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Research

Fit and fatty freshwater fish: contrasting polyunsaturated fatty acid phenotypes between hybridizing stickleback lineages

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Long-chain polyunsaturated fatty acids are biologically important lipids that are unevenly distributed between and throughout environments. This heterogeneity can affect the evolution of metabolic processes, as populations adapt to the resource landscape that they encounter. Here, we compare fatty acid phenotypes of stickleback over two time scales of evolutionary divergence: between two lineages with different metabolic capacities for fatty acid synthesis (i.e. different copy number of the fatty acid desaturase gene; *FADS2*) that independently colonized European freshwaters during the Pleistocene and Holocene; and between two ecotypes within each lineage that have diverged more recently (~150 years) in different habitats (i.e. lake and stream). We measured fatty acid profiles of wild-caught and lab-reared fish for each lineage and ecotype combination after rearing lab fish on a diet deficient in omega-3 long-chain polyunsaturated fatty acids. Since these lineages hybridize in nature, we also measured profiles of lab-reared hybrids and backcrosses raised on the same deficient diet. Wild fish showed strong compositional differences in fatty acids between habitats, lineages and sexes. Common garden fish had generally lower polyunsaturated fatty acid levels than wild fish, and females had lower omega-6:omega-3 than males. Fish from the lineage with fewer *FADS2* copies also had lower levels of docosahexaenoic acid. Overall, we document divergence in fatty acid phenotypes between stickleback lineages with different histories of freshwater colonization, and between ecotypes in the early stages of adaptive population divergence.

Keywords: adaptation, colonization, *FADS2*, long-chain polyunsaturated fatty acids, metabolism

Introduction

The essential compounds required for consumer growth and survival are heterogeneously distributed in nature, leading to potential mismatches between dietary

supply and demand. Long-chain polyunsaturated fatty acids (LC-PUFAs; carbon chain ≥ 20) are biologically important lipids that vary in their distribution and abundance among ecosystems, between habitats and among prey (Hixson et al. 2015). LC-PUFAs include both omega-6 (n-6) and omega-3 (n-3) fatty acids, such as arachidonic acid (ARA; C20:4n-6), eicosapentaenoic acid (EPA; C20:5n-3), docosapentaenoic acid (DPA; C22:5n-6) and docosahexaenoic acid (DHA; C22:6n-3). These compounds play critical roles in endocrine regulation, cognition, immune function and reproductive output (Das 2006, Bell and Tocher 2009), but the optimal n-6:n-3 likely varies among species (Sargent et al. 1995). Imbalanced diets rich in n-6 fatty acids are linked to multiple disease pathologies that can be reduced through increased dietary intake of n-3 LC-PUFAs (Simopoulos 2006). Thus, vertebrates can gain fitness benefits by balancing their consumption and synthesis of PUFAs to achieve lower ratios n-6:n-3 in general (Simopoulos 2002, 2006, Glencross 2009), and by accumulating higher levels of n-3 fatty acids for reproduction in particular (Twining et al. 2018).

Consumers vary widely in their ability to acquire LC-PUFAs from dietary sources, and in their metabolic capacity for synthesis, specifically how they desaturate and elongate fatty acids (Parrish 2009). However, we know little about how LC-PUFA heterogeneity in nature can affect the evolution of consumer acquisition and metabolism. Many marine fish and terrestrial carnivores, for example, have lost the capacity to synthesize LC-PUFAs, likely because they can meet their nutritional requirements directly from dietary sources (Tocher 2010). In contrast, detritivores that feed on low-PUFA diets rely entirely on their internal metabolism to synthesize LC-PUFAs from shorter chain precursors (SC; carbon chain ≤ 18) (Malcicka et al. 2018). Most consumers have an intermediate metabolic capacity. Vertebrates, for example, lack the required enzymes to produce linolenic acid (LIN; C18:2n-6) and α-linolenic acid (ALA; C18:3n-3), but can synthesize LC-PUFAs to varying degrees when these SC-PUFAs are available (Supporting information) (Tinoco 1982).

The diversity of metabolism among species, populations and individuals likely reflects tradeoffs associated with acquiring and synthesizing LC-PUFAs to meet nutritional demands. There is increasing evidence that strong nutritional contrasts lead to the evolution of both consumer metabolism and resource acquisition traits. For example, greater production of n-3 LC-PUFAs in marine compared to freshwater primary producers (Arts et al. 2009) can generate positive selection in freshwater consumers for increased metabolic capacity (Østbye et al. 2018). Fish species that have successfully colonized freshwater environments show substantial variation in their capacity to both acquire DHA-rich prey items (Schmid et al. 2019) and to synthesize DHA (Ishikawa et al. 2019). As another example, the limited production of EPA by terrestrial compared to aquatic primary producers (Twining et al. 2016) can impose contrasting selection pressures on consumers with differential access to aquatic prey. For instance, tree swallows nesting close to water can increase the growth rate of their young by provisioning them with high-quality prey of aquatic origin (Twining et al. 2018), but those that nest further away likely face stronger tradeoffs between resource quality and accessibility.

Ray-finned fishes are useful for studying the evolution of consumer metabolism because they have repeatedly colonized freshwater from marine habitats and have evolved to deal with low LC-PUFA availability of their prey (Arts et al. 2009, Hixson et al. 2015). In vertebrates, LC-PUFA synthesis occurs through desaturation and elongation of precursor fatty acids by enzymes produced by the fatty acid desaturase (*FADS*) and fatty acid elongase (*ELOVL*) gene families (Bláhová et al. 2020) (Supporting information). Most freshwater fish only have the *FADS2* desaturase (Castro et al. 2012, Bláhová et al. 2020), a rate-limiting enzyme in LC-PUFA biosynthesis. The *FADS2* gene has been independently duplicated across multiple freshwater fish species, allowing them to increase *FADS2* expression and hence, their capacity to synthesize LC-PUFAs. In the Pacific marine lineage of threespine stickleback there was an ancient copy-and-paste transposition of *FADS2* from the X-chromosome to chromosome 12, followed by further duplications in the oldest freshwater populations; some now possess up to eight copies, compared to marine populations with only one or two copies (Ishikawa et al. 2019). In general, this metabolic adaptation has allowed freshwater fish to both persist and diversify in comparatively nutrient-poor habitats.

Threespine stickleback provide a practical model to study nutritional adaptation because freshwater populations vary widely in their 1) timing of colonization (Fang et al. 2020), 2) *FADS2* copy number (Ishikawa et al. 2019), 3) adaptations to different resource environments (McGee et al. 2013, Schmid et al. 2019) and 4) foraging performance (Svanbäck and Bolnick 2007, Best et al. 2017). Continental Europe has multiple evolutionarily distinct lineages (Hewitt 2000, Mäkinen and Merilä 2008, DeFaveri et al. 2012, Sanz et al. 2015, Fang et al. 2020), that differ substantially in the timing of freshwater colonization (i.e. pre- and post-glacially) (Fang et al. 2020). Given the northeasterly retreat of glaciers, southern and western freshwater stickleback populations are much older than those in the northeast. For example, marine populations were able to colonize southern Europe from the Mediterranean Sea while northeastern Europe was either under ice, or distant from marine populations. Here, we focused on two lineages: one that colonized before the last ice age, represented by fish from Lake Geneva, and one that colonized after the last ice age, represented by fish from Lake Constance. Consistent with differences in the timing of freshwater colonization, stickleback from Lake Geneva have more *FADS2* copies than those from Lake Constance (Ishikawa et al. 2019) (Fig. 1). Furthermore, because both populations were recently introduced to Switzerland in the past ~150 years where they colonized distinct habitats (Lucek et al. 2010, Hudson et al. 2021a), they are also useful for investigating the early stages of adaptive divergence (Marques et al. 2016). This allows us to quantify differentiation in fatty-acid phenotypes over two distinct evolutionary time scales. Overall, our study builds upon the recent discovery of metabolic adaptations of freshwater stickleback

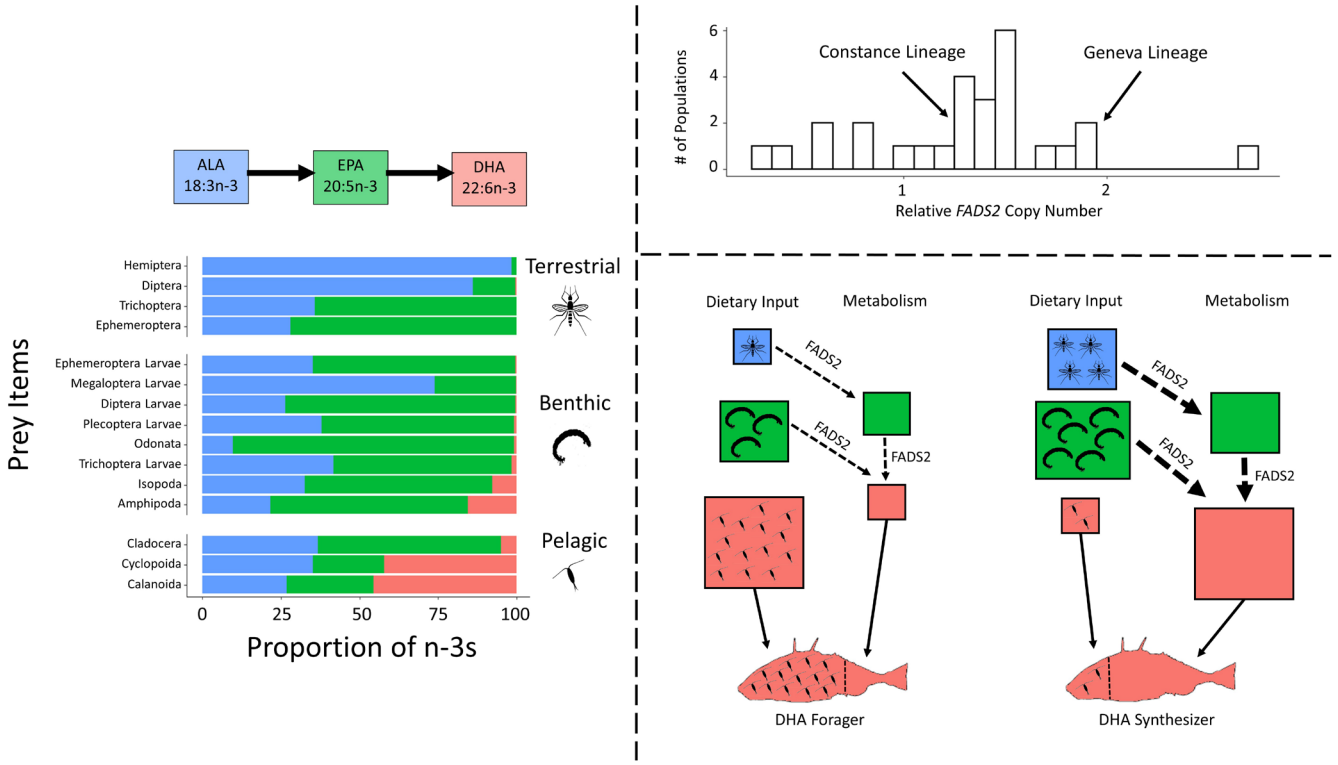

Figure 1. Left panel – relative proportion of n-3 PUFAs collected from the literature (Supporting information) of invertebrate prey, grouped by habitat, with a simplified DHA synthesis pathway above (see the Supporting information for complete pathway). Right panel, top – relative *FADS2* copy number of freshwater female stickleback populations using data from Ishikawa et al. (2019) (Supporting information). Right panel, bottom – two evolutionary strategies for obtaining DHA. Coloured squares represent relative contributions of each PUFA to total muscular concentration in the fish, arrows depict metabolic *FADS2* activity. In the forager strategy, fish metabolism does not evolve and populations adapt to a pelagic diet that is predominantly DHA rich plankton. 2) In the synthesizer strategy, *FADS2* gene duplications allow the fish to convert a greater proportion of dietary precursors into DHA metabolically. Both strategies enable stickleback populations to persist in freshwater habitats, but may have fitness consequences depending on prey availability.

(Ishikawa et al. 2019) by comparing the fatty acid profiles of two divergent lineages (as well as their hybrids and backcrosses), and by quantifying recent divergence associated with lake–stream ecotype formation.

Methods

Experimental system

We focus on two divergent lineages that recently invaded Switzerland, and are widespread in lakes and streams (Hudson et al. 2021a). One lineage originating from the Baltic drainage (nominal species *Gasterosteus aculeatus*) colonized Lake Constance, and the other lineage originating from the upper Rhône (nominal species *Gasterosteus gymnurus*) colonized Lake Geneva (Lucek et al. 2010, Marques et al. 2016, 2019). We hereafter refer them by their respective drainage. Importantly, these lineages differ in *FADS2* copy number, with the Geneva populations exhibiting higher relative copy numbers compared to Constance populations (Ishikawa et al. 2019). The lineages also differ in defensive traits, with Constance fish being fully plated and Geneva being low-plated, and foraging traits, with Constance fish having a more pelagic phenotype and a greater feeding efficiency on zooplankton (Lucek et al. 2013, Best et al. 2017). Both drainages also possess lake and stream adapted ecotypes that have evolved rapidly since their introduction (Lucek et al. 2014b, Marques et al. 2016). These ecotypes exhibit diet differentiation, with lake fish consuming more plankton, and stream fish consuming more benthic or terrestrial invertebrates (Gross and Anderson 1984, Lucek et al. 2012, 2013, Moser et al. 2012). In this study we collected wild individuals from lake and stream sites from both drainage, and conducted two independent common garden rearing experiments using a high n-6:n-3 diet (i.e. a low quality food). In the first experiment, we compared LC-PUFA profiles of stickleback from both lineages and ecotypes, grown in the lab and captured from the field. In the second experiment, we reared lake populations from Constance and Geneva, their hybrids, and backcrosses in the lab. We did this because hybridization between these two lineages is prevalent across Switzerland (Lucek et al. 2010, Roy et al. 2015), and introgression (Marques et al. 2019) might affect LC-PUFA metabolism. We also examined sex-based differences, because the ancestral position of *FADS2* is

on the X-chromosome, and thus female stickleback have at least one additional copy than males (Ishikawa et al. 2019). Overall, our four population contrasts allow us to compare the LC-PUFA metabolism of two divergent stickleback lineages, with known differences in *FADS2* copy number variation, as well as the capacity of different ecotypes to accumulate and synthesize LC-PUFAs in two environments with contrasting resource distributions.

Wild-caught fish

In May 2019 we sampled one lake and one stream population from each drainage using minnow traps. The Geneva sites, Le Grand Canal (Lake; 46°23′48.372″N, 06°53′14.2224″E) and Grand Fossé (Stream; 46°20'52.1052″N, 06°54'37.6416″E) were ~6 km apart, while the Lake Constance sites, Jägershaus Marina (Lake; 47°29'24.9504″N, 09°32'52.5156″E) and Aubach (Stream; 47°19'21″N, 09°33'20.4948″E) were ~30 km apart. Following capture, we euthanized fish with 1 g l^{-1} ethyl 3-aminobenzoate methanesulfonate and dissected fillets from 15 adult individuals per site, determining sex during dissection and from external characteristics (i.e. nuptial colouration in males, gravidness in females). All individuals were sexually mature, and between 30 and 70 mm in standard length. Prior to fatty acid profiling, we freeze-dried these tissues, and stored them at −80°C.

Lab-reared fish

Fish breeding and husbandry

Adult fish were captured from the wild using steel minnow traps and dip nets, transported by car to our lab in drums with portable aerator pumps, and euthanized on the same day of capture with 1 g l^{-1} ethyl 3-aminobenzoate methanesulfonate (MS-222) prior to artificial spawning. To produce clutches in the lab, mature eggs were stripped from female stickleback into petri dishes containing water, and mixed with milt from macerated testes dissected out of the body cavities of males. Eggs were then reared in flow-through incubators until hatching, when juveniles were transferred to aquaria. Stickleback populations were housed in flow-through aquaria (50 l) at densities of 20–40 fish per tank with filtered lake water from Lake Lucerne. Oxygen concentrations are typically maintained at > 8 mg l^{-1}, and the temperature follows the natural seasonal variation in the lake (5–20°C). All juvenile fish were reared on a diet of live artemia nauplii until they were large enough to consume finely chopped frozen chironomid larvae (typically 4–6 weeks post-hatching). Some of the individuals produced are used directly in experiments, while others are maintained as stock populations for future breeding (Supporting information).

Common garden experiment one

To explore differences in LC-PUFA metabolism between stickleback lineages, and whether they differ between populations inhabiting the two freshwater habitats that they occupy in nature (lake–stream contrast), we compared fatty acid profiles of lab-reared populations that were fed the same low n-3 PUFA diet (Supporting information). The fish used in the first common garden experiment were reared from individuals collected from each field site (described in section b) in early April, and bred as described above between 2013 and 2015. Prior to fatty acid profiling, fish (105 individuals total; 28 Geneva lake, 19 Geneva stream, 28 Constance lake, 30 Constance stream) were euthanized and dissected, though they were not sexed in the first common garden experiment.

Common garden experiment two

To investigate the effects of sex and lineage on LC-PUFA phenotype, we conducted a second common garden experiment comparing lake populations representing the two focal lineages. We reared fish from Lake Geneva (Le Grand Canal; 46°23′48.372″N, 06°53′14.2224″E) and Lake Constance (Marina Rheinhof; 47°29'51.5148″N, 09°33'33.2064″E) to produce the fish used in the second common garden experiment from stock populations maintained in husbandry that originate from the same 2013 and 2015 field seasons. The parents of fish from the second experiment were bred in 2017, and individuals in this common garden were two years old when used for fatty acid profiling. There were five treatments based on parental identity: Geneva × Geneva, Geneva × F_1 Hybrid backcross, Geneva × Constance, Constance × F_1 Hybrid backcross and Constance × Constance. All fish were fed the same low n-3 PUFA diet, and muscle tissues were sampled from 13 to 15 individuals per treatment (Supporting information).

Fatty acid profiling, FAMES

We extracted fatty acids in 2:1 dichloromethane/methanol (4×) with the addition of *n*-C19:0 fatty acid as a recovery standard. Resulting extracts were methylated with 1 ml of 14% BF_3 in methanol (two h at 100°C) and quantified by gas chromatography – flame ionization detection (GC-FID). Compounds were identified based on retention times relative to a laboratory standard (Supelco FAME mix C4-C24 18919-1AMP; batch # LC02357). We calculated the concentration of each compound in micrograms per gram (μg g^{-1}) of tissue by dividing peak areas by recovery standard peak area, multiplying this by the total mass of *n*-C19:0 in μg for each sample, and dividing this by the mass of dried tissue.

Statistical analyses

To explore the influence of lineage, sex and habitat we performed type III ANOVAs in R ver. 3.6.1. (<www.r-project.org>), on mean concentration of each PUFA of interest for each population, and on the n-6:n-3 of the same PUFAs (n-6: LIN + ARA + DPA)/(n-3: ALA + EPA + DHA). When one or more of the compounds was undetected, we excluded this individual from the n-6:n-3 analysis. In the second experiment, we tested for differences between crosses and sexes rather than including lineage in the model, as the populations included F_1 hybrid and F_2 backcross individuals. We did not determine the sex of individuals in our first common garden experiment.

Results

Wild populations

We found that ALA, LIN, EPA, ARA, DHA and DPA concentrations differed significantly between wild fish captured from lake and stream habitats (Table 1, Fig. 2), and that LIN concentrations were lower in Geneva lake fish than stream fish. Stream populations had higher ARA than lake populations irrespective of lineage. In Constance, lake fish had higher concentrations of EPA than stream fish, and both ecotypes from Geneva (Supporting information). In stream populations, male fish exhibited higher DHA concentrations than females. We also found that females only had higher EPA than males in lake fish from Constance, but not in the other contrasts (Fig. 2). Finally, we found a significant interaction between lineage, habitat and sex for DPA, where Geneva males had higher concentrations than females, Constance females had higher concentrations than males, and stream fish had higher concentrations than lake fish (Fig. 2).

Common garden 1. Comparison of LC-PUFA production between ecotype and lineage

Rearing individuals in a common garden on a low n-3 diet (Table 2, Fig. 3) caused stark differences in the fatty acid profiles between lineages. In the n-3 metabolic pathway, Geneva fish had substantially more DHA and less ALA and EPA (Fig. 3), than Constance fish. In the n-6 metabolic pathway, Geneva fish also had lower concentrations of the shorter chain fatty acids LIN and ARA than Constance fish (Fig. 3). Except for DHA, which was best explained by lineage, all PUFA concentrations depended on a combination of lineage and ecotype (Table 2). For example, Constance stream fish had the highest EPA concentration, while Geneva stream fish had the lowest (Supporting information).

We found widespread PUFA differences between wild-caught and lab-reared fish (Table 3). Most PUFAs depended strongly on a combination of lineage, ecotype and rearing environment. Interestingly, DHA concentrations were higher in all wild-caught populations (with the exception of Constance stream females) compared to lab-reared fish (Table 3), confirming that our common diet was low in LC-PUFAs. Lab-reared populations also exhibited an abundance of shorter-chain C18 fatty acids compared to wild populations (Supporting information).

Common garden 2. Heritable differences in LC-PUFA production between lineage and sex

We found that female fish consistently had higher ALA concentrations than males (Fig. 4). For LIN and EPA, fish from the Geneva lineage, Geneva backrosses and F_1 hybrids had lower concentrations compared to Constance fish and

Table 1. Statistical tests of PUFA variation in wild-caught fish populations – note ALA not detected in all fish. The table shows sums of squares (Sum Sq), degrees of freedom (df), F-values and p-values (bold = $p < 0.05$) from type III ANOVAs of the six PUFAs of interest with lineage, habitat and sex as factors.

	Sum Sq	df	F-value	p-value
ALA (C18:3n-3)				
Lineage	0.00E+00	1,29	0.00	0.956
Habitat	1.35E+00	1,29	27.62	**< 0.001**
Sex	4.80E-02	1,29	0.99	0.329
Lineage × Habitat	1.33E-01	1,29	2.71	0.110
Lineage × Sex	5.50E-02	1,29	1.12	0.298
Habitat × Sex	2.90E-02	1,29	0.60	0.446
Lineage × Habitat × Sex	5.30E-02	1,29	1.08	0.307
LIN (C18:2n-6)				
Lineage	8.00E-02	1,49	1.18	0.284
Habitat	1.91E+00	1,49	28.32	**< 0.001**
Sex	0.00E+00	1,49	0.07	0.794
Lineage × Habitat	4.10E-01	1,49	6.09	**0.017**
Lineage × Sex	6.00E-02	1,49	0.87	0.357
Habitat × Sex	5.00E-02	1,49	0.81	0.373
Lineage × Habitat × Sex	5.00E-02	1,49	0.70	0.407
EPA (C20:5n-3)				
Lineage	3.00E-02	1,49	1.27	0.265
Habitat	4.50E-01	1,49	18.51	**< 0.001**
Sex	5.00E-02	1,49	2.02	0.162
Lineage × Habitat	7.20E-01	1,49	29.53	**< 0.001**
Lineage × Sex	2.00E-02	1,49	0.94	0.338
Habitat × Sex	4.00E-02	1,49	1.59	0.213
Lineage × Habitat × Sex	5.00E-02	1,49	2.19	0.145
ARA (C20:4n-6)				
Lineage	1.00E-02	1,49	0.37	0.548
Habitat	8.20E-01	1,49	25.14	**< 0.001**
Sex	2.00E-02	1,49	0.58	0.449
Lineage × Habitat	5.00E-02	1,49	1.51	0.226
Lineage × Sex	2.00E-02	1,49	0.75	0.392
Habitat × Sex	4.00E-02	1,49	1.23	0.272
Lineage × Habitat × Sex	8.00E-02	1,49	2.36	0.131
DHA (C22:6n-3)				
Lineage	4.00E-02	1,49	0.92	0.343
Habitat	8.30E-01	1,49	20.14	**< 0.001**
Sex	4.70E-01	1,49	11.43	**0.001**
Lineage × Habitat	1.30E-01	1,49	3.04	0.087
Lineage × Sex	0.00E+00	1,49	0.06	0.813
Habitat × Sex	2.10E-01	1,49	4.98	**0.030**
Lineage × Habitat × Sex	1.60E-01	1,49	3.80	0.057
DPA (C22:5n-6)				
Lineage	0.00E+00	1,49	0.06	0.808
Habitat	6.10E-01	1,49	18.31	**< 0.001**
Sex	0.00E+00	1,49	0.01	0.921
Lineage × Habitat	0.00E+00	1,49	0.00	0.999
Lineage × Sex	9.00E-02	1,49	2.58	0.115
Habitat × Sex	0.00E+00	1,49	0.07	0.793
Lineage × Habitat × Sex	1.60E-01	1,49	4.86	**0.032**
n-6:n-3 *PUFAs*				
Lineage	0.00E+00	1,49	0.01	0.908
Habitat	7.10E-02	1,49	128.02	**< 0.001**
Sex	7.00E-03	1,49	13.46	**0.001**
Lineage × Habitat	2.20E-02	1,49	40.51	**< 0.001**
Lineage × Sex	1.00E-03	1,49	1.34	0.257
Habitat × Sex	7.00E-03	1,49	12.66	**0.001**
Lineage × Habitat × Sex	2.00E-03	1,49	3.86	0.059

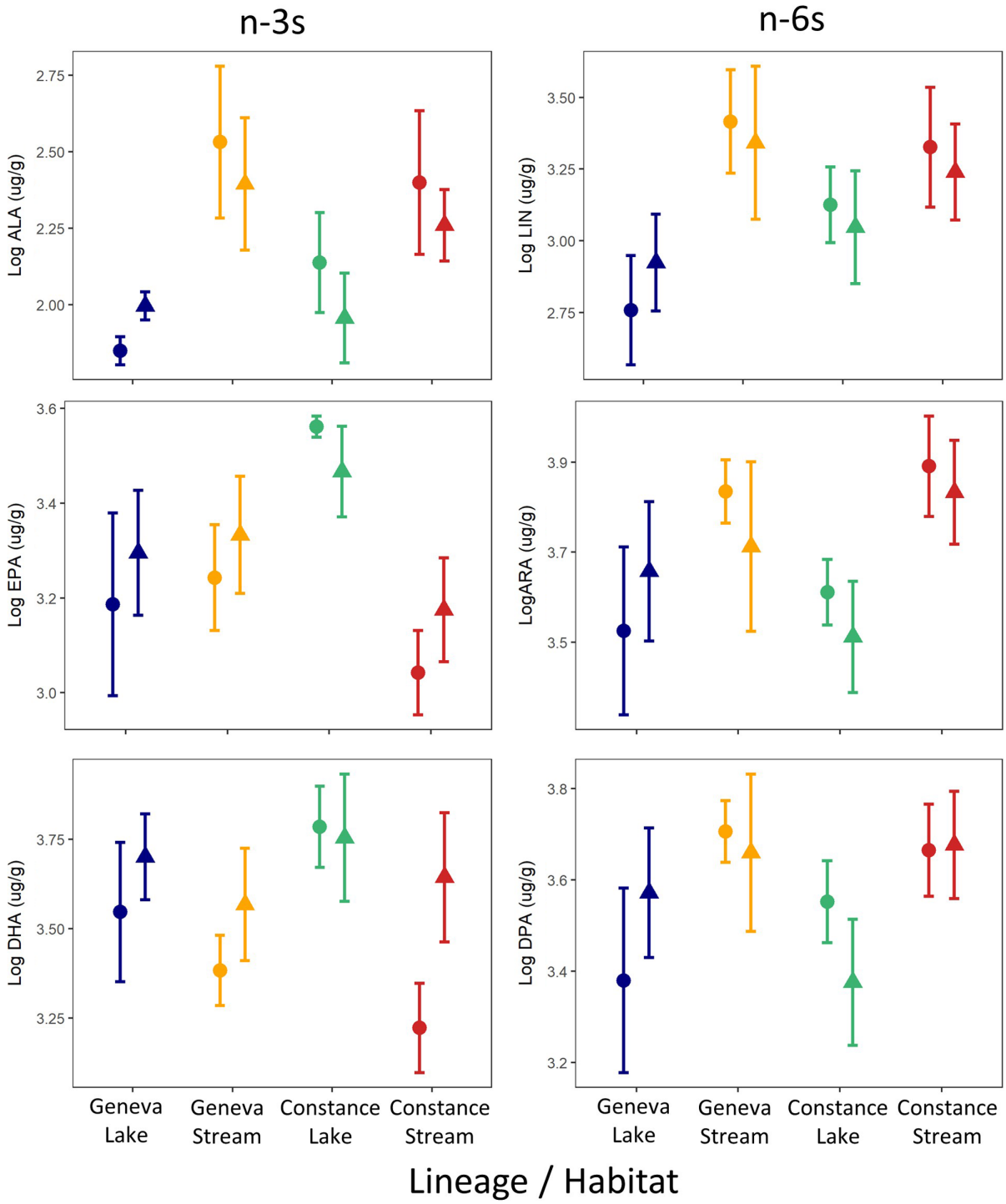

Figure 2. Fatty acid concentrations of n-3 (left panels) and n-6 PUFAs (right panels) for wild caught stickleback populations; males = triangles, females = circles.

Constance backrosses (Supporting information). There were no significant differences among populations or between sexes for ARA or DPA concentrations. Overall mean DHA concentrations varied from 539 μg g^{-1} (Constance males) to 1403 μg g^{-1} (Geneva backcross females), and we found a significant effect of both sex and cross on DHA concentrations (Table 4, Fig. 4). Given that the ancestral copy of *FADS2* is on the X chromosome, we expected that females would have higher DHA concentrations than males, however this was only the case for fish from Constance (post hoc test using Holm correction $p = 0.0044$; Fig. 4). In addition, we expected fish with greater genetic contributions from the Geneva lineage (i.e.

pure Geneva lineage and Geneva backcross individuals) to have higher DHA concentrations when reared in captivity, however this was only evident in males, where the DHA concentrations of Geneva males ($p = 0.0197$) and Geneva backcrosses ($p = 0.0129$) were higher than for Constance lake fish (Fig. 4).

Ratio of n-6:n-3 PUFAs

Our comparison of wild populations revealed the influence of ecotype on n-6:n-3, and the interactions between lineage, habitat and sex highlight possible ecological differences between populations (Table 1, Fig. 5). Constance lake fish had the lowest n-6:n-3 overall (i.e. most favorable), Constance stream fish had the highest (i.e. least favorable). Both stream populations had higher n-6:n-3 than lake populations, and stream males had lower n-6:n-3 than stream females in both lineages. In both experiments, Geneva fish had a significantly lower n-6:n-3 than Constance fish, irrespective of ecotype (Table 2, 4, Fig. 5). In the second experiment, we additionally found that females from all contrasts had a significantly lower n-6:n-3 than males (Table 4, Fig. 5), consistent with the higher DHA ratios of females to males (Fig. 4).

Table 2. Statistical tests of PUFA variation for the first common garden exploring the lineage by habitat contrast. The table shows sums of squares (Sum Sq), degrees of freedom (df), F-values and p-values (bold = $p < 0.05$) from type III ANOVAs of the six PUFAs of interest with lineage and habitat as factors.

	Sum Sq	df	F-value	p-value
ALA (C18:3n-3)				
Lineage	2.14E+00	1,100	54.17	**< 0.001**
Habitat	1.10E-01	1,100	2.87	0.094
Lineage × Habitat	4.30E-01	1,100	10.79	**0.001**
LIN (C18:2n-6)				
Lineage	2.57E+00	1,95	156.10	**< 0.001**
Habitat	3.00E-02	1,95	2.08	0.153
Lineage × Habitat	2.00E-01	1,95	12.28	**0.001**
EPA (C20:5n-3)				
Lineage	7.00E-02	1,101	10.18	**0.002**
Habitat	0.00E+00	1,101	0.35	0.553
Lineage × Habitat	5.00E-02	1,101	7.38	**0.008**
ARA (C20:4n-6)				
Lineage	7.70E-01	1,101	125.65	**< 0.001**
Habitat	0.00E+00	1,101	0.65	0.423
Lineage × Habitat	5.00E-02	1,101	7.33	**0.008**
DHA (C22:6n-3)				
Lineage	1.89E+00	1,97	73.88	**< 0.001**
Habitat	0.00E+00	1,97	0.11	0.747
Lineage × Habitat	4.00E-02	1,97	1.53	0.219
DPA (C22:5n-6)				
Lineage	1.00E-02	1,101	1.48	0.227
Habitat	0.00E+00	1,101	0.12	0.730
Lineage × Habitat	6.00E-02	1,101	6.84	**0.010**
n-6:n-3 *PUFAs*				
Lineage	5.60E-02	1,91	111.57	**< 0.001**
Habitat	0.00E+00	1,91	0.46	0.497
Lineage × Habitat	1.00E-03	1,91	2.07	0.153

Discussion

Overall, our results emphasize the potential fitness relevance of mismatches between the supply of, and demand for, LC-PUFAs in natural populations (Fig. 1). Specifically, we found that the fatty acid composition of wild stickleback is ecotype dependent, and that for n-3 LC-PUFAs such EPA and DHA, there are lineage and sex specific differences in muscular fatty acid concentrations (Fig. 2). When reared on a common diet in the lab, the lineage effects become more obvious, especially with respect to these LC-PUFAs. Such lineage-specific differences provide evidence for divergent evolution in metabolism over a timescale of thousands of generations, likely culminating in differential capacity to synthesize LC-PUFAs (Fig. 3). Within each lineage, we also find that ecologically and genetically differentiated lake and stream populations have contrasting PUFA distributions when raised in a common garden, suggesting much more recent divergence in the expression of fatty acid phenotypes (i.e. over tens to perhaps a hundred generations, and with gene flow). Our study is the first to document divergence in PUFA phenotypes not only between anciently divergent lineages, but also between recently diverged lake–stream ecotypes in one of our lineages (Constance). Furthermore, our hybrid crosses highlight important sex-specific differences in PUFA composition (Fig. 4, 5), and provide additional insight into how introgression between divergent lineages with different colonization histories may influence the capacity for DHA synthesis.

Differences in LC-PUFA production can partly explain the observed differences among lineages, ecotypes and sexes of stickleback reared on a low-quality resource. Fish from the Geneva lineage, which possess more copies of *FADS2*, had higher DHA concentrations in the laboratory compared to fish from the Constance lineage (Fig. 3, 4), and this was independent of their habitat of origin. This is consistent with previous work suggesting that the Geneva lineage has had a longer evolutionary history in freshwater, and are considerably more benthic in their habitat use, foraging mode and morphology (Berner et al. 2010, Lucek et al. 2013, 2014a, Best et al. 2017). The Constance lineage, by comparison, has a much more recent post-glacial colonization history and fewer copies of *FADS2* (Fig. 1). Furthermore, unlike in Lake Geneva, fish in Lake Constance feed predominantly on zooplankton in the pelagic zone of the lake (Lucek et al. 2012, Moser et al. 2012, Alexander et al. 2016), and have high foraging efficiency (Best et al. 2017, Schmid et al. 2019). Such lineage-specific contrasts in foraging habitat use, plankton feeding efficiency, and *FADS2* copy number suggest alternative strategies of meeting LC-PUFA needs. Specifically, we suspect that fish in Lake Geneva are acquiring more of their PUFA from endogenous production, whereas fish in Lake Constance are more likely acquiring PUFA from diet rich in pelagic copepods (Fig. 1).

In wild-caught fish, we observed that ALA and ARA concentrations were higher in stream fish, while EPA and DHA concentrations were higher in lake fish (Fig. 2). These

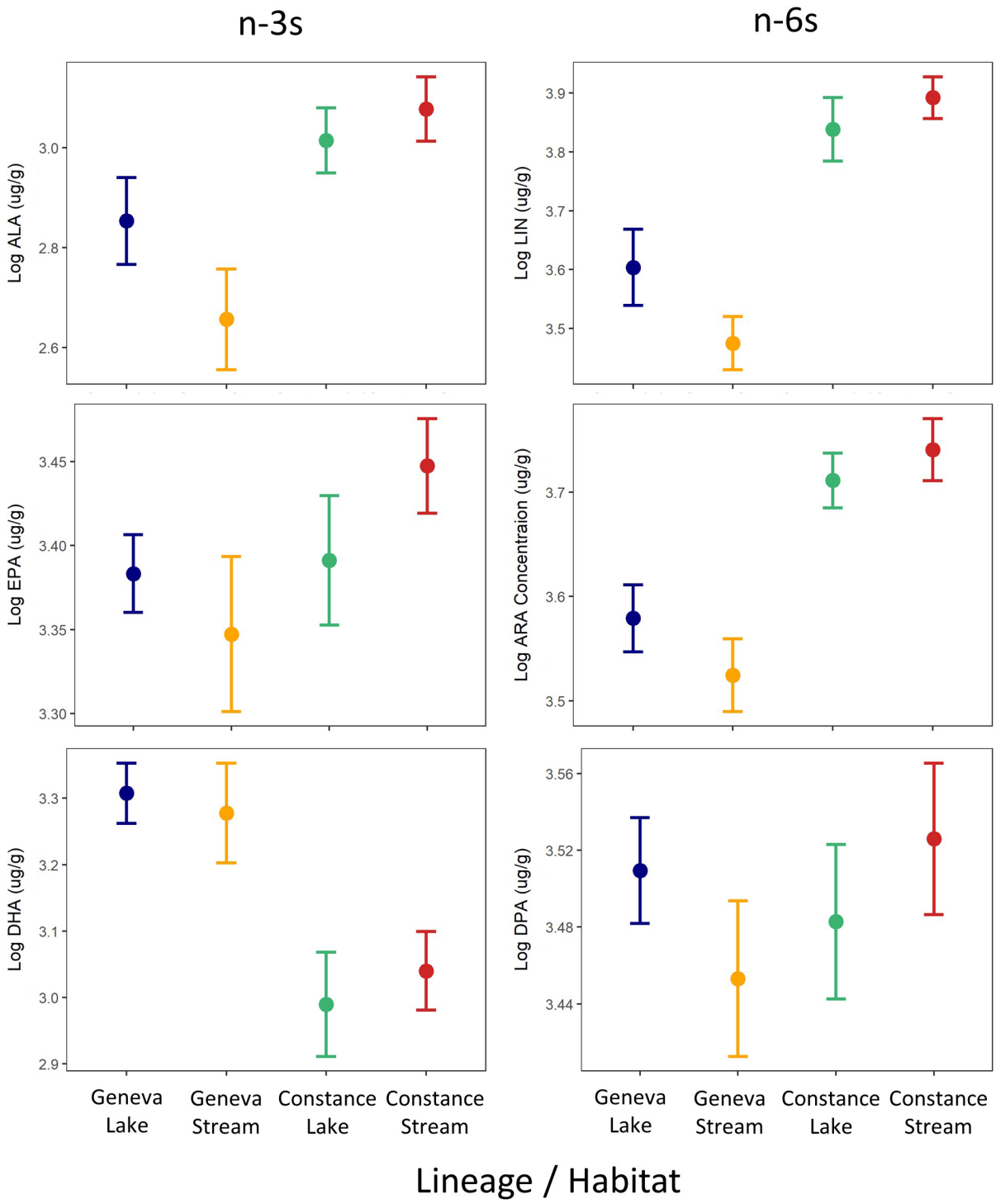

Figure 3. Fatty acid concentrations of n-3 (left panels) and n-6 PUFAs (right panels) from lab-reared stickleback populations in the first common garden experiment; sexes are unknown.

differences are likely explained by variation in diet and PUFA availability between habitats, rather than metabolic processes. The high ALA and ARA concentrations of stream fish could reflect a diet dominated by benthic invertebrates and terrestrial insects (Ahlgren et al. 2009), while the high EPA and DHA concentrations observed in lake stickleback, likely result from a diet of n-3 PUFA rich zooplankton (Lucek et al. 2012, Moser et al. 2012, Hudson et al. 2021a) (Fig. 1). Wild stream populations displayed higher n-6:n-3 (Fig. 5) compared to lake populations as well, suggesting that stream diets are lower n-3 PUFAs, and thus these fish may experience stronger selection for enhanced metabolism. DHA concentrations

did not differ between ecotypes in either lineage in common garden, but EPA concentrations were significantly different between ecotypes in the Constance lineage (Fig. 3).

We observed sex differences in PUFA composition in both lab-raised and wild-caught fish, and stark sex differences in DHA levels for lab-reared fish (Fig. 2, 4). Threespine stickleback have a XX female/XY male sex determination system that lacks a mechanism for global dosage compensation (Leder et al. 2010). This means that X-linked traits will exhibit female-biased expression, as genes on the X-chromosomes are simultaneously expressed. This is the case for *FADS2*, which is ancestrally located on the stickleback X-chromosome (Ishikawa et al. 2019). In the Constance lake population, DHA concentrations of lab raised females were roughly twice that of males (Supporting information), but this sex difference was lower in all other populations contrasts in which the Geneva lineage made a genetic contribution. While increased DHA synthesis is likely advantageous for all freshwater fish, it may be particularly beneficial to females, as eggs and developing embryos have high DHA requirements (Tocher 2010). In contrast to the Constance lineage, fish from the Geneva lineage have additional *FADS2* copies on the X-chromosome (Ishikawa et al. 2019). While these additional copies do increase DHA concentrations, synthesis does not appear to increase linearly with *FADS2* copy number (i.e. Geneva males and females did not significantly differ in DHA, despite females having more copies), and may indicate differences in expression or post-transcriptional regulation between autosomal and X-linked regions of the genome where *FADS2* is located.

Sex differences in wild-caught populations may also indicate ecological differences that result from *FADS2* copy number variation. When we compare DHA concentrations between wild-caught Constance stream fish, males have more than twice that of females so it may be that males compensate for their low biosynthetic ability by targeting different prey (Fig. 1), or that some form of niche partitioning exists in streams. An additional possibility is that hybridization between the multiple lineages that have been introduced to the Constance drainage (Marques et al. 2019) has led to introgression of autosomal *FADS2* copies in stream populations that are differentially expressed between the sexes. As we did not measure relative copy number, we cannot be certain of this, but it is a subject for continued research. Alternatively, since wild females were caught during the breeding season, it is also possible that they exhibited reduced muscular PUFA concentrations because of prior investment into eggs. Lab populations, however, were non-reproductive during this study. In addition, experiments establishing the links between foraging ability, sex, lineage and PUFA composition could help us explain these patterns further.

Freshwater habitats have lower LC-PUFA availability compared to marine habitats, but are these resources actually limiting for stickleback? Fatty acids are typically synthesized de novo by primary producers, and generally accumulate up the food chain culminating in fatty acid distributions that vary within and among freshwater habitats (Müller-Navarra et al. 2004), as well as among prey species

Table 3. Statistical tests for a comparison between PUFA content of wild-caught fish to those from the first common garden. The table shows sums of squares (Sum Sq), degrees of freedom (df), F-values and p-values (bold=$p < 0.05$) from type III ANOVAs of the six PUFAs of interest with lineage and habitat as factors.

	Sum Sq	df	F-value	p-value
ALA (C18:3n-3)				
Lineage	4.90E-01	1,133	11.61	**0.001**
Habitat	7.70E-01	1,133	18.28	**< 0.001**
Captivity	1.22E+01	1,133	290.20	**< 0.001**
Lineage × Habitat	0.00E+00	1,133	0.01	0.917
Lineage × Captivity	5.70E-01	1,133	13.60	**< 0.001**
Habitat × Captivity	1.46E+00	1,133	34.91	**< 0.001**
Lineage × Habitat × Captivity	3.90E-01	1,133	9.38	**0.003**
LIN (C18:2n-6)				
Lineage	1.45E+00	1,148	42.58	**< 0.001**
Habitat	9.80E-01	1,148	28.86	**< 0.001**
Captivity	1.11E+01	1,148	325.04	**< 0.001**
Lineage × Habitat	5.00E-02	1,148	1.47	0.227
Lineage × Captivity	5.60E-01	1,148	16.37	**< 0.001**
Habitat × Captivity	1.48E+00	1,148	43.45	**< 0.001**
Lineage × Habitat × Captivity	5.90E-01	1,148	17.47	**< 0.001**
EPA (C20:5n-3)				
Lineage	8.00E-02	1,154	6.07	**0.015**
Habitat	2.60E-01	1,154	18.79	**< 0.001**
Captivity	4.10E-01	1,154	29.71	**< 0.001**
Lineage × Habitat	3.00E-01	1,154	22.21	**< 0.001**
Lineage × Captivity	0.00E+00	1,154	0.11	0.745
Habitat × Captivity	3.20E-01	1,154	23.58	**< 0.001**
Lineage × Habitat × Captivity	6.90E-01	1,154	50.25	**< 0.001**
ARA (C20:4n-6)				
Lineage	3.80E-01	1,154	24.75	**< 0.001**
Habitat	4.80E-01	1,154	30.86	**< 0.001**
Captivity	1.20E-01	1,154	7.57	**0.007**
Lineage × Habitat	1.00E-01	1,154	6.63	**0.011**
Lineage × Captivity	1.90E-01	1,154	12.11	**0.001**
Habitat × Captivity	5.90E-01	1,154	37.97	**< 0.001**
Lineage × Habitat × Captivity	0.00E+00	1,154	0.28	0.596
DHA (C22:6n-3)				
Lineage	5.10E-01	1,150	14.12	**< 0.001**
Habitat	5.00E-01	1,150	13.88	**< 0.001**
Captivity	6.33E+00	1,150	176.70	**< 0.001**
Lineage × Habitat	4.00E-02	1,150	1.04	0.309
Lineage × Captivity	9.10E-01	1,150	25.46	**< 0.001**
Habitat × Captivity	5.90E-01	1,150	16.47	**< 0.001**
Lineage × Habitat × Captivity	1.90E-01	1,150	5.24	**0.023**
DPA (C22:5n-6)				
Lineage	0.00E+00	1,154	0.04	0.839
Habitat	3.70E-01	1,154	20.48	**< 0.001**
Captivity	2.30E-01	1,154	12.40	**0.001**
Lineage × Habitat	3.00E-02	1,154	1.43	0.234
Lineage × Captivity	1.00E-02	1,154	0.69	0.407
Habitat × Captivity	4.20E-01	1,154	23.23	**< 0.001**
Lineage × Habitat × Captivity	2.00E-02	1,154	1.06	0.305
n-6:n-3 *PUFAs*				
Lineage	1.70E-02	1,124	25.75	**< 0.001**
Habitat	5.80E-02	1,124	87.32	**< 0.001**
Captivity	4.00E-03	1,124	6.74	**0.011**
Lineage × Habitat	1.40E-02	1,124	21.66	**< 0.001**
Lineage × Captivity	1.20E-02	1,124	18.63	**< 0.001**
Habitat × Captivity	6.60E-02	1,124	99.01	**< 0.001**
Lineage × Habitat × Captivity	2.30E-02	1,124	35.22	**< 0.001**

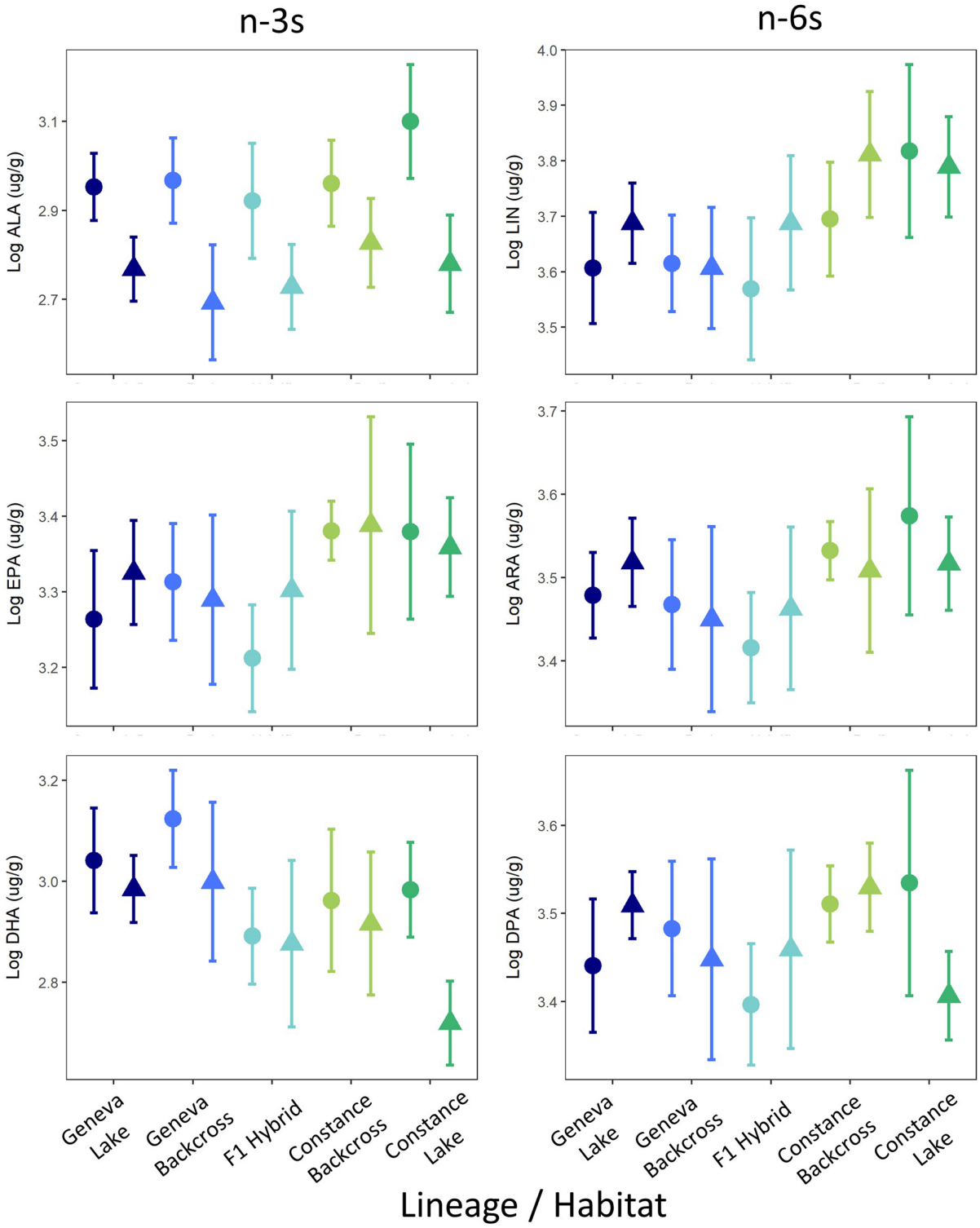

Figure 4. Fatty acid concentrations of n-3 (left panels) and n-6 PUFAs (right panels) from lab-reared stickleback populations in the second common garden; males = triangles, females = circles.

(Hixson et al. 2015). Freshwater copepods, for example, are omnivorous zooplankton with a marine ancestry that preferentially accumulate DHA (Strandberg et al. 2015). In comparison to other stickleback prey however, copepods are small and highly evasive, thus selection on fish foraging efficiency could drive the evolution of fish morphology and behaviours (Schmid et al. 2019). When we compare fatty acid profiles between wild-caught and lab-reared stickleback, there is a

stark difference in their LC-PUFA concentrations, especially for EPA and DHA (Supporting information). Although Constance lake males have a mean concentration of only 538 μg g^{-1} of DHA in the lab, wild-caught males had a mean concentration of 6502 μg g^{-1}. Presumably, this order of magnitude difference is the product of dietary differences between wild and lab-reared fish. This seems to indicate that despite having fewer copies of *FADS2* than the Geneva lineage, Constance lake fish are successful at obtaining LC-PUFAs in the wild by foraging on zooplankton. Comparisons between wild-caught populations further illustrate this point, in that fish from the Constance lineage have a lower n-6:n-3 ratio than fish from the Geneva lineage even though Geneva fish have greater biosynthetic capabilities (Fig. 5). How then, are wild Constance lake fish achieving such high n-3 concentrations? We propose that the expansion into the pelagic zone of the lake (Alexander et al. 2016), morphological adaptations for planktivory (Berner et al. 2010, Lucek et al. 2013, 2014c, Best et al. 2017), and rapid divergence between lake and stream populations in planktonic foraging efficiency (Schmid et al. 2019) can explain this pattern. By exhibiting a limnetic life history strategy, and a more pelagic phenotype (Lucek et al. 2014c, Best et al. 2017) Lake Constance stickleback are capable of obtaining high concentrations of fatty acids, despite lower metabolic capabilities. To explore this finding further, and understand the fitness consequences of foraging differences between the lineages, future studies of foraging performance and competition experiments under variable environmental contexts could be fruitful.

Swiss stickleback populations provide a powerful opportunity to study evolutionary responses to LC-PUFA limitation during freshwater colonization over two different time scales. The lineage contrast allows us to compare traits that have evolved since colonization of freshwater (i.e. increased *FADS2* copy number), to those that have been retained from ancestral marine populations (i.e. efficient plankton foraging seen in Constance lake fish), that help stickleback address the problem of acquiring LC-PUFAs in freshwater. Likewise, as both lineages were introduced within the past ~150 years, we can explore how these evolutionary solutions to LC-PUFA limitation apply across different freshwater habitats (i.e. lakes and streams) with different prey availabilities by comparing fatty acid composition and metabolism between recently diverged lake–stream ecotypes and hybrids between lineages. Specifically, older freshwater lineages (e.g. Geneva) might facilitate adaptation to streams in younger freshwater lineages (i.e. Constance) by contributing genes that are adaptive for LC-PUFA metabolism in freshwater (i.e. additional *FADS2* copies) as introgression between these lineages will increase the standing genetic variation available for selection to act upon. The results of our second common garden experiment show that hybridization and backcrossing produces offspring that have higher DHA concentrations relative to Lake Constance males, which have low *FADS2* copy number (Ishikawa et al. 2019). In stream habitats where PUFA-rich plankton are less abundant (Walks and Cyr 2004, Torres-Ruiz et al. 2007) this could be particularly beneficial.

Table 4. Statistical tests of PUFA variation for the second common garden exploring the lineage by sex contrast. The table shows sums of squares (Sum Sq), degrees of freedom (df), F-values and p-values (bold = $p < 0.05$) from type III ANOVAs of the six PUFAs of interest with population (i.e. parental lineages, F_1 hybrid and backcrosses) and sex as factors.

	Sum Sq	df	F-value	p-value
ALA (C18:3n-3)				
Population	1.20E-01	4,61	1.60	0.186
Sex	8.60E-01	1,61	44.24	**< 0.001**
Sex × Population	8.00E-02	4,61	0.97	0.429
LIN (C18:2n-6)				
Population	3.90E-01	4,61	4.74	**0.002**
Sex	5.00E-02	1,61	2.64	0.109
Sex × Population	7.00E-02	4,61	0.81	0.523
EPA (C20:5n-3)				
Population	1.60E-01	4,61	2.75	**0.036**
Sex	1.00E-02	1,61	0.64	0.425
Sex × Population	4.00E-02	4,61	0.63	0.642
ARA (C20:4n-6)				
Population	1.00E-01	4,61	2.34	0.065
Sex	0.00E+00	1,61	0.01	0.923
Sex × Population	3.00E-02	4,61	0.62	0.653
DHA (C22:6n-3)				
Population	4.30E-01	4,61	4.22	**0.004**
Sex	1.80E-01	1,61	7.02	**0.010**
Sex × Population	1.30E-01	4,61	1.28	0.289
DPA (C22:5n-6)				
Population	6.00E-02	4,60	1.22	0.314
Sex	0.00E+00	1,60	0.01	0.921
Sex × Population	9.00E-02	4,60	1.94	0.115
n-6:n-3 *PUFAs*				
Population	1.10E-02	4,60	6.14	**< 0.001**
Sex	3.50E-02	1,60	80.15	**< 0.001**
Sex × Population	1.00E-03	4,60	0.30	0.874

In this study we did not measure *FADS2* gene expression however, so it could also be valuable to do so. Similar patterns of introgression have been observed between the introduced Constance lineage, and native Swiss populations as the highly plated *Eda* allele has been spreading into historically low plated populations (Lucek 2016, Hudson et al. 2021a). As hybridization continues, it will be prudent to monitor the evolutionary history, abundance, habitat use and phenotype of stickleback populations in other Swiss lakes.

If we consider the role of LC-PUFAs as essential nutrients, differences in availability and abundance can create nutritional constraints that slow the rate that species can colonize new habitats, or act as a barrier and prevent establishment altogether (Ishikawa et al. 2019). For example, organisms that are unable to synthesize DHA from shorter chain n-3 fatty acids cannot subsist on a diet that contains ALA alone. Similarly, prey species containing DHA could be abundant within a habitat, but if predators are unable to effectively capture them, then the resource is essentially absent. When encountering physiological challenges and environmental stressors such as these, over time organisms may evolve adaptations to counteract their negative effects, provided heritable variation is available. Such heritable performance variation in nutrient acquisition or metabolism can therefore shape

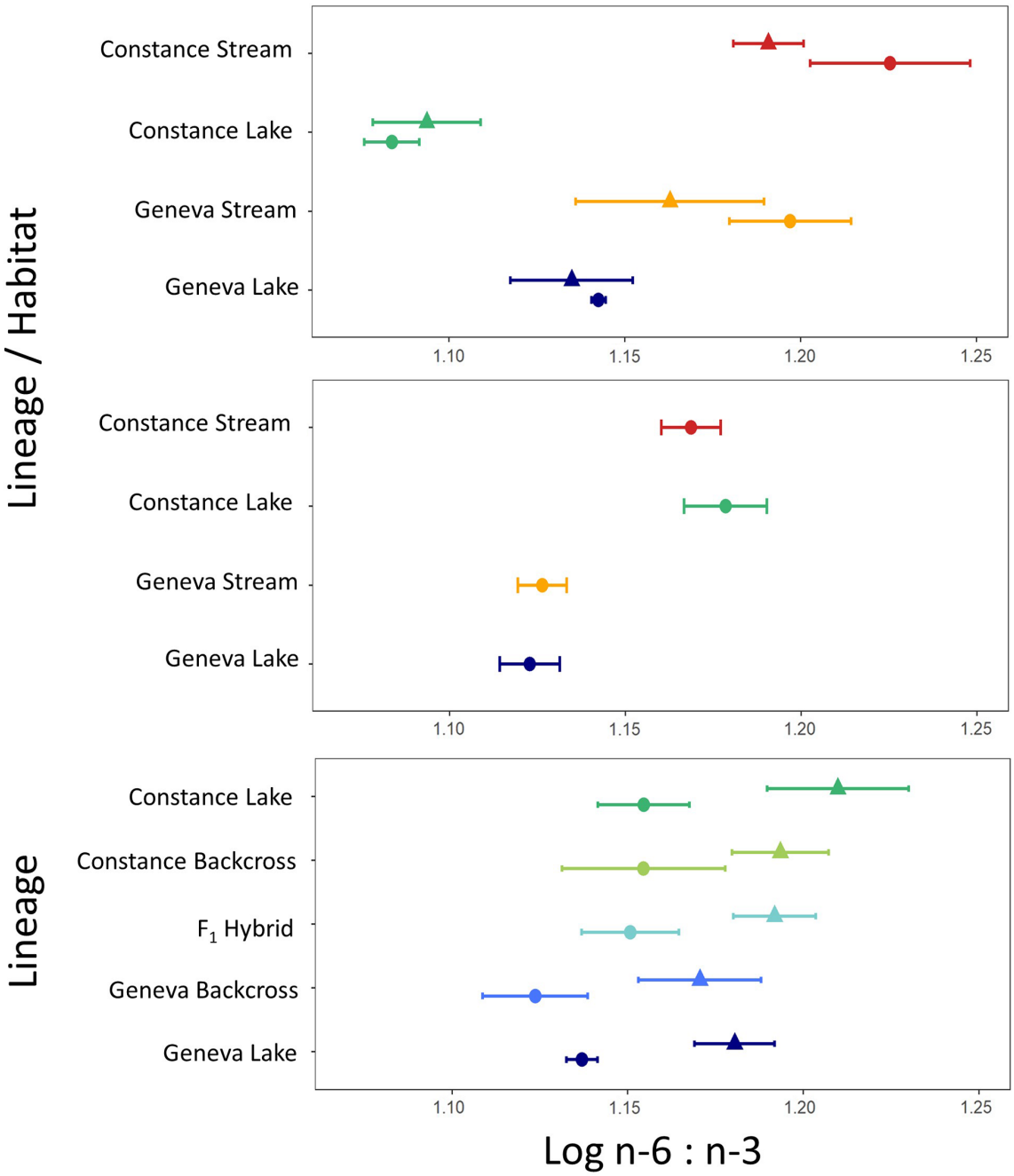

Figure 5. The n-6 to n-3 ratios of wild-caught fish (top panel) compared to lab-reared fish for the lake–stream contrast (common garden experiment one; middle panel) and line cross lineage contrast (common garden experiment two; bottom panel); top and bottom panel, males = triangles, females = circles; middle panel, sexes unknown.

evolutionary trajectories of consumer populations. There is a growing body of evidence that LC-PUFA availability can affect the fitness and health of consumers (Fritz et al. 2017, Twining et al. 2018, Scharnweber and Gårdmark 2020), highlighting the role that nutritional mismatches play in promoting adaptive divergence when environmental variation in nutrient availability interacts with heritable variation in coping ability. To better understand how species adapt to novel conditions, we need to further explore the factors that limit their success (i.e. PUFA availability) and the role of key metabolic adaptations that allow them to overcome such limitations (i.e. *FADS2* gene duplications).

Conclusion

During colonization, species may encounter novel selection pressures, including selection via nutrient limitation. If they can adapt to the novel conditions, such adaptations can accelerate the colonization process further. As stickleback populations

have colonized freshwater habitats and further diversified within them, they have adapted to environments with limited LC-PUFA availability through evolution of metabolism. Here we show variation in fatty acid composition between different populations of introduced freshwater stickleback, and demonstrate that these differences can be partly explained by variation in metabolic adaptations for LC-PUFA synthesis. Namely, lineages that differ in *FADS2* copy number have different capabilities for LC-PUFA biosynthesis, given the same diet. We also identify lineage and sex specific differences in fatty acid composition, and show that hybridization between divergent lineages could facilitate adaptation in LC-PUFA limited environments through introgression of genetic variation from populations with a longer evolutionary history in freshwater. Our study highlights the role of nutrient limitation as a constraint and selection force during colonization.

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Author contributions

Cameron M. Hudson: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing – original draft (lead); Writing – review and editing (equal). **S. Nemiah Ladd**: Conceptualization (equal); Data curation (equal); Formal analysis (supporting); Investigation (equal); Methodology (equal); Visualization (equal); Writing – review and editing (equal). **Miguel C. Leal**: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Visualization (supporting); Writing – review and editing (equal). **Carsten J. Schubert**: Data curation (equal); Funding acquisition (equal); Project administration (equal); Resources (equal); Supervision (equal); Writing – review and editing (equal). **Ole Seehausen**: Funding acquisition (equal); Project administration (equal); Supervision (equal); Writing – review and editing (equal). **Blake Matthews**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (lead); Resources (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing – review and editing (equal).

Data availability statement

Data are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.s4mw6m969> (Hudson et al. 2021b).

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