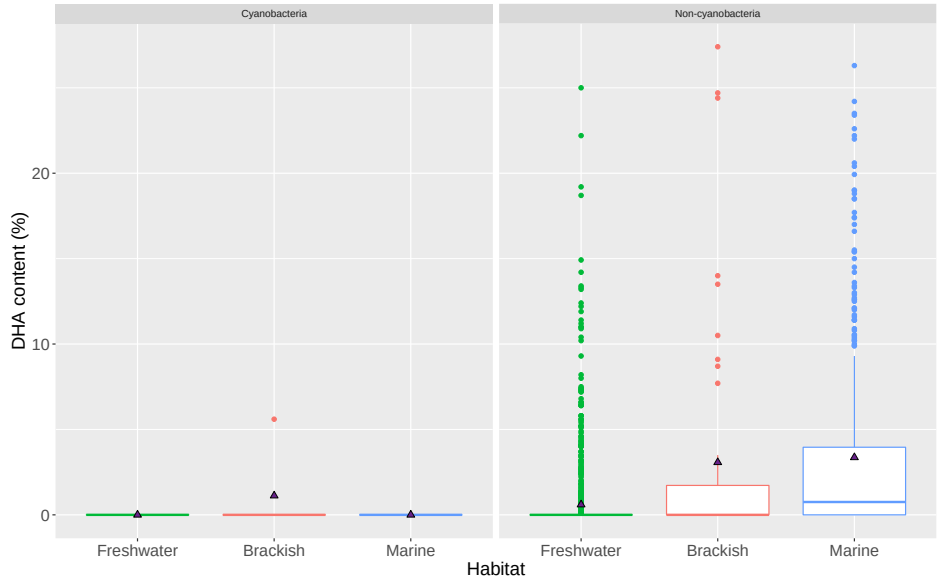

Figure 3. The DHA contents on algae isolated from freshwater (n=1199), brackish (n=57) and marine environments (n=439), separated by taxa (cyanobacteria vs non-cyanobacteria). Triangular points represent the mean DHA contents of each group.

Phylum

The EPA and DHA contents of algae were significantly influenced by phylum (**Table S4-S5, Fig. 4-5**). Variation in EPA and DHA contents exists between algae of different phyla isolated from the same habitats as well as between species of the same phyla sampled from different environments (**Fig. 4-5**). Rhodophyta have the highest median EPA contents in marine and brackish habitats (**Fig. 4**). Moreover, Rhodophyta and Glaucophyta have the highest mean EPA contents (**Fig. 4**). Charophyta, Chlorophyta, Cyanobacteria and Tracheophyta have minimal mean and median EPA contents (**Fig. 4**). In a majority of phyla, average DHA contents are less than corresponding mean EPA contents (**Fig. 4-5**). Average DHA content is highest in Miozoa (~15% of total fatty acids) (**Fig. 5**). Moreover, Miozoa sampled from freshwater, brackish and marine habitats all contain the highest median DHA contents compared to other phyla sampled from such environments (**Fig. 5**). Within phyla, large variation in EPA and DHA contents exists between classes, although no statistical tests were performed to test the significance of such variation (**Fig. S1-S2**). For example, within Haptophyta, the median EPA content of Coccolithophyceae and Pavlovophyceae differs by ~20% (**Fig. S1**). Moreover, median DHA contents of Dinophyceae and Oxyrrhidophyceae, classes of Miozoa, differed by ~10% (**Fig. S2**).

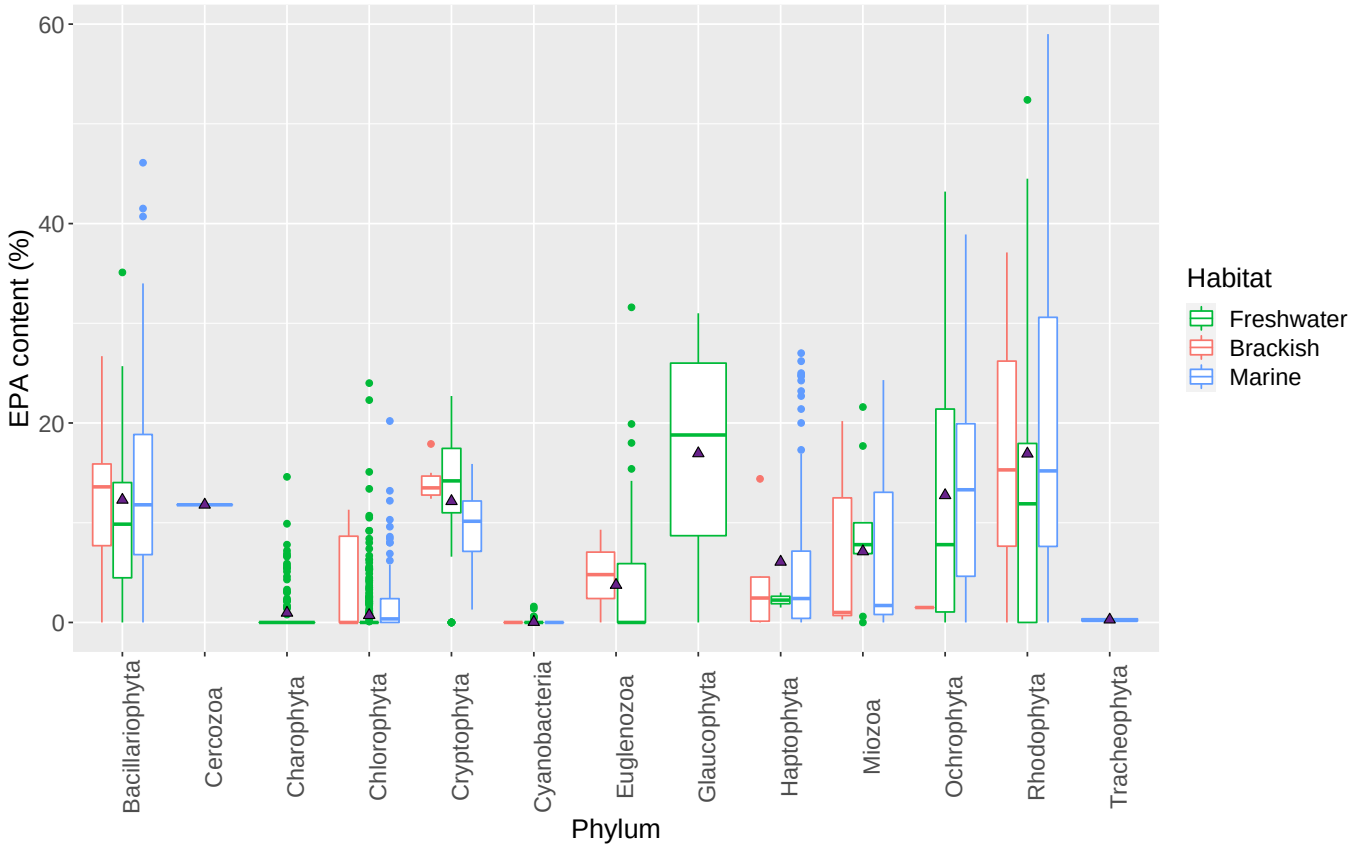

Figure 4. The EPA contents of species in 13 major algae phyla, separated by habitat of origin, being either freshwater (n=1202), brackish (n=57) or marine (n=478) in nature. Triangular points represent the mean EPA contents of each phylum.

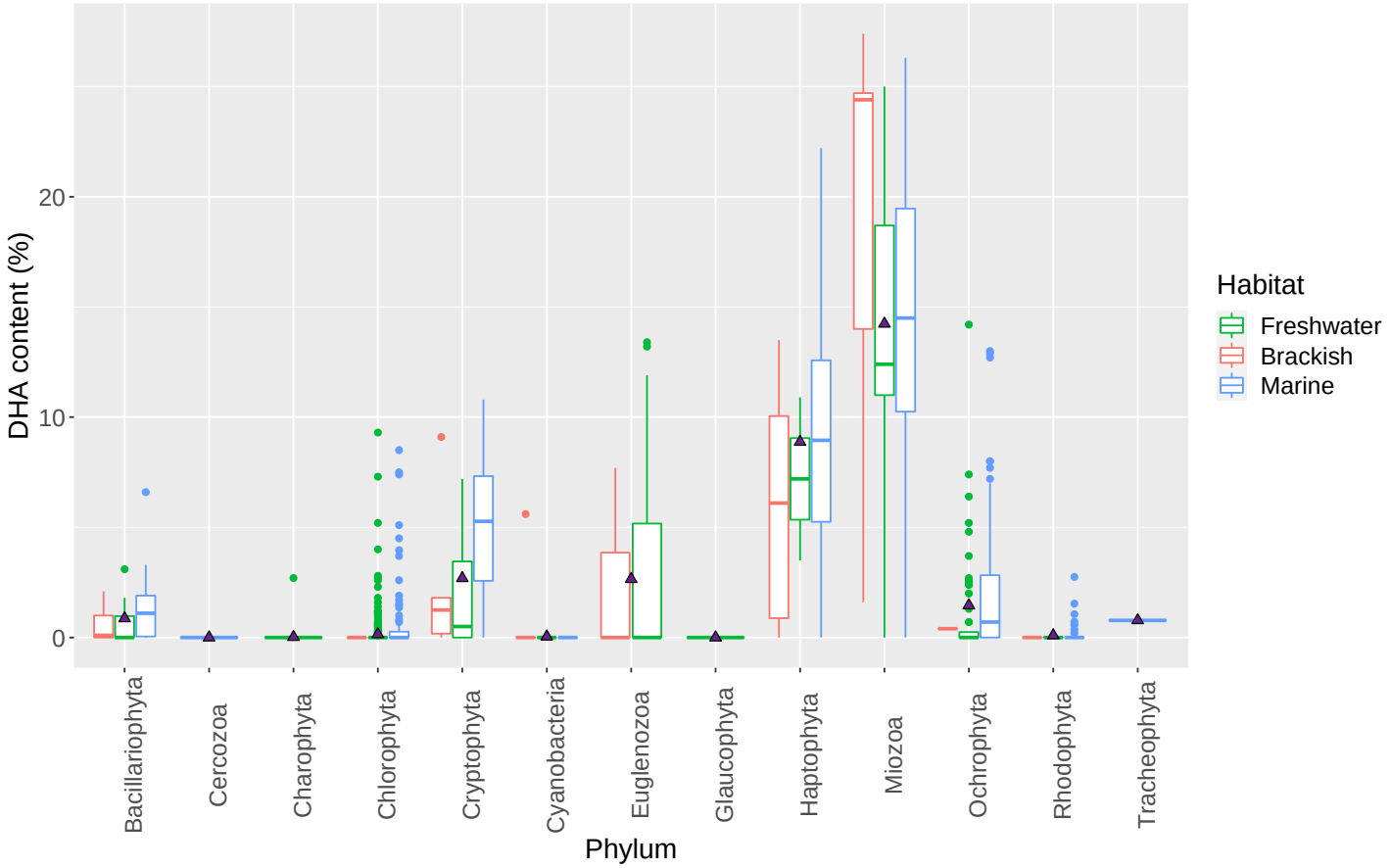

Figure 5. The DHA contents of species in 13 major algae phyla, separated by habitat of origin, being either freshwater (n=1196), brackish (n=57) or marine (n=431) in nature. Triangular points represent the mean DHA contents of each phylum.

Discussion

Habitat

A summary analysis was performed to clearly show how EPA and DHA contents of algae differ between habitats and taxa. Overall, results show an effect of habitat on n-3 LC-PUFA levels (**Table S1-S2, Fig. 2-3**), a conclusion supported by Cañavate (2018), who found impacts of habitat on algal fatty acid profile variation. Saltwater ecosystems contained more EPA and DHA compared to freshwater environments, a result found in previous meta-analyses (e.g., Ishikawa *et al*., 2019). An increase in salinity from 1mM to 300mM increased the n-3 PUFA content of all tested membrane lipids in *Suaeda salsa* L. (Sui *et al*., 2010). As double bonds maintain membrane fluidity (Los and Murata, 2004), the increased concentration of unsaturated fatty acids was hypothesised to aid in the protection of photosystem II under high salinities by maintaining membrane fluidity to permit the activation of membrane proteins involved in photosynthesis (Sui *et al*., 2010). Moreover, a threshold level of NaCl is required by marine algae to maintain a necessary degree of infiltration (Sang *et al*., 2012). Therefore, increased EPA and DHA in saltwater environments is likely partially associated with salinity tolerance. This may also explain EPA and DHA content variation between brackish and marine environments, the latter of which is characterised by a higher salinity and therefore may require more n-3 PUFAs for membrane fluidity maintenance. The brackish algae dataset was relatively small compared to marine and freshwater alternatives, which is surprising given the importance of these environments for many organisms, including migratory fish crucial in aquaculture. Expanding this dataset will enhance our knowledge of dietary limitations in these environments and will

provide a more precise answer for the size of variation between saltwater habitats. Highly saline environments increase EPA and DHA synthesis difficulties.

Disparities in the EPA and DHA contents of primary producers affect higher trophic levels (Ishikawa *et al*., 2019; Taipale *et al*., 2019). Our results support previous conclusions that freshwater environments have the lowest dietary availability of EPA and DHA. Therefore, in terms of three-spined stickleback on North Uist, freshwater populations will likely have divergent dietary selection pressures compared to anadromous populations, which predominantly inhabit marine and brackish environments with algal communities rich in EPA and DHA. Freshwater lochs on North Uist also vary in pH. Gutierrez (2009) and Sang *et al*. (2012) showed growth rates in a majority of investigated algae were highest at alkaline compared to acidic pHs. Therefore, the dietetic availability of n-3 LC-PUFAs may also vary between freshwater lochs, prompting further divergent selection pressures and potential differential metabolic evolution between corresponding populations. To answer such questions, plankton samples from lochs on North Uist were profiled to understand EPA and DHA variation in food chains between freshwater environments (see chapter 3).

Cyanobacteria

Significant variation was found between cyanobacteria and non-cyanobacteria of freshwater and marine environments in corresponding EPA and DHA contents (**Table S1-S3, Fig. 2-3**). Non-cyanobacteria consistently possess more EPA and DHA in all environments compared to cyanobacteria. Moreover, results suggest the majority of cyanobacteria do not possess capacities

for *de novo* n-3 LC-PUFA synthesis. Therefore, the dietary availability of EPA and DHA will differ between aquatic environments depending on the cyanobacterial composition of communities. This conclusion suggests phosphorus concentrations may influence dietary EPA and DHA availability between aquatic habitats, as high phosphorus levels can result in increased cyanobacterial blooms and in turn decreased n-3 LC-PUFA synthesis and content at the base of food chains (Taipale *et al*., 2019). However, anthropogenic eutrophication also increases the abundance of other algae taxa with n-3 LC-PUFA synthesis capabilities (Taipale *et al*., 2019). Therefore, increases in phosphorus may not affect the relative availability of ALA, EPA and DHA drastically. Moreover, other factors may also influence the growth and abundance of cyanobacteria. Wynne and Stumpf (2015) observed cyanobacterial blooms in Summer and Autumn in Western Lake Erie between 2002-2014, suggesting cyanobacteria may grow better in different seasons.

Taxonomy

EPA and DHA contents of algae were also significantly influenced by phyla (**Table S4-S5**). Cañavate (2018) suggested phylogeny, particularly the phylum and class, exerted the greatest influence on algal fatty acid profile variation, with habitat (salinity) having negligible effects. Our taxonomic-based data tentatively supports this conclusion, as a greater variation in EPA and DHA content seemingly exists between phyla than within phyla between habitats (**Fig. 4-5**). This suggests differences in EPA and DHA availability between habitats is driven more by the distribution of taxa than by a direct response to salinity. Further statistical analyses should be performed to confirm this observation. Moreover Lang *et al*. (2011) concluded fatty acids could

be used as chemotaxonomic markers for identifying algae at the levels of phyla and class due to their patterns of distribution at these ranks, further suggesting phylogeny is the predominant driver of fatty acid variation at higher taxonomic levels. Large variation in the EPA and DHA content of algae between classes of the same phylum (**Fig. S1-S2**) may suggest class has a larger effect on n-3 LC-PUFA variation compared to phylum, although a statistical analysis must be performed to confirm this observation. Many other studies suggest *de novo* n-3 LC-PUFA synthesis is limited to certain algal taxa (Mühlroth *et al*., 2013; Taipale *et al*., 2019; Twining *et al*., 2021). Results suggest all analysed phyla, including cyanobacteria, contain species with the capacity to *de novo* synthesise EPA and DHA, contrary to previous statements.

Conclusion

Overall, this summary analysis suggests an influence of both habitat and phylogeny on EPA and DHA contents of algae. The n-3 LC-PUFA contents of algae are lowest in freshwater environments. As the fatty acid composition of zooplankton is partially dependent on the fatty acid composition of their algae foodstuffs (Ravet *et al*., 2010), freshwater zooplankton likely have lower n-3 LC-PUFA contents compared to zooplankton in more saline environments. Therefore, dietary selection pressures for improved DHA biosynthesis are likely apparent in freshwater fish populations, as observed in three-spined stickleback (Ishikawa *et al*., 2019). Further analyses will need to be performed to understand whether variation between environments is driven by responses to salinity or by differential taxonomic compositions of local species pools.

Chapter 3: Fatty acid variation in plankton and three-spined stickleback (*Gasterosteus aculeatus*)

Introduction

DHA deficiency affects the survival of fishes in freshwater environments (Ishikawa *et al*., 2019). Divergent nutritional selection pressures have resulted in differential metabolic adaptive evolution among and between marine and freshwater three-spined stickleback populations (Ishikawa *et* al., 2019; Ishikawa *et al*., 2021; Hudson *et al*., 2022). Numerous studies have highlighted the importance of understanding the dietary availability of n-3 LC-PUFAs in studied environmental systems, as well as the feeding behaviours of ecotypes, to provide evidence of potential differential nutritional selection pressures driving divergent evolution of correlated physiological traits (Ishikawa *et al*., 2019; Ishikawa *et al*., 2021). Moreover, Hudson *et al*. (2022) showed fatty acid phenotypes of three-spined stickleback can be used to predict divergent evolution between populations. The fatty acid profiles of freshwater, resident and anadromous three-spined stickleback ecotypes inhabiting five lochs on North Uist were investigated to understand the underlying variation between them. Plankton samples from two freshwater lochs were collected to examine disparities in the strength of dietary selection pressures between freshwater stickleback populations.

Study Species

The three-spined stickleback are a phenotypically diverse species complex occupying environments varying in salinity across the northern hemisphere (Bell and Foster, 1994). Stickleback successfully colonised and radiated into freshwater environments after Pleistocene glacial retreat (Bell and Foster, 1994; Ishikawa *et al*., 2019). The ecology (Hudson *et al*., 2022) and associated genomics of fatty acid traits in three-spined stickleback have been well characterised in recent years, particularly through landmark studies investigating population-level variation in *Fads2* copy number (Ishikawa *et al*., 2019; Ishikawa *et al*., 2021), making this an ideal species to investigate divergent fatty acid phenotypes between environmentally variable freshwater environments.

Three ecotypes of the three-spined stickleback co-occur on North Uist, all of which were investigated. Anadromous fish migrate into coastal brackish lagoons and multiple freshwater lochs (such as Trosavat) during the breeding season, but do not reside in such habitats throughout the year (MacColl *et al*., 2013). In contrast, resident saltwater ecotypes inhabit and co-occur in coastal saltwater lagoons at all times (MacColl *et al*., 2013). Anadromous and resident populations are reproductively isolated from one another (Dean *et al*., 2019). Finally, freshwater populations reside in lochs across the island (MacColl *et al*., 2013). Dean *et al*. (2019) has shown North Uist stickleback populations have evolved from an admixture of multiple ancestral lineages. Considerable body size variation is exhibited between ecotypes (MacColl *et al*., 2013). For example, the mean length of freshwater fish from Scad was 31.3 mm compared to anadromous fish from Duin, which had an average length of 68.8 mm (MacColl *et al*., 2013). Similarly to freshwater populations, resident fish from Duin had a mean length of 34.6 mm (MacColl *et al*., 2013). Such disparities may be due to dietary n-3 LC-PUFA scarcity in loch,

compared to marine environments. Fatty acid biosynthesis is energetically costly in mammals (DiRusso and Black, 2004), therefore, if similar costs are apparent in fish, the limited nutritional availability may restrict growth rates due to a greater allocation of resources to perform such cellular procedures. This variation makes these three-spined stickleback populations ideal to test for an association between fatty acid resource heterogeneity and morphological phenotypic divergence.

Experimental system

The island of North Uist (57.57ºN, 7.28ºW) (**Fig. 6**), located in the Scottish Western Isles, is composed of lochs varying in levels of interconnectivity (MacColl *et al*., 2013; Dean *et al*., 2021). The Outer Hebridean archipelago itself was covered in ice during the last glacial maximum c. 25 ka and supported an independent ice cap throughout Pleistocene cold stages, although this merged with the mainland ice sheet (Ballantyne and Hallam, 2001; MacColl *et al*., 2013; Hall *et al*., 2021). Deglaciation of surrounding islands (South Uist and Harris) at similar ice sheet altitudes to North Uist (predicted through three-dimensional reconstructions of ice cover) occurred between 13.2 – 16.4 ka (Ballantyne and Hallam, 2001; Stone and Ballantyne, 2006; Ballantyne, 2010). Glacial retreat from North Uist likely occurred within this time period, resulting in rising sea levels (MacColl *et al*., 2013). MacColl *et al*. (2013) theorised these events subsequently permitted the colonisation of novel freshwater environments by anadromous three-spined stickleback. The generalised hypothesis of postglacial freshwater invasion by marine and euryhaline fishes in northern hemisphere environments is supported throughout the literature (e.g., Campbell and Williamson, 1979; McPhail, 1993; McPhail, 1994; Bell and Foster, 1994).

Only five other freshwater species are indigenous to the Outer Hebrides, a subset of which occur within North Uist lochs (Campbell and Williamson, 1979). MacColl *et al.* (2013) estimated the evolutionary period for stickleback residing in North Uist freshwater lochs was less than 16000 years.

North Uist lochs lie over differing substrates, yielding variation in nutrient availability and water chemistry across the island (MacColl *et al*., 2013). Waterston *et al.* (1979) classified such aquatic habitats into three categories on the basis of water quality and vegetation. Firstly, machair lochs, defined by calcareous sands which influence water alkalinity, lie on the Western side of the isle (Waterston *et al*., 1979; MacColl *et al*., 2013). The nutritional quality of such machair lochs is determined by distance and relative position of these water bodies from the coast, ranging from oligotrophic (nutrient deficient) to eutrophic (nutrient rich) conditions (MacColl *et al*., 2013). Secondly, oligotrophic lochs consisting of peaty sediment and characterised by acidic pH waters occur across the eastern side of the island (Waterston *et al*., 1979; MacColl *et al*., 2013). Large disparities in surface geology result in a latitudinal axis of variation in water chemistry between freshwater lochs from east (acidic pH and alkaline metal scarcity) to west (alkaline pH and high alkaline metal concentrations) (MacColl *et al*., 2013). This variation has resulted in differential productivity between lochs, a factor hypothesised to drive divergent evolution between populations (MacColl *et al*., 2013). Overall, freshwater habitats on North Uist are poor (Waterston *et al*., 1979). Finally, brackish lochs differing in the amount of marine (saltwater) input form a second axis of variation between fresh- and saltwater water bodies (Waterston *et al*., 1979; MacColl *et al*., 2013). The significant variance in environmental parameters among and between freshwater and saltwater lochs makes North Uist a unique and ideal system to study

both the natural variation in the dietary availability of PUFAs as well as the resultant FA variation in corresponding three-spined stickleback populations. Five lochs were selected for this study to investigate how the fatty acid profiles of stickleback vary across both axes of variation (**Table 2-3**). Moreover, anadromous fish from Duin raised in two different environments were analysed to compare fatty acid profiles. One anadromous population was reared in saltwater aquaria fed diets containing n-3 LC-PUFAs. The second population was introduced to a freshwater pond in Nottinghamshire as juveniles in 2018 (**Table 3**). Host is an alkaline loch located on the western side of North Uist connected to the sea by a stream containing two separate populations at either end. Bottom of Host and top of Host stream stickleback were captured to compare fatty acid profiles between Host populations (**Table 3**).

Table 2. Information regarding the sample sites utilised in this study, including their pH, salinity and the three-spined stickleback populations present.

Loch	*Code*	*pH (measured 2022)*	*Salinity*	*Ecotypes Present*	*Coordinates*
Scadavay	Scad	6.22	Freshwater	Freshwater	57°58”N; 7°24”W
a’Bharpa	Bhar	6.25	Freshwater	Freshwater	57°57”N; 7°30”W
Trosavat	Tros	7.04	Freshwater	Anadromous and Freshwater	57°56”N; 7°32”W
an Dùin	Duin	8.17	Saltwater	Anadromous and Resident	57°64”N; 7°21”W
Hosta	Host	8.2	Freshwater	Freshwater	57°63”N; 7°49”W

Table 3. The three-spined stickleback populations analysed in this study, with corresponding sampling locations and sample sizes.

Population	*Sampling location*	*Sample size (n)*
Scad	Loch Scadavay, 57.585495, -7.237532	6
Bhar	Loch a'Bharpa, 57.570861, -7.301476	6
Tros (anadromous)	Loch Trosavat, 57.584080, -7.413829	3
Tros (freshwater)	Loch Trosavat, 57.584080, -7.413829	6
Duin (anadromous)	Loch an Dùin, 57.642391, -7.208284	6
Duin (anadromous)	University of Nottingham aquarium	7
Duin (anadromous)	Freshwater pond, 53.073000, -1.258185	5
Duin (resident)	Loch an Dùin, 57.642391, -7.208284	6
Host (loch)	Loch Hosta, 57.627467, -7.490194	6
Host (top of stream)	Hosta stream, 57.625787, -7.491650	9
Host (bottom of stream)	Hosta stream, 57.626955, -7.498578	9

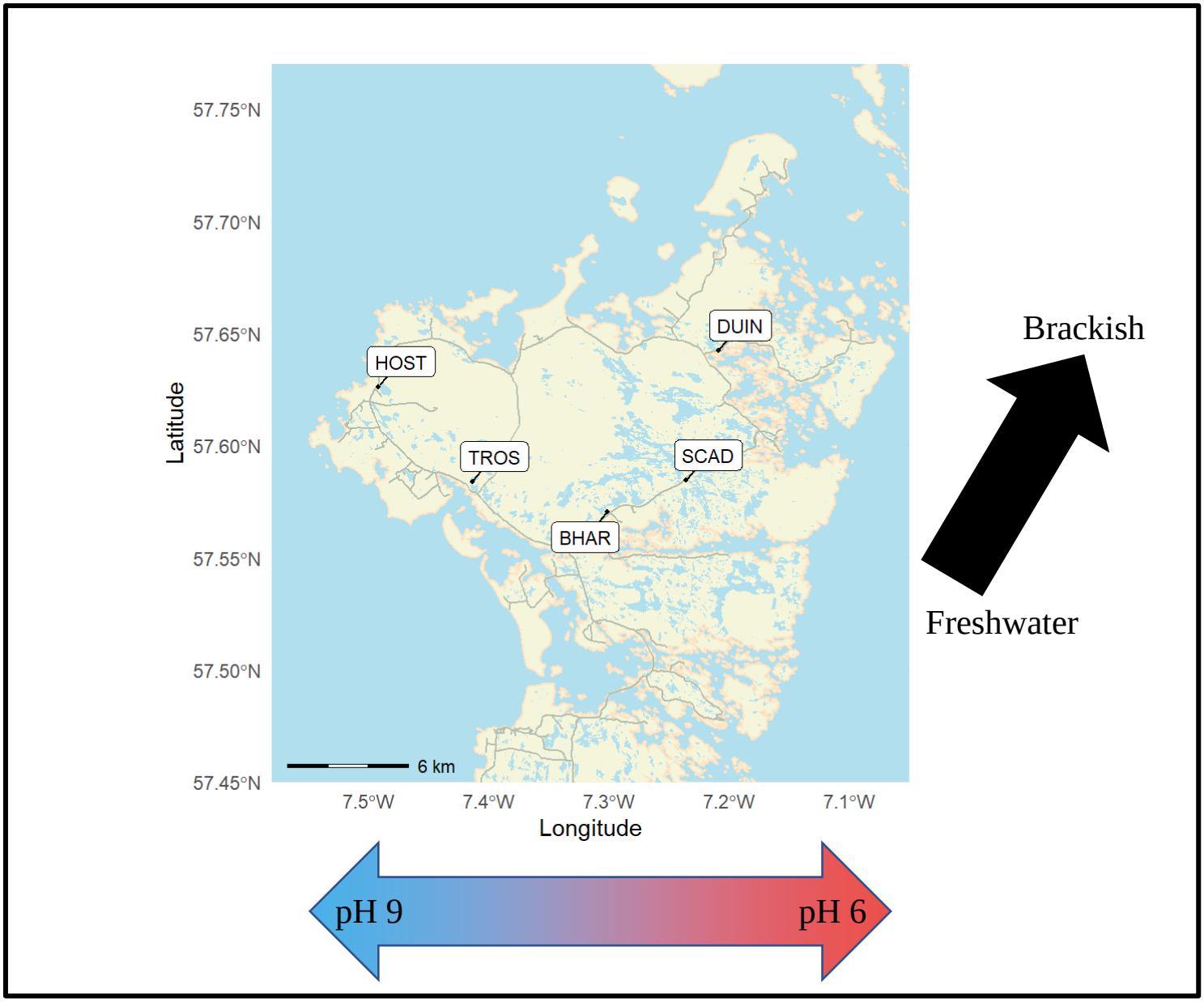

Figure 6. The isle of North Uist. From east to west, freshwater lochs transition across an axis of variation from pH 6 to pH 9. Lochs also transition from freshwater to brackish lochs with increasing marine input as distance from the coast decreases, forming a second axis of variation in salinity. Stickleback and plankton were sampled from labelled lochs.

Hypotheses

Hypotheses regarding the fatty acid compositions of Host and Bhar plankton are based on the environmental parameters of each loch. Unlike Bhar, which has peaty sediment and is characterised by oligotrophic conditions, Host is a machair loch close in proximity to the ocean, defined by eutrophic conditions (MacColl *et al*., 2013). Therefore, it is hypothesised the plankton in Host have a higher concentration of total fatty acids, as well as higher concentrations of individual essential dietary fatty acids (LIN and ALA) and key n-3/n-6 LC-PUFAs, particularly EPA and DHA, compared to Bhar plankton. In addition to the nutritional conditions of lochs, hypotheses regarding the fatty acid phenotypes of freshwater stickleback populations are further based on mean lengths of fish measured by MacColl *et al*. (2013). For example, the Host freshwater three-spined stickleback population had a mean length of 40.6 mm compared to Bhar conspecifics, which had a mean length of 30.8 mm, variation which may be explained by nutritional disparities resulting in divergent resource allocation. Where no adaptive divergent evolution has occurred between freshwater populations, greater concentrations of n-3/n-6 LC-PUFAs, notably EPA and DHA, are expected in lochs characterised by eutrophic conditions and higher quality plankton. Therefore, the Host (alkaline) freshwater three-spined stickleback population is, in this scenario, hypothesised to express fatty acid phenotypes comprising greater concentrations of EPA and DHA compared to conspecifics inhabiting acidic lochs defined by oligotrophic conditions (Scad and Bhar). Similar or greater amounts of EPA and DHA in these populations would provide evidence for divergent physiological adaptive evolution between freshwater populations.

Coastal saltwater lagoons are shallow (Mathieu-Resuge *et al*., 2019). In turn, resident populations likely rely on DHA-poor benthos as prey items (Ishikawa *et al*., 2021). Considering this, it is hypothesised the resident three-spined stickleback population inhabiting Duin, a brackish lagoon, will exhibit low concentrations of EPA, DHA and total fatty acids, in parallel with freshwater populations. High amounts of fatty acids will provide evidence of either evolutionary enhanced physiological capabilities for DHA biosynthesis or retention, or an unexpected richness of prey. As anadromous fish were sampled during breeding seasons, it is hypothesised such populations contain higher concentrations of total and key individual fatty acids compared to both freshwater and resident ecotypes, which would suggest strategies to store fatty acids when residing in n-3-rich marine environments, as observed in other anadromous species (Miller *et al*., 2009; Twining *et al*., 2021). To further support this hypothesis, Duin anadromous populations sampled from North Uist were reared in an aquarium and freshwater pond environment to understand the effect of environment on fatty acid profile variation in anadromous stickleback. The aquarium population were fed diets containing n-3 LC-PUFAs. Stream environments are also defined by shallow depths and in turn, poor dietary DHA availability (Ishikawa *et al*., 2021). Three-spined stickleback populations inhabiting the top and bottom of Host stream were further sampled to identify variation in fatty acid profiles between Host loch, top of Host stream and bottom of Host stream populations. As the top of Host stream will receive input from the eutrophic lake, it was hypothesised the bottom of Host stream population would have lower concentrations of fatty acids, particularly EPA and DHA, compared to both Host loch and top of Host stream populations. Deviations from these results might suggest the bottom of Host stream receives marine input.

Methods

Stickleback sampling

In May 2021 and 2022, populations of three-spined stickleback occupying five lochs and a stream (Host) on North Uist were sampled using metal mesh minnow traps retrieved the day after initial placement. Bhar stickleback were the only population captured in 2022. Following capture, fish were euthanised using an overdose of tricaine methane sulphonate (MS222). Destruction of the brain confirmed death. Samples were washed, wrapped in clingfilm and stored in a local freezer. After transport to the laboratory in Nottingham, fish were stored at -80^{o}C for up to 12 months until commencing fatty acid analyses.

Lab-reared and Pond A fish

During fieldwork, mature eggs were extracted from Duin anadromous females and fertilised by mixing with testes from Duin anadromous males. Artificially fertilised clutches were transported to saltwater aquaria at the University of Nottingham. Once hatched, juveniles were reared on *Artemia*, a diet progressively replaced with bloodworms of varying sizes (chopped or whole) depending on age and consumption capabilities. Packets of bloodworms contain 45 mg/kg of n-3 LC-PUFAs (Peregrine Livefoods). Families were separated into tanks, with ~30 individuals per tank maximum. Aquarium room temperature ranged between 13-18^{o}C. This range of temperatures may result from changes in outdoor temperature. Water tests were consistently performed to ensure healthy tanks were maintained. Moreover, juvenile Duin fish were

introduced to a freshwater pond, that previously contained no stickleback, in north Nottinghamshire in autumn 2018 and allowed to live naturally. Adult fish were collected from the pond in autumn 2021 and euthanised and stored for fatty acid analyses using the same procedure as for wild-caught fish. A total of 69 individuals were analysed during this study (**Table 8**).

Plankton sampling

In May 2022, plankton samples were collected from two freshwater lochs varying in environmental parameters to understand how dietary selection pressures vary across spatially proximate freshwater environments. Host, an alkaline eutrophic loch, and Bhar, an acidic oligotrophic loch, were selected for plankton sampling as they represent the opposing extremes of environmental conditions on North Uist. Therefore, they are the best candidates to unveil the presence and subsequent size of variation in the nutritional quality of plankton and, in turn, the dietary availability of n-3 LC-PUFAs between lochs. The accessibility of necessary equipment (rowing boats, oars and rowlocks), as well as the weather, further influenced the loch selection process. A plankton net with a connected collection bottle was attached to the back of a rowing boat. The boat was rowed for 30 minutes in Host and 60 minutes in Bhar. Rowing was periodically stopped to concentrate the plankton and change collection bottles. The presence of zooplankton, (mainly copepods and *Daphnia*), was confirmed by examination of samples under a dissecting microscope. Plankton samples were stored at -80^{o}C for fatty acid analyses, following extraction of excess water.

Sample preparation

Preceding pulverisation, the standardised lengths and weights of fish were measured. The sex of each individual was also determined. As wild-caught fish were captured during the breeding season, sex was initially identified through external characteristics such as gravidness in females and bright colouration in males. However, sex was confirmed through a minimal dissection of individuals to identify reproductive organs. Subsequently, samples were individually pulverised under liquid nitrogen. Plankton samples from each loch were also separately pulverised. Between samples, the mortar and pestle used for pulverisation was washed to avoid cross-contamination.

After pulverisation, fish and plankton samples were separated into subsamples weighing ~300 mg to avoid procedural difficulties generated by larger sample weights. Due to cost, one subsample was analysed per fish. However, all subsamples from either one or two individuals per population (excluding Bhar) were analysed to confirm the validity of the procedure, as the same or similar amounts of fatty acids between subsamples of the same individual suggests no technical- or human-based error occurred during procedures, including ensuring effective homogenisation of particles during pulverisation. Host and Bhar plankton were subset into 12 and 8 subsamples respectively. All samples were stored at -20^{o}C for subsequent lipid extractions and reweighed once removed from the freezer to retrieve an accurate sample weight measure.

Lipid extraction

Initially, samples were homogenised in 2 ml water and transferred to a glass test tube, with a further 1 ml of water being used to collect remaining remnants. Lipids were extracted in 2:1 chloroform/methanol, a method first developed by Folch *et al.* (1957). Briefly, 3 ml of methanol

was added to the sample solution which was vortexed for 30 seconds and allowed to stand for five minutes. Subsequently, 5 ml of chloroform and 1 ml chloroform containing ~0.01 g C19:0 fatty acid as a recovery standard were added, followed by equal vortex and standing stages. After centrifugation, the bottom organic phase containing lipids, including fatty acids, was extracted and added to a pre-weighed solvent resistant tube. The remaining solution was re-extracted in 1.5 ml methanol and 3 ml chloroform, with the resultant bottom layer being added to the original extract. Samples were evaporated to dryness under nitrogen, reweighed to calculate percentage fat, suspended in hexane and stored at -20^{o}C for methylation.

Fatty acid methylation

200 µl of each lipid sample was added to 700 µl 10M KOH and 5.3 ml methanol, vortexed for 30 seconds and added to a 55^{o}C water bath for 90 minutes, with periodic mixing every 20 minutes for the first hour. Samples were subsequently added to an ice bath for 10 minutes and 580 µl 12M H_2SO_4 was added to halt reactions. A salt precipitate formed. Samples were vortexed and the 90-minute water bath and 10-minute ice bath procedures were repeated. 3 ml of hexane was added thereafter and, after centrifugation, the top layer of each sample solution (~3ml in volume) containing fatty acid methyl esters (FAMEs) was transferred to solvent resistant tubes, 1 ml of which was used for gas-chromatography mass-spectrometry (GCMS) analysis.

GCMS analysis

A FAME selected ion monitoring (SIM) scan method was used. A calibration curve was generated with the laboratory standard (FAME37 mix) at varying concentrations (1:80 – 1:2). The retention times of molecules in the standard were utilised to identify compounds in samples.

In each instance of GCMS usage, the sample injection sequence was random to avoid potential human or technical errors influencing results. All plankton samples were ordered randomly and profiled in a single run. Every ten samples, a standard was run to avoid retention time shifts, and a hexane blank was subsequently run to wash the GC column. The GCMS provides results as amount of FAME. Molecular weight differences between fatty acids and their associated FAMEs can influence results regarding the amount of each compound present in samples. Therefore, results were converted from amounts of FAMEs to amounts of fatty acids by dividing and multiplying by respective molecular weights. Moreover, the final 1 ml solution analysed on the GCMS did not contain the total amount of fatty acids present in the original sample as only 0.2ml of the 3ml lipid sample was used during fatty acid methylation and 1ml of the final 3ml FAME solution was used for fatty acid analyses. Therefore, the total concentration of each fatty acid per sample was calculated and results were subsequently converted to nanograms per milligram (ng/mg) stickleback or plankton. The concentration of total fatty acids in each sample was calculated by adding the concentrations of all identified fatty acids together, excluding C19:0, as this was artificially added during lipid extractions. For analyses of the total amount of DHA in individual fish, corresponding concentrations were multiplied by the milligram weight of each fish and values were subsequently converted to micrograms.

Statistical analyses

Using R ver. 4.1.2, the difference in concentrations of individual n-3/n-6 LC-PUFAs and total fatty acids was tested between plankton and freshwater stickleback from Host and Bhar via Wilcoxon and two-sample t-tests. Moreover, the influence of loch (habitat), ecotype and sex on the concentrations of DHA, EPA and total fatty acids was determined through gamma and

gaussian distributed generalised linear models (GLMs). As a relatively low number of male fish were sampled from the majority of analysed populations compared to females, any inferences regarding sex differences in fatty acid phenotypes will be loose and will require further investigation. The influence of weight on the total amount of DHA was also determined through GLMs.

Results

Replicates

Replicates of either one or two individuals from each analysed population (excluding Bhar due to insufficient weights) were profiled to confirm the strength of the methodology and the validity of all subsequent results. In total, replicates of 10 individuals were profiled. In the majority of individuals, replicates had similar concentrations of DHA, EPA and total fatty acids, with the largest variation between replicates in individual fatty acids being approximately 0.2 ng/mg (**Fig. 7-8**). The largest variation in total fatty acids was between Scad replicates, which differed by approximately 2.5 ng/mg (**Fig. 9**). The similarities in fatty acid concentrations between replicates suggest results from subsequent analyses are reliable, the methodology used was sound and samples were adequately homogenised.

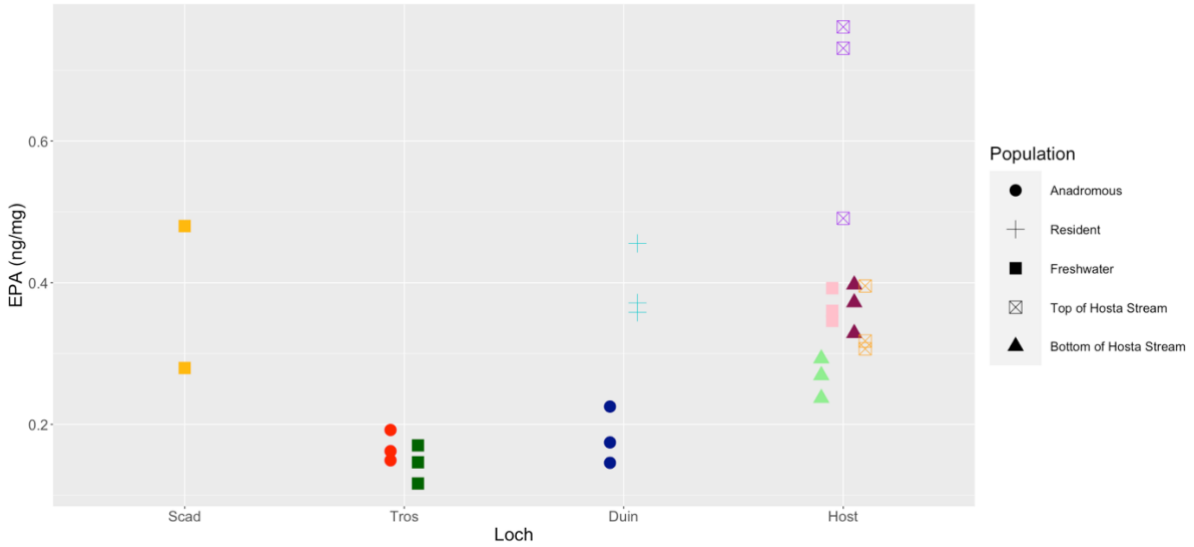

Figure 7. The EPA concentrations of replicates. Each colour represents a different individual (10 total), with between 2-3 replicates per fish. Individuals are categorised by loch and population (point shapes).

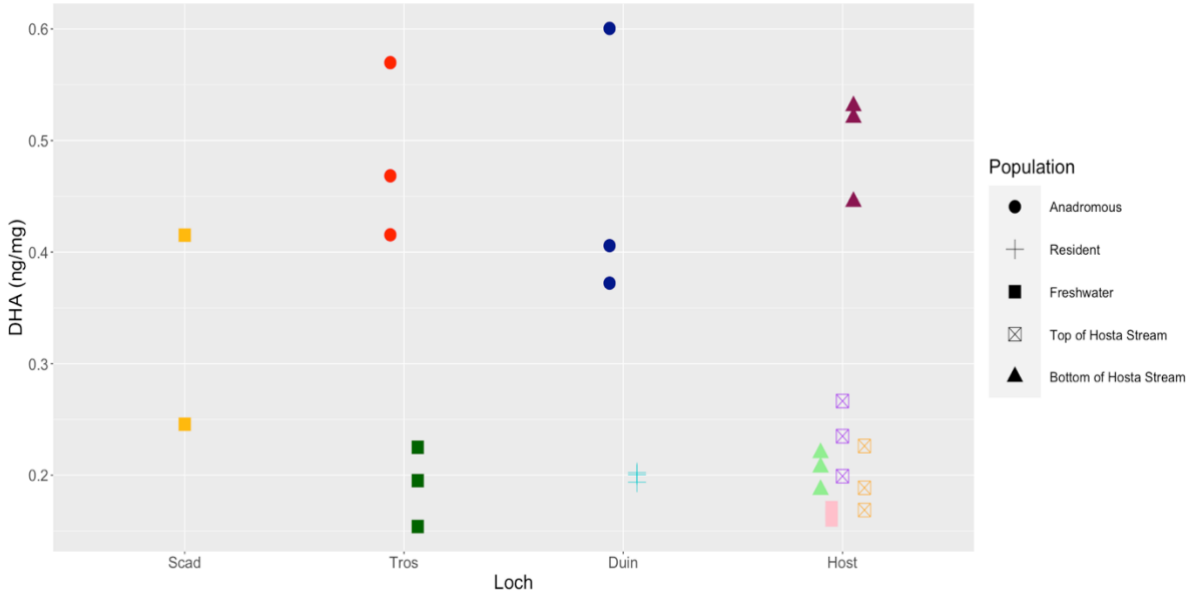

Figure 8. The DHA concentrations of replicates. Each colour represents a different individual (10 total), with between 2-3 replicates per fish. Individuals are categorised by loch and population (point shapes).

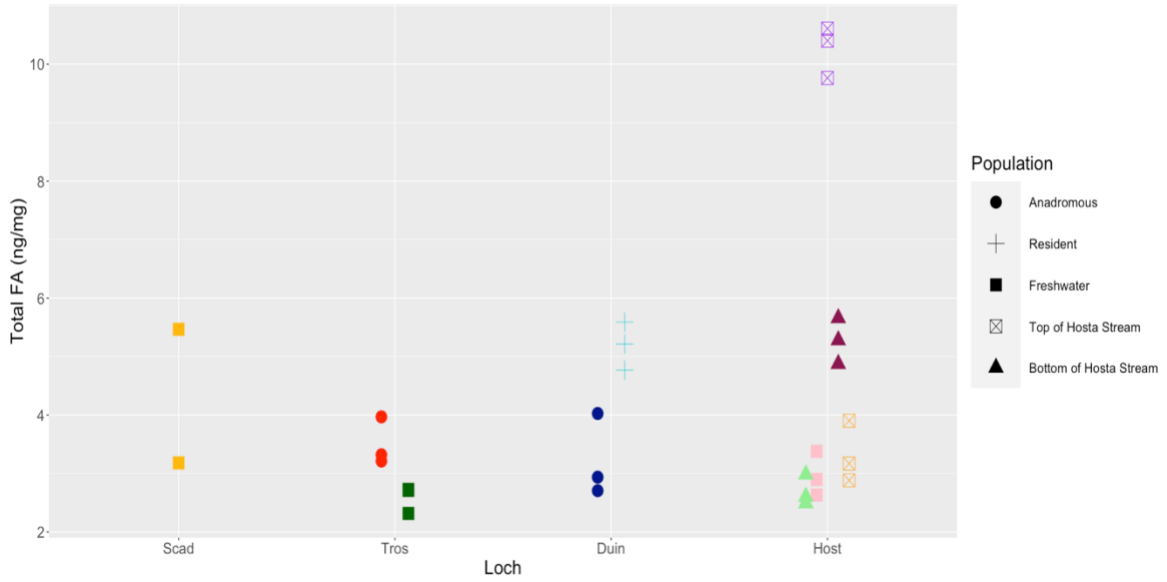

Figure 9. The total fatty acid concentrations of replicates. Each colour represents a different individual (10 total), with between 2-3 replicates per fish. Individuals are categorised by loch and population (point shapes).

Host and Bhar plankton and three-spined stickleback

The concentrations of total fatty acids and all individually analysed n-3/n-6 PUFAs (LIN, ALA, ARA, EPA, DHA) significantly varied between Host and Bhar plankton (**Fig. 10-12**). In all cases, Host plankton had higher concentrations of analysed fatty acids compared to Bhar plankton. Moreover, Host plankton had a median total fatty acid concentration three times greater than Bhar plankton. In terms of corresponding stickleback, the concentrations of total fatty acids, as well as EPA and DHA, significantly varied between freshwater populations. However, unlike respective plankton, Bhar stickleback had greater concentrations of EPA, DHA and total fatty acids compared to Host conspecifics (**Fig. 13**). Moreover, a significant difference was discovered in the relationship between total DHA and individual fish weight between lochs, with the majority of Bhar stickleback, which weigh less than Host conspecifics, containing higher amounts of DHA (**Table S6**, **Fig. 14**).

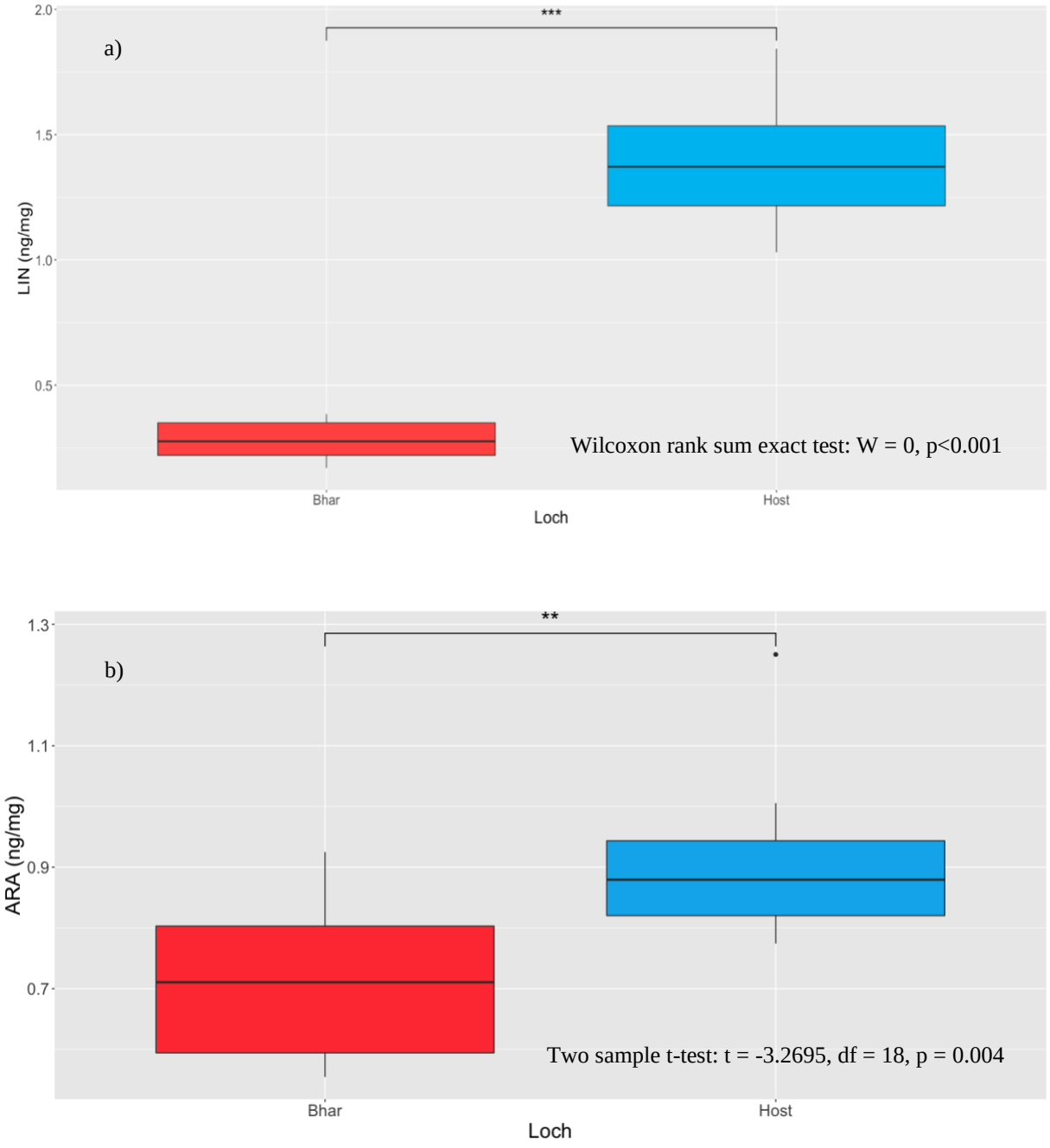

Figure 10. Significant variation in the concentrations of a) LIN and b) ARA between plankton of an acidic (Bhar, n=8) and alkaline (Host, n=12) freshwater loch.

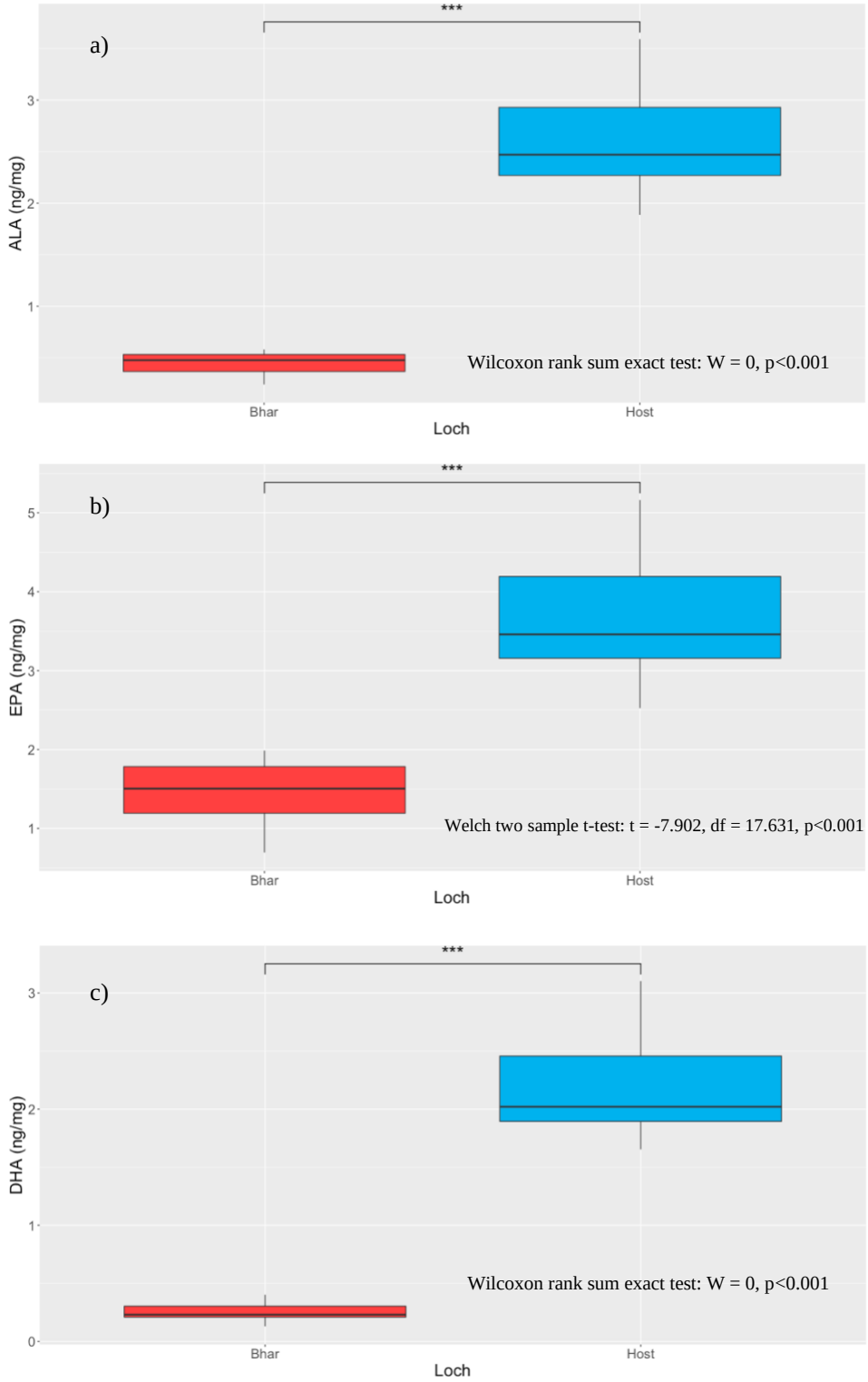

Figure 11. Significant variation in the concentrations of a) ALA, b) EPA and c) DHA between plankton of an acidic (Bhar, n=8) and alkaline (Host, n=12) freshwater loch.

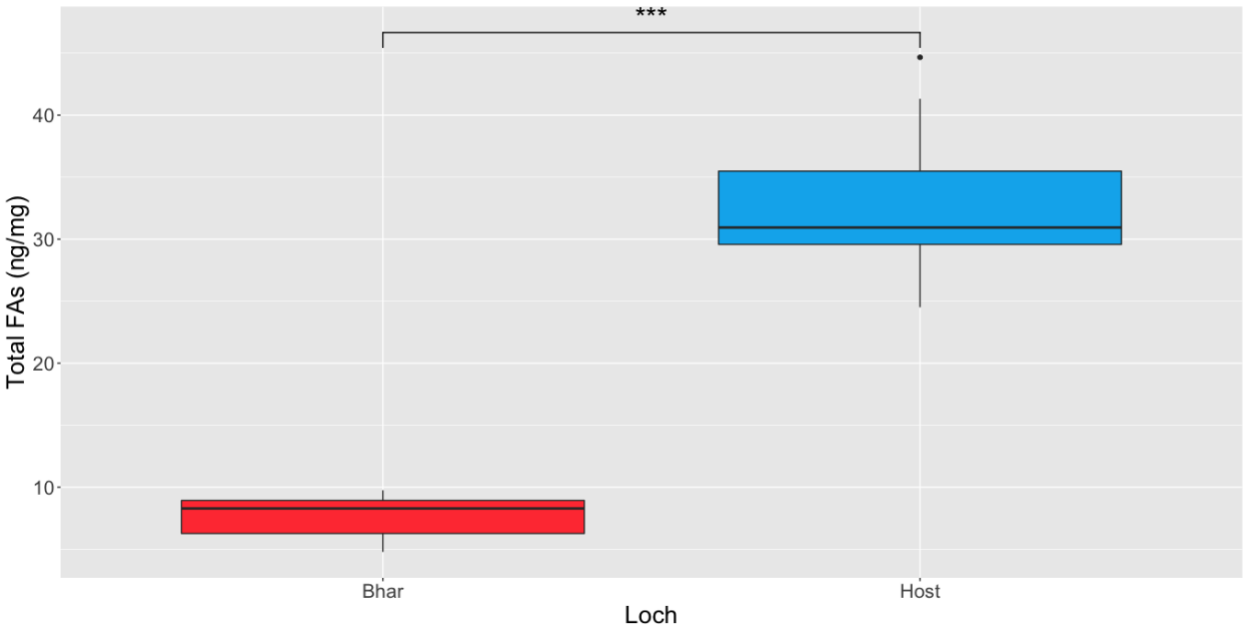

Figure 12. Significant variation in the concentration of total fatty acids between plankton of an acidic (Bhar, n=8) and alkaline (Host, n=12) freshwater loch (Wilcoxon rank sum exact test: W = 0, $p<0.001$).

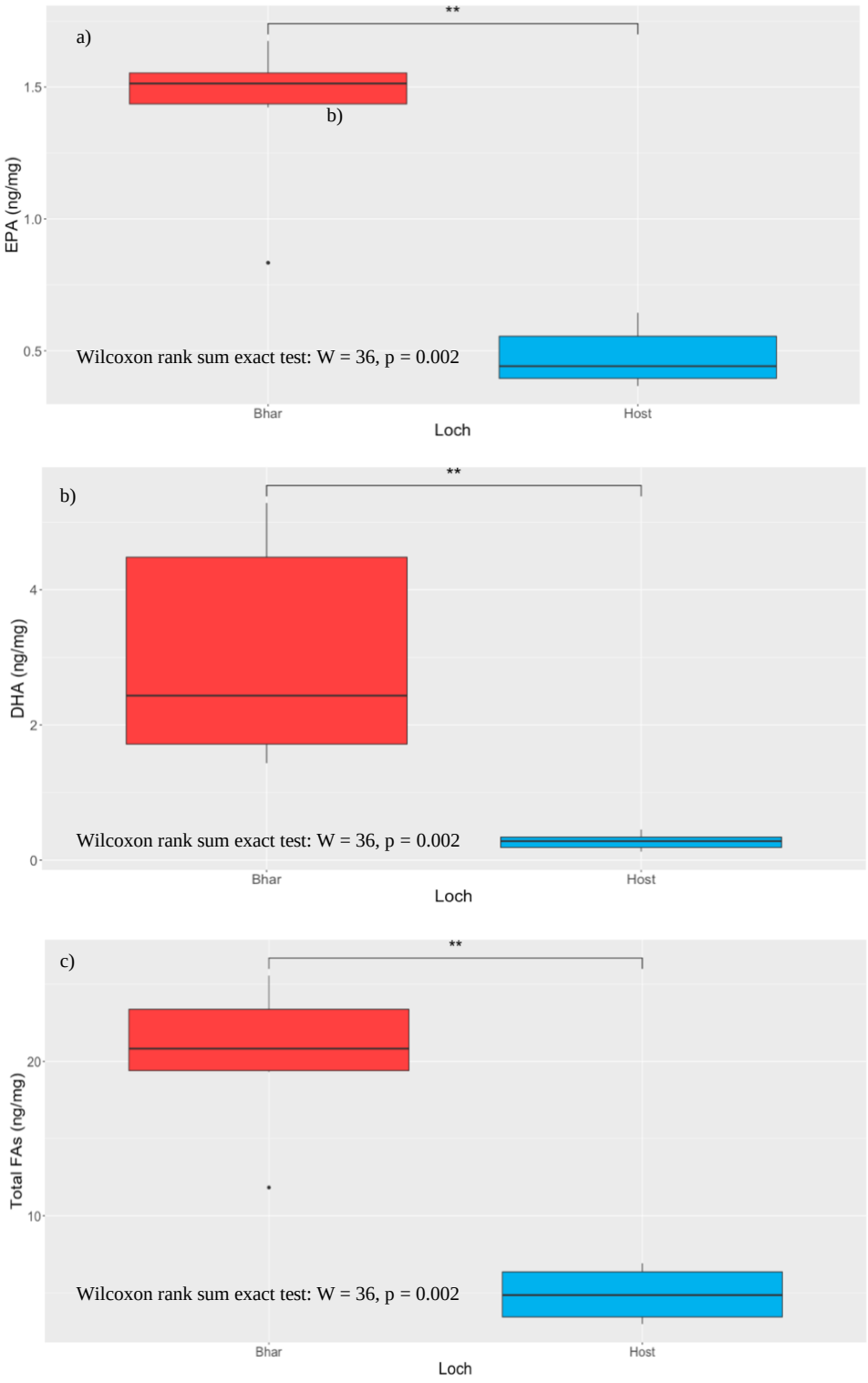

Figure 13. Significant variation in the concentrations of a) EPA b) DHA and c) total fatty acids between freshwater stickleback of an acidic and alkaline loch.

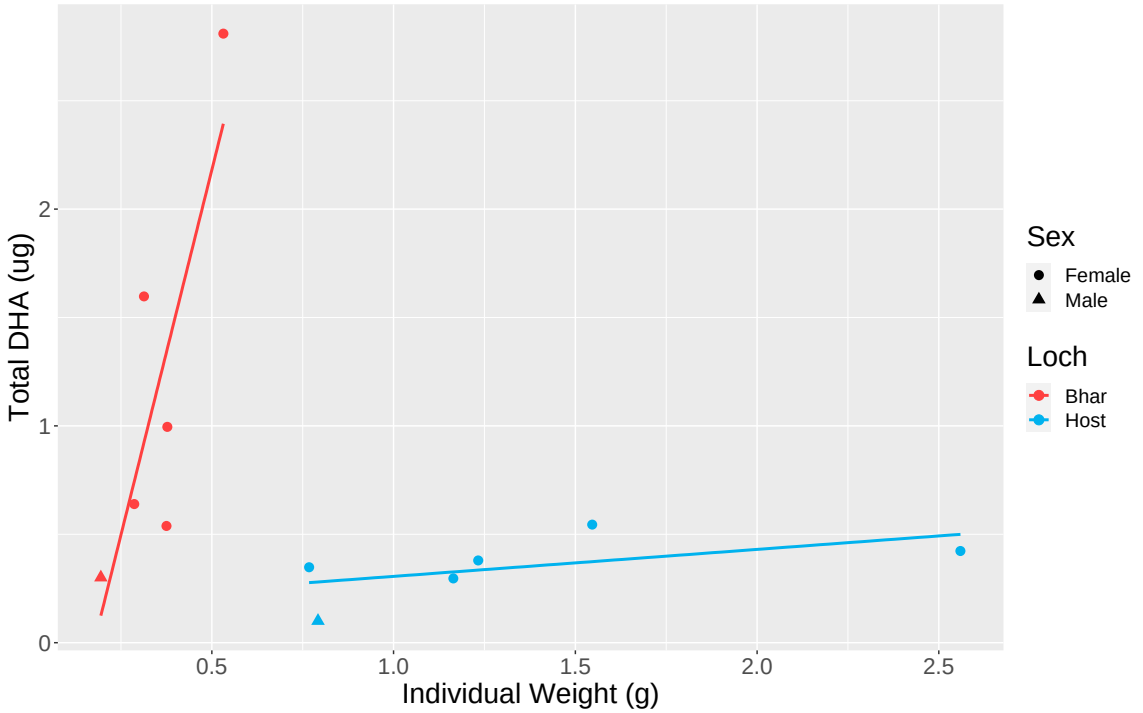

Figure 14. Total amounts of DHA in freshwater three-spined stickleback occupying an acidic oligotrophic (Bhar) and alkaline eutrophic (Host) loch.

Loch-residing stickleback populations

EPA, DHA and total fatty acid concentrations of fish varied significantly between lochs. In each case, Bhar three-spined stickleback possessed higher median concentrations compared to every other analysed loch-residing stickleback population profiled (**Fig. 15, Table S7-S9**). No significant difference was found in the concentration of EPA between sexes or ecotypes, although, the median EPA concentration was higher in a majority of freshwater populations compared to either anadromous or resident alternatives (**Fig. 15, Table S7**). In addition to significant variance between habitats, the concentrations of DHA also differed significantly between ecotypes. Anadromous fish have a higher median DHA concentration compared to resident stickleback and a majority of freshwater populations, although, the Bhar freshwater population possessed the highest median concentration (**Fig. 15, Table S8**). No significant difference was found between the DHA concentrations of males and females. Concentrations of total fatty acids significantly varied between ecotypes and between sexes (**Fig. 15, Table S9**). Finally, absolute amounts of DHA varied significantly with individual weights and significant differences were found in the relationship between total DHA and weight between freshwater lochs (**Fig. 16, Table S10**). The majority of Bhar stickleback had greater amounts of DHA compared to other freshwater conspecifics.

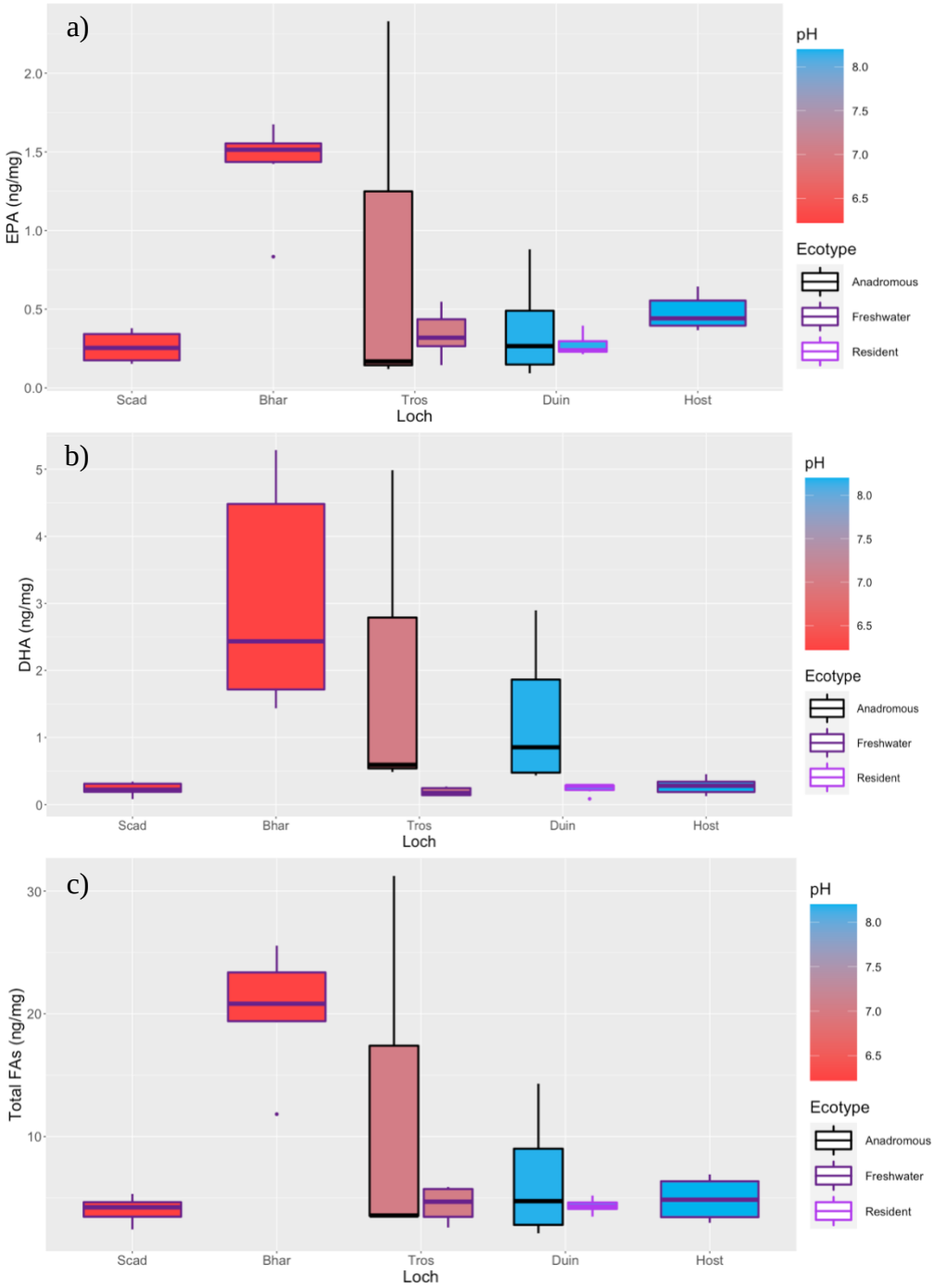

Figure 15. a) EPA, b) DHA and c) total fatty acid concentrations of loch-inhabiting stickleback populations. Three ecotypes reside in sampled lochs which drastically vary in environmental parameters, including pH and nutritional conditions. pH transitions from 6.22 (Scad) to 8.2 (Host).

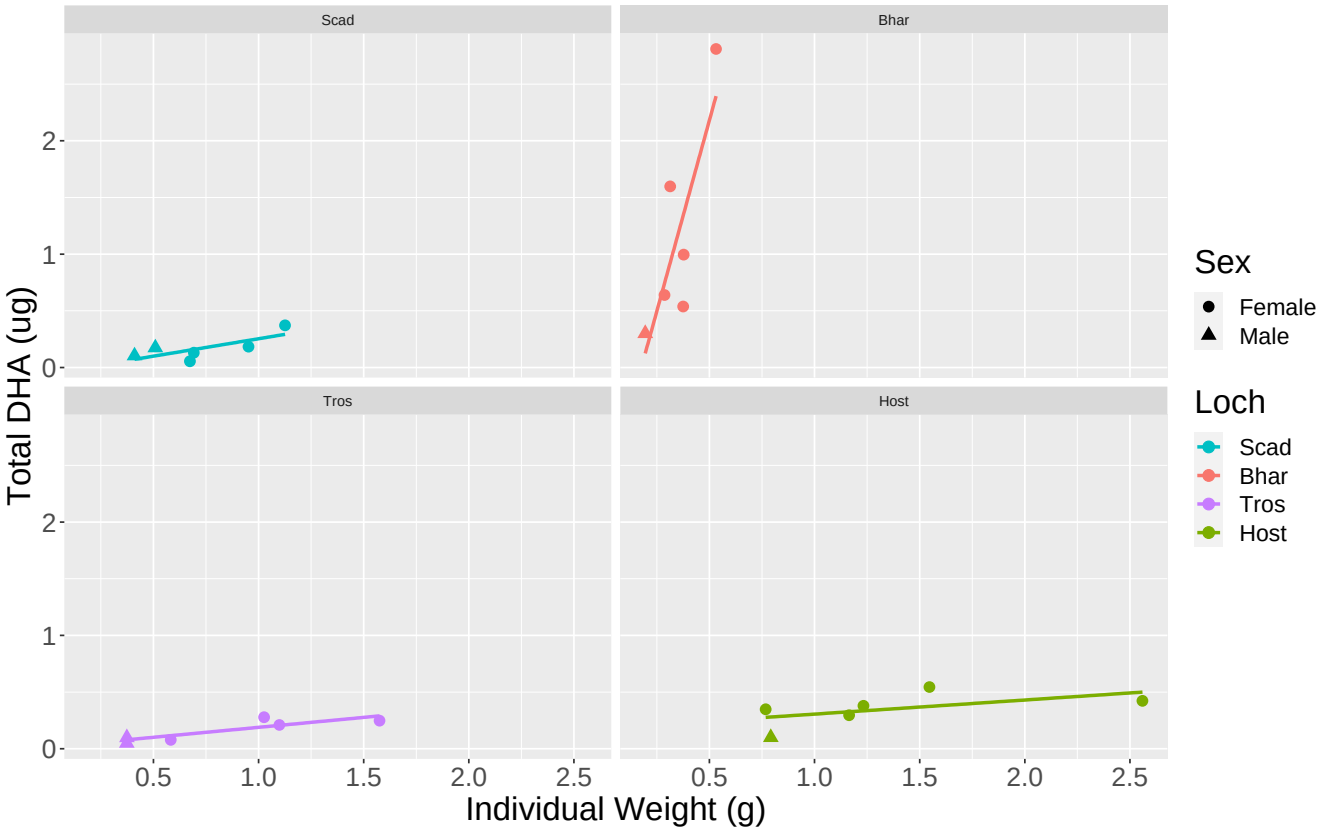

Figure 16. The total amount of DHA in freshwater stickleback inhabiting lochs varying in environmental parameters, particularly pH. Each point represents an individual. Bhar individuals were sampled from lochs in May 2022. All other populations were sampled between May-June 2021 and stored in -80ºC freezers until analysis.

Duin wild-caught, lab-reared and pond fish

DHA concentrations varied significantly between populations (**Fig. 17, Table S12**). The wild-caught anadromous population possessed a higher median concentration of DHA compared the saltwater resident population as well as the Duin anadromous genotype lab-reared and freshwater pond populations (**Fig. 17**). Moreover, wild-caught anadromous and resident populations had higher DHA concentrations than lab-reared and freshwater pond anadromous stickleback (**Fig. 17**). Concentrations of total fatty acids also differed significantly between populations, with freshwater pond fish having a greater median concentration compared to wild-caught anadromous, lab-reared anadromous and saltwater resident fish (**Fig. 17, Table S13**). Although insignificant, median EPA and total fatty acid concentrations are higher in lab-reared and freshwater pond anadromous fish compared to wild-caught anadromous and resident populations (**Fig. 17**). EPA, DHA and total fatty acid concentrations did not vary significantly between sexes (**Table S11-S13**). Unlike respective DHA concentrations, no significant differences in the absolute amounts of DHA were found between populations. Although, within the wild-caught anadromous population, large variation was observed between males (**Fig. 18**). Total DHA did significantly vary between individual fish weight and a significant difference was found in the relationship between total DHA and weight between sexes (**Fig. 18, Table S14**).

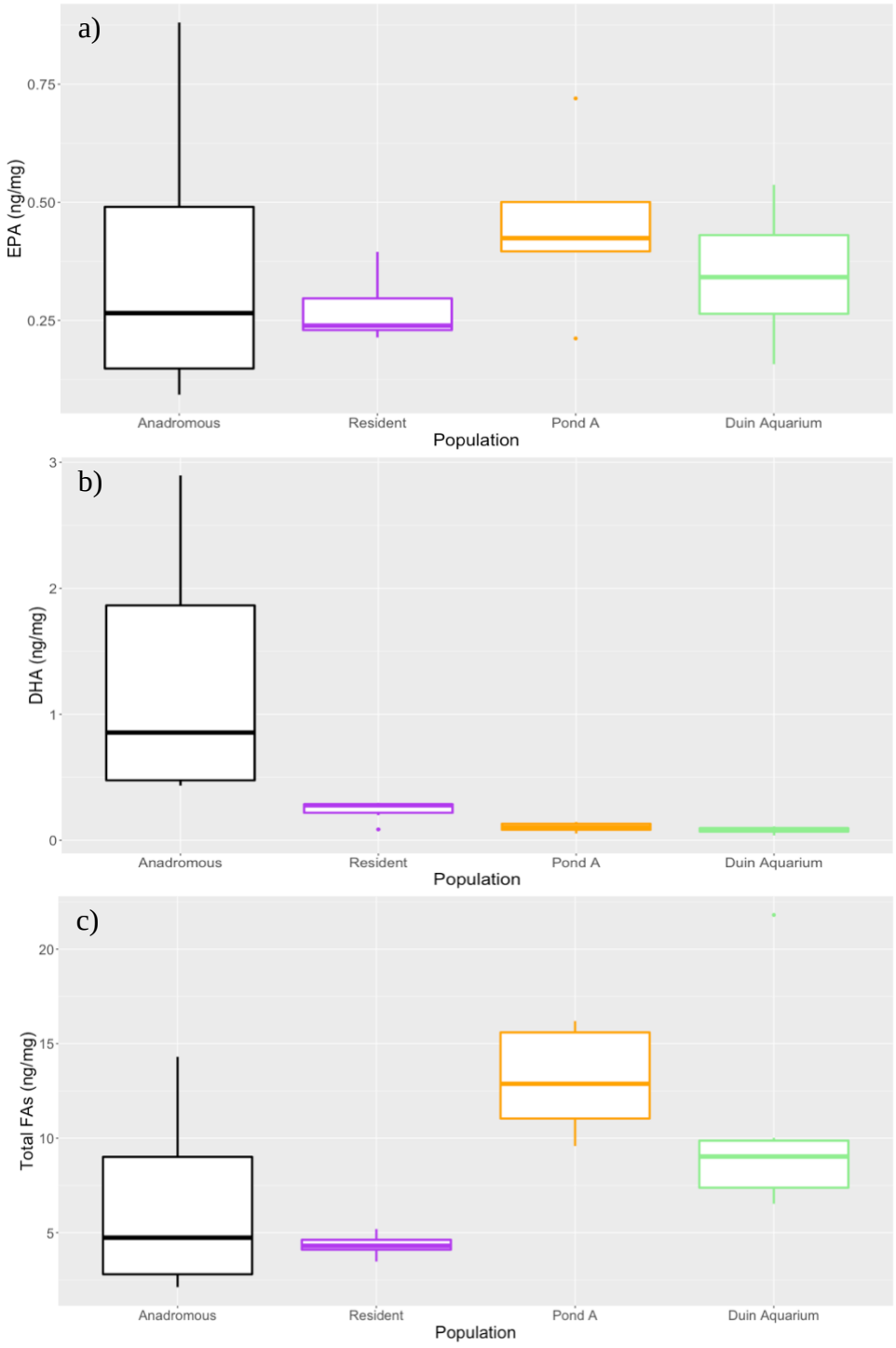

Figure 17. a) EPA, b) DHA and c) total fatty acid concentrations of wild-caught Duin resident and anadromous populations as well as Duin anadromous genotype populations reared either in an aquarium or freshwater pond.

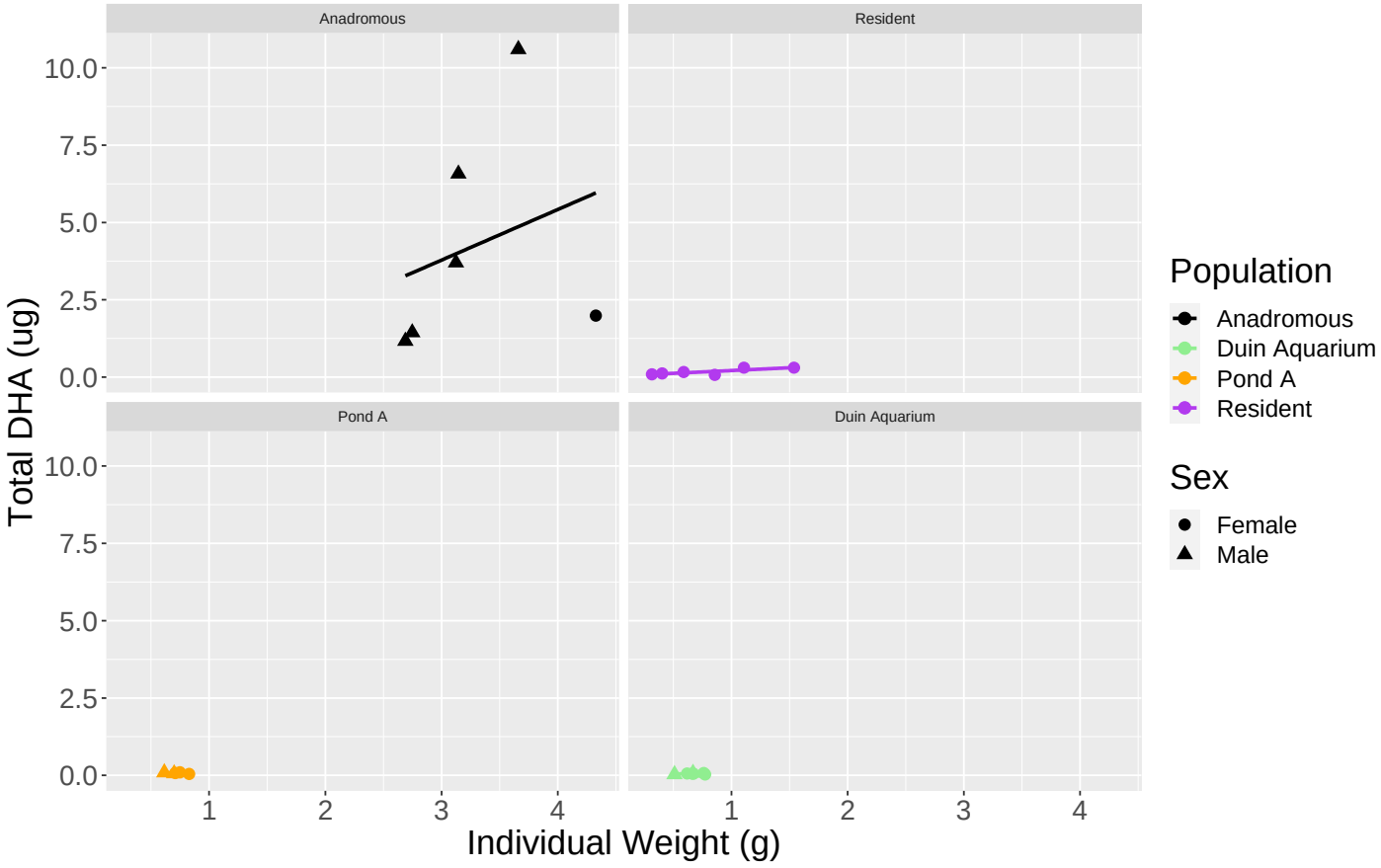

Figure 18. The total amount of DHA in wild-caught Duin resident and anadromous populations as well as Duin anadromous genotype populations reared either in an aquarium or freshwater pond. Each point represents an individual.

Host loch and stream populations

EPA concentrations significantly varied among Host loch and stream populations (**Fig. 19, Table S15**). The median EPA concentration of the bottom of Host stream population was lower compared to Host loch and top of Host stream stickleback. Differences between these populations in concentrations of DHA were insignificant, although, stickleback captured from the bottom of Host stream had the lowest median concentration (**Fig. 19, Table S16**). Concentrations of total fatty acids differed significantly between populations and sexes, with stickleback captured from the top of Host stream possessing a higher median concentration compared to Host loch and bottom of the Host stream fish (**Fig. 19, Table S17**). The total amount of DHA significantly varied with individual weight (**Fig. 20, Table S18**). Interestingly, although having a lower median DHA concentration, the bottom of Host stream population comprised stickleback possessing the highest amounts of DHA. A significant difference in the relationship between weight and total DHA was found between populations (**Fig. 20, Table S18**). The amount of DHA in bottom of Host stream stickleback seemingly increases at a greater rate with increasing individual fish weight compared to other Host populations (**Fig. 20**). However, significant results from the total DHA analysis, testing whether the effect of weight on total DHA differs by population, are likely resulting from the large outlier in the bottom of Host stream population (**Fig. 20, Table S18**). Considering its large weight, sex and high total amount of DHA, it is likely a gravid female which has not yet spawned. Therefore, differences in the effects of population on the relationship between weight and total DHA are likely smaller and may be insignificant.

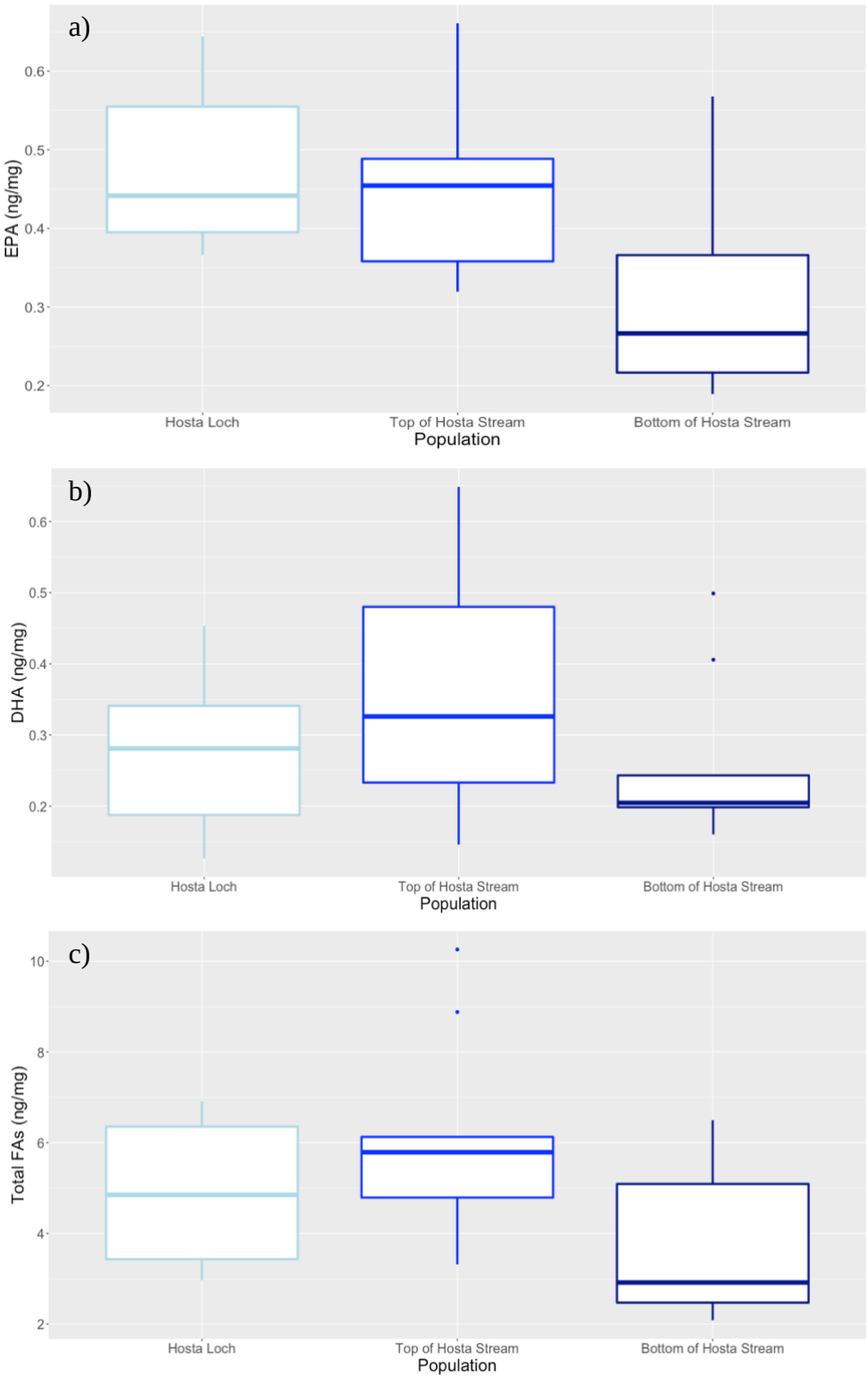

Figure 19. a) EPA, b) DHA and c) total fatty acid concentrations of stickleback populations residing in Host loch and stream environments.

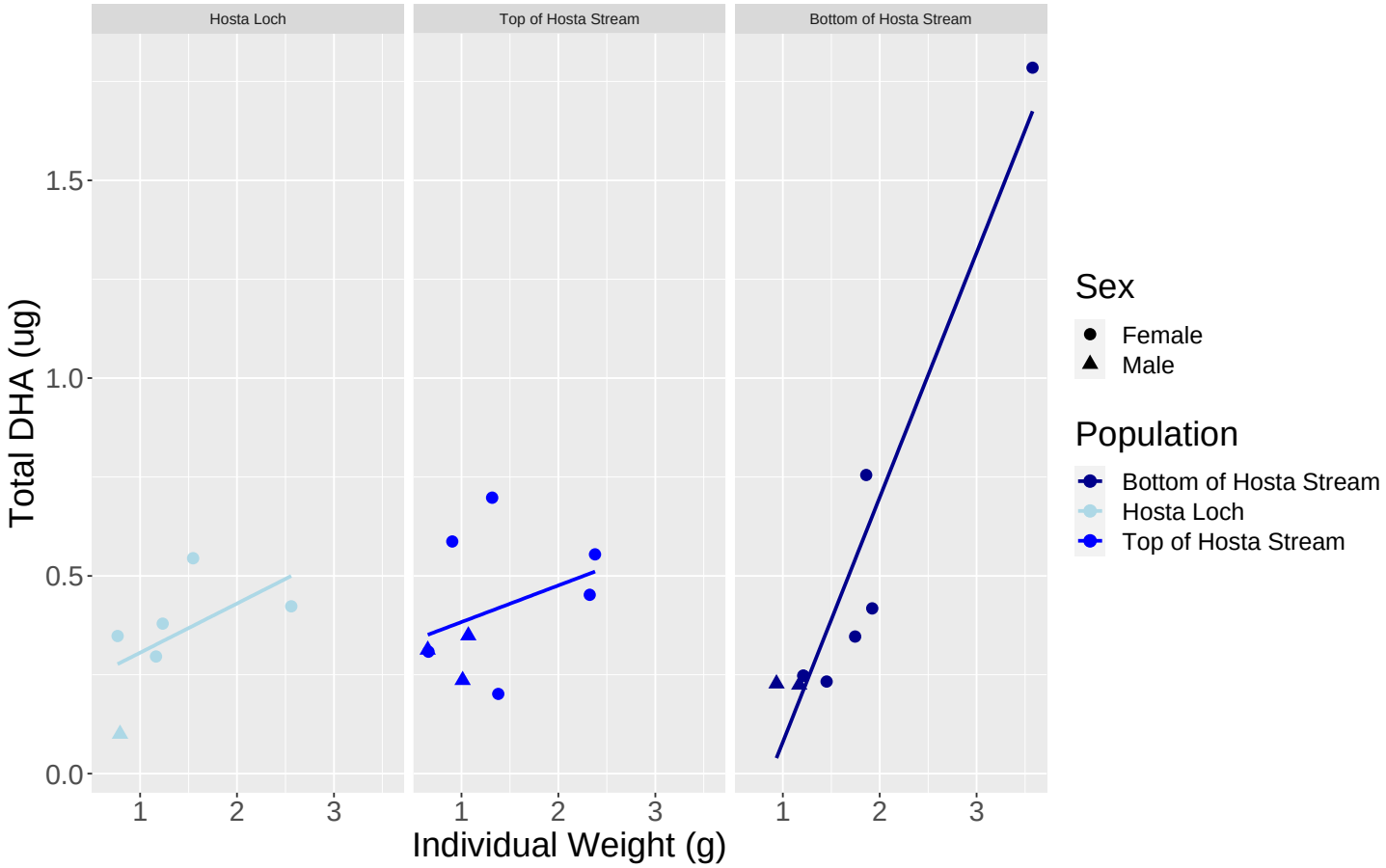

Figure 20. The total amount of DHA in stickleback populations inhabiting Host loch and stream environments. Each point represents an individual.

Discussion

Plankton

Results of plankton analyses show a differential availability of fatty acids in food chains between deep freshwater loch environments on North Uist. Host plankton have higher concentrations of LIN, ALA, ARA, EPA, DHA and total fatty acids compared to Bhar plankton (**Fig. 10-12**). Therefore, divergent dietary selection pressures for improved DHA biosynthetic or retention capabilities may be acting upon corresponding freshwater three-spined stickleback populations to compensate for dietary variations.

Previous ecological studies have rarely investigated differences in the dietary availability of n-3 LC-PUFAs, particularly DHA, between deep freshwater environments. Depth seemingly drives differences in the biomass of different stickleback prey items between freshwater environments, with deep-lake habitats having higher abundances of DHA-rich plankton compared to shallower alternatives, including streams (Ishikawa *et al*. 2021). As both Host and Bhar are relatively deep, (max approx. 10 m), other environmental factors must be driving the significant variation in planktonic essential fatty acid concentrations. Two primary environmental differences occur between both lochs, being nutritional conditions and pH. Bhar is acidic (pH 6.25) and oligotrophic, defined by low net primary production. Opposingly, Host is alkaline (pH 8.2) and eutrophic. Both environmental parameters have been found to influence phytoplankton species composition in freshwater lakes (Lafrancois *et al*., 2004), which could affect the nutritional value of zooplankton. Kolarova and Napiórkowski (2022) further observed an effect of water pH on primary production and in turn zooplankton community composition and biomass in freshwater

environments. Moreover, acidification of freshwater environments is hypothesised to result in decreased nutritional qualities of phytoplankton and altered population dynamics of zooplankton (Ramaekers *et al*., 2022). Overall, pH and nutrient conditions are significant candidates for explaining the environmental difference resulting in planktonic disparities in n-3/n-6 LC-PUFA concentrations. Freshwater zooplankton fatty acids are also often directly or indirectly negatively correlated with water temperature (Gladyshev *et al*., 2011; McMeans *et al*., 2015). Measured temperature was highest in Host (15.2ºC) compared to Bhar (14.3ºC), although these lochs were measured on different days. The influence of temperature on the dietary n-3/n-6 LC-PUFA variation cannot be evaluated in this study. A detailed study, consisting of repeated measurements of abiotic variables and fatty acid analyses on plankton collected on different days over a certain time period, should be performed. This will assess the effects of environmental parameters, including pH, temperature and nutrient conditions, as well as biotic factors, such as seston composition, on the dietary availability of n-3 LC-PUFAs in North Uist lochs. Moreover, analyses on plankton in future years and different seasons will aid in understanding how the dietary availability of n-3 LC-PUFAs in lochs varies over long time periods. Performing these studies will answer whether Host houses consistently higher quality plankton and would confirm hypotheses regarding divergent dietary selection pressures.

Stickleback

The concentrations of EPA, DHA and total fatty acids were reversed in Host and Bhar stickleback (**Fig. 13**), relative to plankton. Moreover, variation in the relationship between individual weight and total DHA between lochs suggest, at any weight, Bhar stickleback will

have more DHA compared to Host conspecifics (**Fig. 14**). This suggests food web deficiencies in relation to DHA availability in Loch a'Bharpa may have prompted the divergent evolution of compensatory metabolic adaptations for either improved biosynthetic or retention capabilities. *Fads2* copy number variation was discovered between stickleback species varying in capabilities to colonise DHA deficient freshwaters (Ishikawa *et al*., 2019). Moreover, variable evolution of *Fads2* copy number has occurred between three-spined stickleback populations occupying different freshwater habitats defined by disparities in DHA availability (Ishikawa *et al*., 2021; Hudson *et al*., 2022). Neo- and multi-functionalisation of this enzyme has also improved DHA biosynthetic capabilities in fish, permitting freshwater colonisation (Matsushita *et al*., 2020). Therefore, *Fads2* is a strong candidate gene to test for genomic and metabolic differences between Host and Bhar populations in DHA biosynthetic capabilities. As EPA and DHA affect organismal growth, these populations may be exhibiting countergradient variation, in which genetic effects on fatty acid traits combat environmental influences, reducing body size phenotypic divergence between populations across the pH gradient (Conover and Schultz, 1995; Marcil *et al*., 2006). Countergradient variation is assumed where homogenous non-extreme phenotypes are selected for between distinct environments (Marcil *et al*., 2006).

The dietary availability of n-3 LC-PUFAs is dynamic, with a multitude of environmental parameters, including seasonality, temperature and light intensity, all affecting either the EPA and DHA contents or the community composition of primary producers at the base of food webs (see Chapter 2). He *et al*. (2015) observed different metabolic strategies in the Nile tilapia during periods of high versus low lipid availability. These involved increasing lipid storage capabilities when the availability of these molecules was high and higher fatty acid metabolism when lipid

availability was low via the differential expression of genes (He *et al*., 2015). Therefore, the fatty acid variation between the stickleback populations may be resulting from metabolic strategies instead of from the metabolic adaptation of fatty acid traits. The plankton samples and Bhar stickleback were obtained in 2022, however, the Host population was sampled in 2021. As there is no information on the fatty acid content of fresh Host stickleback, it is difficult to hypothesise potential metabolic strategies utilised by these populations. The freshness of stickleback samples may also contribute to or explain the variation between stickleback samples.

Life-history differences between populations may also explain the variation in concentrations and amounts of n-3 LC-PUFAs. Firstly, eggs and embryos are dependent on large amounts of DHA to survive gestation, as this molecule is required for important growth processes, including neurogenesis and visual development (Dyall and Michael-Titus, 2008; Innis, 2008; Tocher, 2010). Moreover, during female stickleback spawning, clutches can be produced up to every three days (Baker *et al*., 2008). This short time frame means variation could reflect the different reproductive states of females. Host stickleback may have already laid clutches containing significant amounts of DHA before being captured whilst Bhar females were still gravid and had not laid eggs, meaning they still possessed high amounts of DHA. Futhermore, some females may be sexually mature whereas others could be immature. Similarly, females in both populations may produce different egg sizes. The size of eggs can significantly influence the growth and survival of fry (Baker *et al*., 2008). Egg size differs drastically between three-spined stickleback populations (Baker, *et al*., 2008). Divergence in egg size in Alaskan stickleback populations is hypothesised to be predominantly controlled by local environmental parameters (Baker *et al*., 2008). Therefore, Bhar females may invest more into producing larger, higher-

quality eggs to improve fry survival in the more unfavourable environment. This would require increased capabilities to acquire or store DHA and could be a selection pressure prompting metabolic evolution. Other life-history traits, such as clutch size and mass, vary between stickleback populations (Baker *et al*., 2008) and similarly to egg size, may select for increased n-3 LC-PUFA biosynthetic capabilities. However, these traits are predominantly affected by body size (Baker *et al*., 2008). As freshwater populations on North Uist are characterised by small body sizes (MacColl *et al*., 2013), these traits may not explain the variation between Host and Bhar in the amounts and concentrations of DHA and EPA.

Habitat had a significant influence on EPA, DHA and total fatty acid concentrations as well as the total amounts of DHA, with Bhar stickleback in each case possessing the highest median concentration of all lochs and the highest amounts of DHA among freshwater lochs (**Fig. 15-16**). It is important to note Bhar was the only wild-caught stickleback population sampled in 2022 which was analysed in this study. All other wild-caught populations were sampled from North Uist in 2021. Studies have suggested freezing fish tissue at -80^{o}C immediately or as soon as possible after capture is often the best available method to limit alterations to the lipid and fatty acid content of samples (Rudy *et al*., 2016). Moreover, Rudy *et al*. (2016) found no fatty acids predominantly fluctuated in quantity relative to others, suggesting stored stickleback samples would not have disproportionate alterations to amounts of analysed n-3 LC-PUFAs. As our sample preparation procedures followed this protocol, storage should not have affected the concentrations and amounts EPA and DHA in samples. However, it is important to test the effect of storage on the fatty acid contents of three-spined stickleback, as Rudy *et al*. (2016) showed responses to storage methods and durations in terms of alterations to sample fatty acid content

were species-specific. The concentration of FAMEs in fish characterised by the highest lipid quantities (Charr and Trout) were highly impacted by storage conditions (Rudy *et al.*, 2016). Additionally, the digestive tracts of stickleback should be removed when preparing future samples for fatty acid analyses, as gut contents may unknowingly contribute to lipid and fatty acid compositions, as exhibited in cod larvae (Lochmann *et al.*, 1996).

DHA concentrations were further influenced by ecotype (**Fig. 15**). Excluding the Bhar freshwater population, anadromous fish had greater median DHA concentrations compared to both freshwater and resident ecotypes. Anadromous fish migrate and inhabit nutritionally poor shallow lake and freshwater environments to spawn. Therefore, increased DHA concentrations in this ecotype may reflect the evolution of improved metabolic capabilities or a physiological strategy involving the accumulation of DHA in n-3 LC-PUFA-rich environments, a tactic employed by other migratory consumers (Miller, *et al.*, 2009; Twining *et al.*, 2021).

DHA concentrations in Duin populations are dependent on environment (**Fig. 17, Table S12**). Anadromous wild-caught populations had a higher median concentration of DHA compared to Duin anadromous genotypes reared in the aquarium or a freshwater pond. Moreover, both freshwater pond and Duin aquarium anadromous fish possess higher median concentrations of EPA compared to respective concentrations of DHA and have the largest median total fatty acid concentrations (**Fig. 17**). The amount of DHA in wild-caught anadromous populations is high, whereas amounts in pond and aquarium populations are negligible (**Fig. 18**). These results suggest Duin anadromous fish are ineffective at or have an inability to biosynthesise DHA from EPA, confirming results from wild-caught populations may reflect a physiological strategy to

store and ration resources from DHA-rich marine environments instead of the evolution of metabolic traits associated with n-3 LC-PUFA synthesis (Twining *et al.*, 2021). The inclusion of either a Duin resident or freshwater aquarium population would have aided in supporting the hypothesis regarding the ineffective DHA biosynthetic capabilities of anadromous fish. Greater concentrations and amounts of DHA in either control population would confirm the presence of ecotype differences in metabolic capacities to acquire DHA. Interestingly, Duin aquarium fish are fed n-3 LC-PUFA enriched bloodworms, yet still have low concentrations and amounts of DHA, suggesting anadromous stickleback may only store DHA if environmental or reproductive pressures require it. Further fatty acid analyses on lab reared fish from populations originating from North Uist is required to determine if similar trends between wild and artificially reared populations exist in concentrations and amounts of DHA.

Duin anadromous males seemingly have greater amounts of DHA compared to female conspecifics (**Fig. 18**). This is surprising, as *Fads2*, involved in DHA biosynthesis, is ancestrally an X-linked gene (Ishikawa *et al.*, 2019). As X-inactivation does not occur in fish, females simultaneously express *Fads2* on both chromosomes, predicted to result in greater concentrations of DHA compared to males, a favourable scenario given the essential fatty acid demands of female gametes (Tocher, 2010; Hudson *et al.*, 2022). Hudson *et al.* (2022) observed higher DHA concentrations in males of wild-caught lake and stream population, hypothesised to result from divergent foraging behaviours or niche partitioning between sexes to compensate for differential biosynthetic capabilities. However, my result is based on only a single female, and it is possible she had recently spawned, resulting in reduced amounts of DHA due to significant preceding investments in mature gamete production, a scenario also postulated by Hudson *et al.* (2022) in

response to high male DHA concentrations. Moreover, within the wild-caught anadromous population, large variation was observed in the amount of DHA between males (**Fig. 18**). This may be resulting from individuals feeding on different combinations of prey differing in PUFA content (Hudson *et al*., 2022). Variation in sperm quality and sperm competition may also explain large differences in the amounts of DHA in Duin anadromous males. Lipid metabolism is the predominant energy production method for spermatozoa in animals and mammals possess high amounts of DHA in these cells (Zaniboni *et al*., 2006). Zbinden *et al*. (2004) showed male competitor body size might control sperm competition risk and in turn, ejaculate size in stickleback. Males increased ejaculate size when an image of a large stickleback was displayed compared to when a small picture was shown (Zbinden *et al*., 2004). As anadromous ecotypes are characterised by large body sizes (MacColl *et al*., 2013) the risk of sperm competition could have resulted in differential investment into sperm to increase their quality. Unlike Duin anadromous populations, all freshwater and Host stream populations follow expected trends in amounts of DHA, with males often having less DHA compared to female conspecifics (**Fig. 20**).

Regarding Host loch and stream populations, the bottom of Host loch stickleback had lower median EPA, DHA and total fatty acid concentrations compared to Host loch and top of Host stream populations (**Fig. 19**). The relative input of marine and loch environments to respective stream populations is unknown, hindering the ability to comprehend corresponding stickleback data fully. Fatty acid analyses of prey items collected from the top and bottom of Host stream are required to hypothesise differential adaptive metabolic evolution between stickleback populations.

Conclusion

Overall, plankton analyses have showed divergent selection pressures for DHA biosynthetic capacities are likely acting on freshwater environments differing in environmental parameters, plankton quality and the dietary availability of n-3/n-6 LC-PUFAs. EPA, DHA and total fatty acid concentrations were reversed in Host and Bhar stickleback populations relative to plankton, a characteristic of countergradient variation. Moreover, variation in concentrations and amounts of DHA between Duin anadromous genotypes reared in different environments and wild-caught equivalents suggest ineffective DHA biosynthesis capabilities might be present in anadromous stickleback. Although comparisons of EPA, DHA and total fatty acid concentrations between loch-inhabiting populations suggest Bhar stickleback may have evolved capacities for increased DHA biosynthesis, further investigation into the effect of storage on stickleback fatty acids is required to confirm results attained from fish are valid.

Chapter 4: General Discussion

Overview of study

Overall, our results have showcased variation in the dietary availability of n-3 LC-PUFAs, particularly EPA and DHA, between aquatic environments varying in salinity, pH and nutritional conditions. Environmental and taxonomic variation in the EPA and DHA contents of algae from previous studies were synthesised to assess the dietary limitations encountered by freshwater consumers. Freshwater algae have lower EPA and DHA contents compared to marine and brackish equivalents, driven either by responses to salinity or by the taxonomic composition of local species pools. As phytoplankton directly influence the population dynamics and nutritional value of zooplankton (Taipale *et al*., 2019; Taipale *et al*., 2020), freshwater fish will struggle to meet physiological n-3 LC-PUFA requirements through dietary intake, prompting metabolic evolution. pH also affects the growth and n-3 LC-PUFA content of phytoplankton, with evidence suggesting acidic pH's negatively impact the growth and triacylglycerol content of numerous algae species (Gutierrez, 2009). This will negatively impact the fitness and nutritional value of zooplankton further, which may suggest why variation in the nutritional quality of plankton exists between Host and Bhar.

Ishikawa *et al*. (2021) highlighted that metabolic evolution, specifically of *Fads2* copy number, can arise between freshwater three-spined stickleback populations inhabiting lakes and streams and, in turn, exploiting different prey items which vary in DHA content (Hudson *et al*., 2022). This study has shown fatty acid variation exists between freshwater stickleback populations

residing in loch environments inhabited by plankton of differing quality. This variation in nutritional quality may have resulted in divergent metabolic adaptive evolution, a hypothesis support by the large amount of DHA in Bhar stickleback compared to other freshwater populations.

Fatty acid variation was also shown between ecotypes, with results suggesting populations rely on different physiological or metabolic adaptive strategies to survive in poor freshwater and potentially poor saltwater habitats (Waterston *et al*., 1979; Ishikawa *et al*., 2021; Hudson *et al*., 2022). Freshwater and resident populations, which continuously inhabit lochs, including Duin, a shallow loch likely possessing a relatively high proportions of DHA-poor benthos (Ishikawa *et al*., 2021; Hudson *et al*., 2022), probably possess metabolic adaptations, possibly including increased *Fads2* copy number, for improved DHA biosynthesis capabilities. In contrast, anadromous stickleback, which predominantly inhabit EPA and DHA-rich marine environments and migrate to spawn, may rely on storage and rationing of resources collated in such environments to survive breeding seasons (Miller *et al*., 2009; Twining *et al*., 2021), instead of evolving enhanced biosynthetic capacities. Comparisons between anadromous fish grown in different environments supports such conclusions. Further fatty acid analyses should be performed on stickleback, particularly males, from each analysed population to validate apparent sex-linked patterns observed in this data.

Future perspectives

This research has provided the basis for a plethora of further investigation. Genomic analyses combining long- and short-read sequencing data from North Uist stickleback will be performed to identify copy number variation in *Fads2* among and between freshwater, anadromous and resident populations. If *Fads2* duplications are present, functional characterisations of each copy, including the identification of amino acid domain, will be required to determine the range of substrate specificities present (Garrido *et al*., 2019). Moreover, simultaneous investigations will be made into the presence of regulatory, coding sequence or structural variation in *Elovl*. Diet trials among and between ecotypes, using similar methodology to Ishikawa *et al*. (2019), will provide evidence on the impact of genomic differences between populations. During diet trials, RNA-seq may be performed on individuals from each treatment group to measure the expression levels of candidate genes involved in n-3 LC-PUFA synthesis and more generally lipid homeostasis. This would aid in discovering whether n-3 LC-PUFA biosynthesis and storage capabilities are under selection in different populations as well as environments characterised by differences in dietary n-3 LC-PUFA availability. Collective results will answer hypotheses regarding differential metabolic evolution postulated from results found in this study. Further fatty acid analyses on other stickleback populations occupying different fresh- and saltwater habitats on North Uist will permit the mapping of fatty acid variation across the island as well as an understanding of the relationship between such differences and environmental gradients, including pH. Using this data in line with findings from genomic analyses will support or reject hypotheses regarding the presence of countergradient variation on North Uist. Benthic invertebrate samples have also been collected from numerous freshwater and saltwater lochs on North Uist, including Bhar and Host. Conducting fatty acid analyses on benthos will permit the understanding of specific differences between lochs in all critical trophic levels of food webs in

the dietary availability of n-3/n-6 LC-PUFAs. Finally, sectioning and staining of sampled fish to identify regions of lipid deposition and performing fatty acid analyses on Duin anadromous and freshwater stickleback throughout the breeding season, will reveal the adaptive strategies employed.

Conclusion

Here, we have shown three-spined stickleback fatty acid variation exists among and between fresh- and saltwater environments. Our study showcases how fatty acid profiles, particularly the contents of EPA and DHA, can be used to predict the evolution, presence and level of fatty acid associated genomic metabolic adaptations. My results support previous conclusions regarding algal and stickleback n-3 LC-PUFA variation between habitats varying in salinity and taxonomy (algae) as well as sexes (stickleback). Importantly, this study highlights the importance of understanding differences in the nutrient quality of prey, especially plankton, between specific habitats when conducting comparative ecological or evolutionary studies regarding fatty acid phenotypes and associated genomic variation between populations of the same species. Knowing this information will aid in uncovering answers to questions surrounding the origins of species diversification. Results from this study will add to a small pool of research investigating metabolic adaptive evolution through an ecological lens.

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Supplementry material

Table S1. A GLM of EPA content variation in algae with habitat/isolation source and taxa as factors. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	-2.342	0.369	-6.356	**<0.001**
Freshwater	-0.949	0.321	-2.958	**0.003**
Marine	0.180	0.332	0.543	0.587
Non-cyanobacteria	4.419	0.212	20.813	**<0.001**

Table S2. A GLM of DHA content variation in algae with habitat/isolation source and taxa as factors. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened. The GLM algorithm did not converge, meaning predictor variables perfectly separated from the response variable.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	-2.643	0.597	-4.429	**<0.001**
Freshwater	-13.915	0.518	-26.856	**<0.001**
Marine	-12.153	0.538	-22.574	**<0.001**
Non-cyanobacteria	15.9844	0.346	46.143	**<0.001**

Table S3. Wilcoxon tests of EPA and DHA content variation between cyanobacteria and non-cyanobacteria of freshwater, brackish and marine environments. The sample sizes, Wilcoxon test statistics and p-values are noted, with significant results ($p<0.05$) emboldened.

Habitat	*Fatty acid*	*n*	*W*	*p-value*
Freshwater	EPA	1205	46259	**<0.001**
	DHA	1199	51975	**<0.001**
Brackish	EPA	57	35	**0.007**
	DHA	57	99	0.343
Marine	EPA	486	810	**<0.001**
	DHA	439	1557	**<0.001**

Table S4. A GLM of EPA content variation in algae with phylum as a factor. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Terms	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	2.508	0.273	9.199	**<0.001**
Cercozoa	-0.040	2.821	-0.014	0.989
Charophyta	-2.553	0.369	-6.911	**<0.001**
Chlorophyta	-2.833	0.291	-9.740	**<0.001**
Cryptophyta	-0.011	0.411	-0.026	0.979
Cyanobacteria	-5.792	0.364	-15.898	**<0.001**
Euglenozoa	-1.186	0.387	-3.069	**0.002**
Glaucophyta	0.322	0.889	0.362	0.718
Haptophyta	-0.704	0.438	-1.606	0.108
Miozoa	-0.546	0.500	-1.093	0.275
Ochrophyta	0.037	0.341	0.108	0.914
Rhodophyta	0.320	0.394	0.812	0.417
Tracheophyta	-3.793	1.644	-2.308	**0.021**

Table S5. A GLM of DHA content variation in algae with phylum as a factor. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened. The GLM algorithm did not converge, meaning predictor variables perfectly separated from the response variable.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	-0.141	0.576	-0.244	0.807
Cercozoa	-27.162	5.955	-4.561	**<0.001**
Charophyta	-3.710	0.780	-4.758	**<0.001**
Chlorophyta	-1.782	0.614	-2.900	**0.004**
Cryptophyta	1.130	0.869	1.301	0.193
Cyanobacteria	-3.019	0.773	-3.906	**<0.001**
Euglenozoa	1.114	0.816	1.365	0.172
Glaucophyta	-27.162	1.878	-14.467	**<0.001**
Haptophyta	2.324	0.948	2.453	**0.014**
Miozoa	2.796	1.055	2.652	**0.008**
Ochrophyta	0.515	0.734	0.702	0.483
Rhodophyta	-2.213	0.891	-2.484	**0.013**
Tracheophyta	-0.108	5.955	-0.018	0.986

Table S6. A GLM of total DHA variation between Host and Bhar freshwater three-spined stickleback populations with loch and weight as factors. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard error*	*t-value*	*p-value*
Intercept	-1.969	0.741	-2.657	**0.029**
Host	0.363	0.902	0.402	0.698
Weight	5.590	2.049	2.728	**0.026**
Host x Weight	-5.198	2.079	-2.500	**0.037**

Table S7. A GLM of EPA concentration variation between loch-inhabiting wild-caught stickleback populations with loch, ecotype and sex as factors. The estimates of coefficients, standard error, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Terms	*Estimate of Coefficients*	*Standard Error*	*t-value*	*p-value*
Intercept	1.096	0.479	2.287	**0.029**
Duin	-2.341	0.525	-4.458	**<0.001**
Host	-1.081	0.328	-3.292	**0.002**
Scad	-1.719	0.331	-5.198	**<0.001**
Tros	-1.470	0.331	-4.444	**<0.001**
Freshwater	-0.788	0.410	-1.922	0.064
Resident	-0.058	0.383	-0.152	0.880
Male	0.257	0.236	1.089	0.285

Table S8. A GLM of DHA concentration variation between wild-caught loch-inhabiting stickleback populations with loch, ecotype and sex as factors. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	3.305	0.499	6.617	**<0.001**
Duin	-3.293	0.547	-6.016	**<0.001**
Host	-2.395	0.342	-6.996	**<0.001**
Scad	-2.651	0.345	-7.690	**<0.001**
Tros	-2.819	0.345	-8.178	**<0.001**
Freshwater	-2.212	0.427	-5.177	**<0.001**
Resident	-1.449	0.399	-3.634	**<0.001**
Male	0.240	0.246	0.976	0.337

Table S9. A GLM of total fatty acid concentration variation between wild-caught loch-inhabiting stickleback populations with lochs, ecotype and sex as factors. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimates of Coefficients*	*Standard Error*	*t-value*	*p-value*
Intercept	3.748	0.394	9.516	**<0.001**
Duin	-2.324	0.432	-5.383	**<0.001**
Host	-1.457	0.270	-5.395	**<0.001**
Scad	-1.719	0.272	-6.322	**<0.001**
Tros	-1.631	0.272	-5.998	**<0.001**
Freshwater	-0.797	0.337	-2.366	**0.024**
Resident	0.044	0.315	0.139	0.891
Male	0.485	0.194	2.506	**0.018**

Table S10. A GLM of total DHA variation between freshwater stickleback populations with loch and individual weight as factors. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimates of Coefficients*	*Standard Error*	*t-value*	*p-value*
Intercept	-1.969	0.680	-2.894	**0.011**
Host	0.363	0.828	0.438	0.667
Scad	-0.946	0.911	-1.038	0.315
Tros	-1.057	0.800	-1.322	0.205
Weight	5.590	1.882	2.970	**0.009**
Host x Weight	-5.198	1.909	-2.723	**0.015**
Scad x Weight	-4.129	2.041	-2.023	0.060
Tros x Weight	-4.320	1.934	-2.234	**0.040**

Table S11. A GLM of EPA concentration variation between wild-caught, lab-reared and freshwater pond populations from Duin with population and sex as factors. The estimates of coefficients, standard error, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	3.441	0.823	4.179	**<0.001**
Duin Aquarium	-0.170	0.831	-0.204	0.840
Pond A	-0.812	0.661	-1.230	0.234
Resident	0.242	1.322	0.183	0.857
Male	-0.858	0.661	-1.298	0.210

Table S12. A GLM of DHA concentration variation between wild-caught, lab-reared and freshwater pond populations from Duin with population and sex as factors. The estimates of coefficients, standard error, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	-0.057	0.303	-0.188	0.853
Duin Aquarium	-2.540	0.314	-8.077	**<0.001**
Pond A	-2.335	0.324	-7.203	**<0.001**
Resident	-1.381	0.365	-3.782	**0.001**
Male	0.314	0.269	1.169	0.257

Table S13. A GLM of total fatty acid concentration variation between wild-caught, lab-reared and freshwater pond populations from Duin with population and sex as factors. The estimates of coefficients, standard errors, t-values and p-values of coefficients are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	1.720	0.304	5.668	**<0.001**
Duin Aquarium	0.569	0.315	1.806	0.087
Pond A	0.790	0.325	2.432	**0.025**
Resident	-0.252	0.366	-0.689	0.499
Male	0.152	0.269	0.564	0.579

Table S14. A GLM of total DHA variation between wild-caught, lab-reared and freshwater pond populations from Duin with population and sex as factors. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	-4.414	1.002	-4.406	**<0.001**
Duin Aquarium	0.704	0.854	0.825	0.421
Pond A	0.949	0.840	1.129	0.274
Resident	1.665	0.839	1.985	0.064
Weight	1.161	0.235	4.932	**<0.001**
Male	-0.484	0.279	-1.735	0.101
Weight x Male	0.847	0.179	4.735	**<0.001**

Table S15. A GLM of EPA concentration variation between stickleback populations inhabiting Host loch and stream environments, with sex and population as factors. The estimates of coefficients, standard errors, t-values and p-values of coefficients are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	3.288	0.433	7.591	**<0.001**
Host Loch	-1.117	0.481	-2.325	**0.031**
Top of Host Stream	-1.040	0.467	-2.227	**0.038**
Male	-0.284	0.288	-0.989	0.335

Table S16. A GLM of DHA concentration variation between stickleback populations inhabiting Host loch and stream environments, with sex and population as factors. The estimates of coefficients, standard error, t-values and p-values of coefficients are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	-1.303	0.155	-8.394	**<0.001**
Host Loch	0.039	0.234	0.165	0.871
Top of Host Stream	0.373	0.210	1.772	0.092
Male	-0.248	0.212	-1.171	0.255

Table S17. A GLM of total fatty acid concentration variation between stickleback populations inhabiting Host loch and stream environments, with sex and population as factors. The estimates of coefficients, standard errors, t-values and p-values are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	0.309	0.044	6.977	**<0.001**
Host Loch	-0.092	0.050	-1.845	0.080
Top of Host Stream	-0.118	0.045	-2.610	**0.017**
Male	-0.068	0.023	-2.944	**0.008**

Table S18. A GLM of total DHA variation between stickleback populations inhabiting Host loch and stream environments, with population, weight and sex as factors. The estimates of coefficients, standard error, t-value and p-value of coefficients are noted, with significant results ($p<0.05$) emboldened.

Term	*Estimate of Coefficient*	*Standard Error*	*t-value*	*p-value*
Intercept	-2.107	0.317	-6.642	**<0.001**
Host Loch	0.596	0.461	1.294	0.213
Top of Host Stream	1.200	0.406	2.953	**0.009**
Weight	0.743	0.167	4.458	**<0.001**
Male	-0.382	0.190	-2.012	0.060
Host Loch x Weight	-0.401	0.289	-1.387	0.183
Top of Host Stream x Weight	-0.648	0.253	-2.562	**0.020**

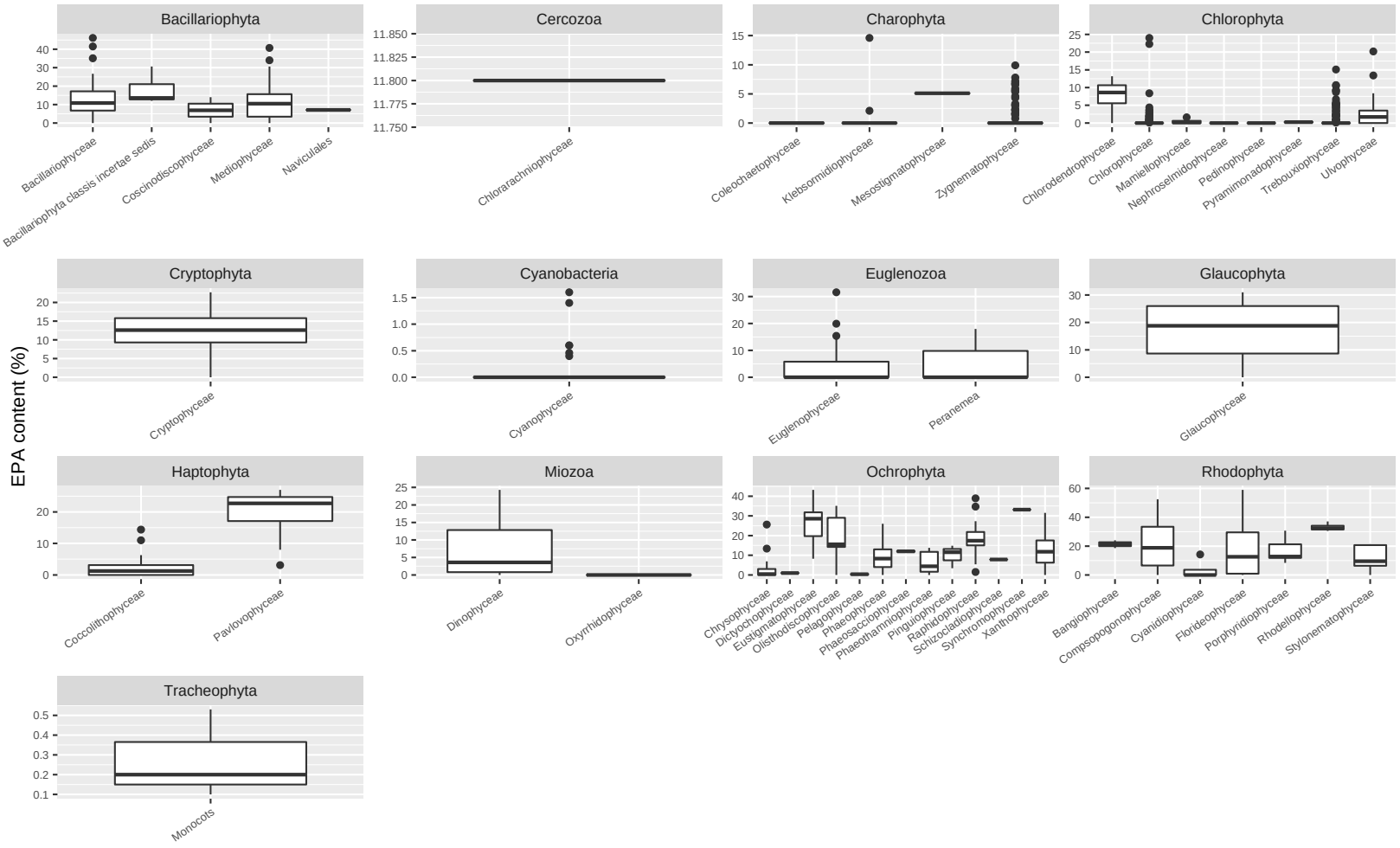

Figure S1. The EPA contents of species in 48 algae classes, grouped by corresponding phylum.

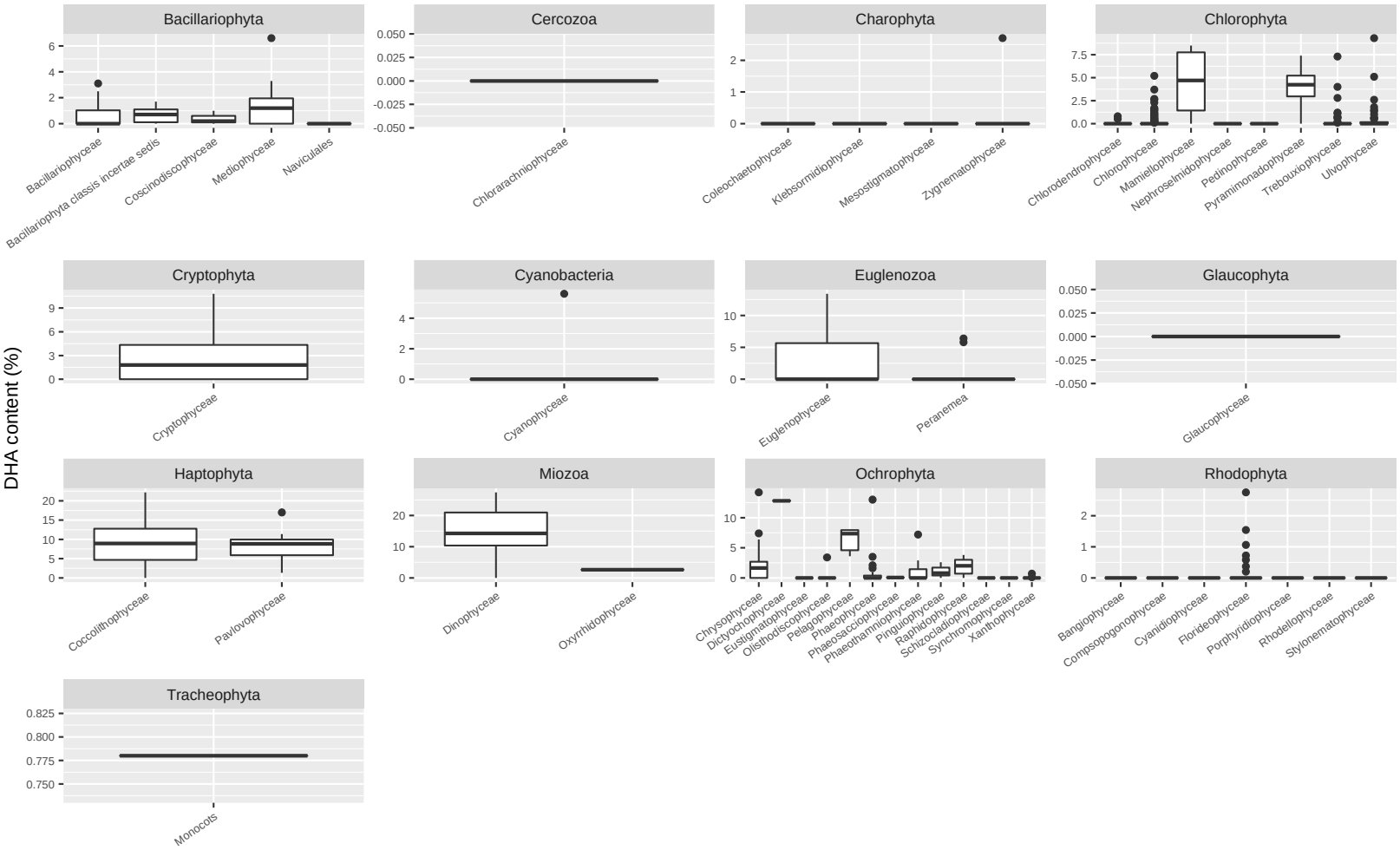

Figure S2. The DHA contents of species in 48 algae classes, grouped by corresponding phylum.