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Genome-phenotype-environment associations identify signatures of selection in a panmictic population of threespine stickleback

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Abstract

Adaptive genetic divergence occurs when selection imposed by the environment causes the genomic component of the phenotype to differentiate. However, genomic signatures of natural selection are usually identified without information on which trait is responding to selection by which selective agent(s). Here, we integrate whole-genome sequencing with phenomics and measures of putative selective agents to assess the extent of adaptive divergence in threespine stickleback occupying the highly heterogeneous lake Mývatn, NE Iceland. We find negligible genome wide divergence, yet multiple traits (body size, gill raker structure and defence traits) were divergent along known ecological gradients (temperature, predatory bird densities and water depth). SNP based heritability of all measured traits was high ($h^2 = 0.42–0.65$), indicating adaptive potential for all traits. Environment-association analyses further identified thousands of loci putatively involved in selection, related to genes linked to, for instance, neuron development and protein phosphorylation. Finally, we found that loci linked to water depth were concurrently associated with pelvic spine length variation - supporting the conclusion that divergence in pelvic spine length occurred in the face of gene flow. Our results suggest that whilst there is substantial genetic variation in the traits measured, phenotypic divergence of Mývatn stickleback is mostly weakly associated with environmental gradients, potentially as a result of substantial gene flow. Our study illustrates the value of integrative studies that combine genomic assays of multivariate trait variation with landscape genomics.

KEYWORDS
adaptive divergence, environmental gradients, *Gasterosteus aculeatus*, gene flow, genome scans, landscape genomics

1 | INTRODUCTION

Elucidating the genetic basis of adaptive divergence in natural populations is an enduring goal of evolutionary biology (Stinchcombe & Hoekstra, 2008). Doing so can provide insight into evolutionary processes occurring in the wild, including the mechanisms associated with adaptive divergence, and the extent to which divergence takes place in the face of gene flow (Räsänen & Hendry, 2008; Rudman et al., 2018). Genetically, adaptive divergence is expected to manifest as blocks of differentiation across the genome, at regions containing genes that contribute to adaptation to divergent local environments (Nosil et al., 2009). Genome scan studies that test these expectations have identified genomic regions associated with adaptation to divergent ecological niches in numerous species (e.g., Campbell-Staton et al., 2021; Marrano et al., 2018; Slate et al., 2002). This has been termed a "reverse ecology" approach, whereby loci associated with adaptation may be identified without measuring the traits themselves (Li et al., 2008). However, genome scan studies on wild populations are seldom able to provide precise information on which aspects of the phenotype selection is acting on, or which environmental factors are imposing selection (MacColl, 2011).

A comprehensive view on the genomic mechanisms associated with adaptive divergence requires studies that combine phenotypic, environmental and genomic data. Accordingly, integrative approaches that combine association mapping with landscape genomics or selection scans to map gene-phenotype-environment associations could be a powerful means to infer the genomic basis of adaptation (Jones et al., 2013). Association mapping studies (e.g., genome-wide-associations [GWA]; Santure & Garant, 2018) identify specific loci that underlie divergent traits, whereas landscape genomic studies can aid in determining loci associated with adaptive divergence, under the assumption that loci should be correlated with environmental variation that is directly or indirectly causing selection (Coop et al., 2010; Eckert et al., 2010). Combining association mapping with landscape genomics can strengthen the identification of genomic signatures of selection by allowing inference on whether causal variants of phenotypic variation are concurrently associated with environmental variation. This would be especially true in cases where correlations between phenotype and environment are mirrored in genetic polymorphisms, where at some quantitative trait loci, allele frequencies differ between groups that inhabit different environments.

In the absence of dispersal barriers, many populations remain connected by gene flow during the process of adaptive divergence, often along environmental clines (Feder et al., 2012; Räsänen & Hendry, 2008). Gene flow is expected to constrain divergence, swamping locally adapted alleles and breaking up favourable allele combinations through recombination (Yeaman & Whitlock, 2011). Whilst in cases of substantial gene flow there may be little genome-wide divergence, responses to natural selection may be present at specific genomic regions (islands of divergence; Wolf & Ellegren, 2017). Identifying genomic divergence in the presence of gene flow is a major challenge because most genome scan approaches require grouping individuals, which is not usually possible when individuals remain connected (de Villemereuil et al., 2014; Narum & Hess, 2011). Our perspective on adaptive divergence may therefore be biased towards studies where physical barriers to gene flow have facilitated divergence. Although such studies have provided great insight into evolutionary processes, studying processes of divergence in populations connected by gene flow can greatly improve our understanding of the relative roles of natural selection and gene flow in adaptive divergence (Richardson et al., 2014).

Here, we employ GWA and landscape genomic approaches to map gene-phenotype-environment associations in threespine stickleback (*Gasterosteus aculeatus*) that inhabit Mývatn, a highly environmentally heterogeneous lake in NE Iceland. Threespine stickleback is a well-established model system in evolutionary biology (Hendry et al., 2013; Reid et al., 2021). Within freshwater systems, there is evidence for repeated adaptive divergence at both phenotypic and genomic levels (Härer et al., 2021; Hendry et al., 2009; Hudson et al., 2021), most commonly across the benthic-limnetic axis (e.g., Härer et al., 2021) but also across a range of other selective agents (e.g., predation; Reimchen, 1992, 2000; Reimchen & Nosil, 2002). However, most of the studies focus on simple environmental contrasts (e.g., benthic vs. limnetic or lake vs. stream), and only few studies have aimed to test intralacustrine divergence across environmental gradients.

Mývatn is a large (37 km^2) and geologically young lake, formed after a volcanic eruption ca. 2300 years ago. The lake is highly heterogenous, with temperature, water depth, invertebrate, and vertebrate (including stickleback) densities varying over space and time (Einarsson et al., 2004; Ives et al., 2008). Stickleback habitats in this lake can crudely be divided to five main types, across which stickleback vary phenotypically (Kotrschal et al., 2012; Kristjánsson et al., 2002; Millet et al., 2013). Previous work found that male stickleback had relatively larger brains in a "lava" (warm) than a "mud" (colder) habitat (Kotrschal et al., 2012), relatively longer spines in the north basin than the south basin (Millet et al., 2013), and divergence in gill raker morphology and diet among some of the habitats (Kristjánsson et al., 2002; Millet et al., 2013). Evidence for population genetic divergence of stickleback across the lake is mixed. Using samples collected between 1999 and 2002 (Ólafsdóttir et al., 2007) found evidence for genetic divergence using a suite of nuclear and mitochondrial markers between stickleback inhabiting the "lava" and "mud" habitats (microsatellites: F_{ST} = 0.08; mtDNA: F_{ST} = 0.223), suggesting the presence of two contrasting morphs. In contrast, using samples collected in 2009 and 12 nuclear microsatellite loci (seven of which were the same as in Ólafsdóttir et al., 2007; Millet et al., 2013) found little evidence for neutral genetic divergence of stickleback across five habitat types (average pairwise F_{ST} = 0.004), suggesting extensive gene flow.

Given the known phenotypic divergence in traits typically under selection in stickleback, coupled with spatial variation in possible selective agents, our main goal here was to identify genomic signatures of selection in Mývatn stickleback occupying different environments. When information on fitness is not available, one common method to identify genomic signatures of selection is to identify as

genomic regions which are disproportionately divergent between groups compared to the rest of the genome (Hoban et al., 2016). We extended this definition to strengthen our identification of signatures of selection: we expected that genomic regions that bear a signature of selection should be both divergent across ecological axes, and contain loci associated with variation in divergent traits. We further measured single nucleotide polymorphism (SNP)-based additive genetic variation of divergent traits to gain insight into the evolutionary potential of traits that are spatially divergent.

2 | MATERIALS AND METHODS

2.1 | Study system and sampling

Lake Mývatn is composed of two basins (North and South basin) that are connected by two narrow channels and vary in a range of abiotic and biotic conditions (Einarsson et al., 2004). The lake is spring fed, with geothermal hot springs (up to c. 23°C) feeding the north-east of the lake and cold-water springs (c. 5°C) feeding the south-eastern parts. Most part of the lake follows the ambient temperature, which in summer is around 12–13°C (Millet et al., 2013). The lake is shallow (1–4 m), but with some deeper areas (up to c. 7 m) caused by historical diatomite mining in some parts of the North basin (Ólafsson, 1979). Productivity, as well as benthic, epibenthic and pelagic invertebrate abundance and community structure, also varies through space (Bartrons et al., 2015). Based on the combination of water temperature and depth, as well as vegetation and substrate, the habitats occupied by stickleback have previously been classified as warm, rocky shore, cladophorales, pondweed and mined (Millet et al., 2013, see below). In addition, long-term monitoring data shows that stickleback population density varies in both space and time (Einarsson et al., 2004; Phillips et al., 2023), with the North basin having higher densities than the South, and with periodically strong dispersal from the North to the South basin (Phillips et al., 2023).

The stickleback population of Mývatn has been surveyed each year since 1991 as part of an ongoing long-term monitoring of population demographics (Phillips et al., 2023). This sampling is done during the third week of June and August each year by laying five unbaited minnow traps at predetermined locations over two 12 h periods (see Millet et al., 2013 for details). During monitoring, stickleback are counted to estimate catch-per-unit-effort (CPUE) and frozen for later analysis. For phenotyping and genotyping, a random subset of individuals (c. $N = 100$ per site for each of the day and night catches) have been stored since 2009. To study patterns of spatial divergence, we used stickleback from nine sites collected in June of 2012 due to the availability of detailed ecological data for this time point.

2.2 | Ecological data

To characterize the environment that stickleback occupy, we focused on a set of ecological variables which represent putative selective agents. First, we used the same five habitat classifications (warm, mined, pondweed, cladophorales, and rocky shore) previously described in Millet et al. (2013; see Data S1 for details). We also collated data on ecological variables likely to reflect selective agents. These were: water temperature, water depth, stickleback CPUE, piscivorous bird density, and zooplankton abundances and community composition. These were chosen because temperature can affect metabolic processes, development, tolerance to parasite infections (Franke et al., 2017; Karvonen et al., 2013), as well as key life history traits (Kim et al., 2017; Mehlis & Bakker, 2014), whilst depth can affect sensory processes (Veen et al., 2017) invertebrate availability, and stickleback visibility to predators (Rypel et al., 2007). Stickleback CPUE was used as a measure of intraspecific competition (Bolnick, 2004), piscivorous bird density as a measure of predation pressure (Vamosi & Schluter, 2004), and invertebrate data as a measure of prey abundance and composition (Bolnick & Ballare, 2020). Note that although Mývatn stickleback are also subject to predation by brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*), available data on trout or charr abundances from Mývatn were not at the same spatial scale as the stickleback data (Phillips, Guðbergsson, & Ives, 2022). Hence, we did not integrate those data into our current analysis and note that fish predation is still a likely agent of selection. In general, it should be noted that our measures are only proxies for selection imposed by several correlated ecological factors.

Temperature and water depth of each site were used as per Millet et al. (2013). Average temperature at each site was measured between 30 June 2011 and 18 August 2011 with a temperature logger (iButton Maxim Integrated Products), placed at mid-depth and recording at 3-h intervals. CPUE for each site was estimated using count data from the long-term monitoring study from June 2012. To measure piscivorous bird density, we used data collected during the waterfowl census conducted each year at Mývatn, during which all waterfowl observed from predetermined vantage points with known survey areas are counted (Gardarsson, 1979). We used count data collected between 15 May and 10 June 2012 on the following species known to predate on stickleback: horned grebe (*Podiceps auritus*), red-breasted merganser (*Mergus serrator*), great northern diver (*Gavia immer*), red throated diver (*Gavia stellata*) and goosander (*Mergus merganser*). Note that the Arctic tern (*Sterna paradisaea*) is abundant at Mývatn, and predates on stickleback, but this species is not counted during the bird census. We calculated the density of piscivorous birds (summed across all taxa) in each surveyed segment of the lake (number/m^2; Figure S1).

We used invertebrate data from Bartrons et al. (2015), which were collected by conducting surveys of the epibenthic and zooplanktonic community. Crustaceans (including *Daphnia*, copepods and epibenthic cladocerans) as well as rotifers are important food sources for stickleback in Mývatn (e.g., Kristjánsson et al., 2002). Although chironomid larvae are a main food source for Mývatn stickleback, data on midge larval abundance were not of sufficient spatial resolution to be used (see Bartrons et al., 2015). However, the benthic community is spatially correlated with the epibenthic and zooplankton

community in the South basin (Bartrons et al., 2015), suggesting that measures of pelagic and epibenthic zooplankton may serve as a proxy measure for the benthic community in Mývatn. Briefly, three transects were conducted between June–July 2012, during which integrated vertical tows of the whole water column were made at each of 31 sites, spaced 500–600 m apart (see Figure S2 for distribution, and Bartrons et al., 2015 for more details on sampling and sample processing). Each pooled sample of 15 L was filtered through 63-μm mesh and counted in entirety under a binocular microscope. We used data from the second transect (25 July 2012) as the spatial resolution in this transect was the greatest. We used data collected from the closest site to each stickleback sampling site (distance to closest zooplankton site: min = 290 m, max = 1365 m). All stickleback sites were within 2.55 km of the nearest site used to collect invertebrate data, which was the distance at which zooplankton communities were found to be spatially autocorrelated. We used number per litre (n/L) of each taxon at the sites closest to stickleback sites.

To summarize variance between sites for use in downstream analyses, we ran a principal components analysis (PCA) using the native stats package in R version 4.1.2 (R Core Team, 2021). This summarized the invertebrate data in four general axes, described in detail in Table S1. PC1 (zPC1) described the overall abundance of crustaceans and rotifers, and explained 29% of the variation; PC2 (zPC2) described the negative covariance of rotifer sp. and *Alona* sp. with planktonic and epibenthic crustaceans, which explained 17% of the variation; PC3 (zPC3) described the negative covariance of the rotifer *Keratella* and the cladocerans *Acroperus harpae* and *Chydorus sphaericus* with *Daphnia longispina*, and explained 15% of the variation; and PC4 (zPC4) described the negative covariance of the cladocerans *Eurycercus lamellatus* and *Macrothrix hirsuticornis* with *Daphnia longispina*, *Cyclops abyssorum* and *Asplancha*, explaining 11% of the variation in the data. Overall invertebrate abundance (described in zPC1), was highly negatively correlated with stickleback CPUE. We therefore used only CPUE and not zPC1 for downstream analyses.

2.3 | Phenotypic data

We randomly selected individuals from each habitat type for phenotyping and genome sequencing from the frozen subset of the long-term monitoring samples. Individuals were thawed, weighed on an electronic balance (wet mass, nearest mg) and their total length (TL) measured using a ruler (to the nearest mm). The right pectoral fin was then cut and stored in 96% ethanol for DNA isolation. We measured traits typically under selection in stickleback: body size, defence traits (armour plate number and length of spines) and dietary traits (gill raker morphology and gut length; Härer et al., 2021; Hendry et al., 2009; Reid et al., 2021). Specifically, for each individual we measured the following 10 traits: total length (TL), total gut length (gut length), number of lateral armour plates (plate number), length of the first dorsal spine (DS1), length of the second dorsal spine (DS2), length of the pelvic spine (PS), length of the second gill raker on the first gill arch (GRL2), length of third gill raker on the first gill arch (GRL3), gap width between second and third gill rakers (GRW), and number of long gill rakers on the first gill arch (GRN) (see below). Note that we measured the second and third gill rakers, rather than the first (which is usually used in studies of stickleback trophic phenotype), because in some cases gill arches broke during dissection. After measurement of TL, each individual was dissected to remove the stomach and the gut, and any tapeworm (*Schistocephalus solidus*) parasites. Gut length (from the sphincter at the end of the oesophagus to the end of the digestive tract) was measured (to the nearest mm) using a ruler.

To aid morphological measurements, fish were stained with alizarine red using standard protocols (Millet et al., 2013). Fish were bleached using a 1:1 ratio of 3% H_2O_2 and 1% KOH and then stained in a solution of alizarin red and 1% KOH (Bell, 1982). After staining, digital images were taken of the left side of the fish with a Canon EOS 600D digital camera, with mm paper for scale. From these images, plate number was counted and the length of the spines (DS1, DS2 and PS) measured to the nearest hundredth of a millimetre. After imaging, we dissected the first gill arch and, where necessary, re-stained before mounting it between two glass plates and photographing using a digital camera (Nikon Coolpix 4500) mounted to a stereomicroscope (Leica MZ12) with mm paper for scale. We used the digital images of gill arches to measure GRL2, GRL3 and GRW (in mm) and counted GRN. All measurements were taken from the digital images were done using segmented tool in ImageJ (Schneider et al., 2012).

2.4 | Whole genome resequencing and SNP detection

Genomic DNA was isolated and purified from the ethanol stored fin clips using Macherey-Nagel nucleomag tissue kit, following the manufacturer's protocol. Paired end, PCR-free 150-bp insert libraries were then prepared for whole genome sequencing using the DNBSeqTM platform by BGI-Hongkong, generating an average of 40 million cleaned reads per individual (min = 33.4 million, max = 41.8 million, equating to an average depth of coverage of 10×). The clean paired-end reads were aligned to the threespine stickleback genome assembly version 5 (Nath et al., 2021) with BOWTIE2 (version 2.4.1) using default parameter settings (Langmead & Salzberg, 2012), and sorted and indexed using SAMTOOLS (version 1.10) (Li et al., 2009). Variants were discovered using the short variant discovery pipeline of GATK (van der Auwera et al., 2013). SNPs and indels were quality filtered according to GATKs best practices guidelines, using the following hard filters: QualByDepth > 2.0, FisherStrand bias < 60.0, RMSMappingQuality < 40.0, MappingQualityRankSumTest < −12.5, ReadPosRankSumTest < −8.0, StrandOddsRatio > 3.0, variant quality score < 30.0. For all following analyses, we removed mitochondrial variants, indels and multiallelic variants, as well as variants identified on either of the sex chromosomes (Peichel et al., 2020). We then filtered the remaining autosomal SNPs for genotype depth less than six or greater than

100; minor allele counts less than four; missingness <20%. The sex of individuals was confirmed using the proportion of reads with depth greater than eight mapped to the X versus Y chromosome (Peichel et al., 2020).

2.5 | Statistical analyses

We conducted a series of analyses to test for (1) phenotypic and genomic divergence in relation to geographic location and ecological variables, (2) genomic architecture of traits, and (3) associations of genomic variation with environment.

2.5.1 | Phenotypic divergence

Phenotypic divergence was analysed using multivariate and univariate Bayesian linear mixed models. All traits were standardized to have a mean of zero and standard deviation of one to improve comparability and model convergence. All models described below were fit with a Gaussian error distribution in the MCMCglmm package in R (Hadfield, 2010). Fixed effects were given weakly informative flat priors, random effects were given default inverse Wishart priors, and we fit full unstructured covariance matrices for the multivariate models. Each model was run for a total of 1,020,000 iterations with a burnin of 20,000 iterations and thinning of 1000 iterations, which resulted in low autocorrelation. Convergence of models was assessed by examining traceplots to visualize sampling mixing and by assessing effective sample sizes and autocorrelation.

Effects of site and habitat

To investigate the extent of phenotypic variation among sites and habitats, we compared three linear mixed effects models with different random effects structures using the deviance information criterion (DIC) to identify the model with the most support, at ΔDIC = 2 (Spiegelhalter et al., 2014): a model with no random effects (i.e., a "null" model), a model that included site as a random effect, and a model that included habitat type as a random effect. These models were designed to identify whether there was phenotypic variance between all habitats or all sites. We grouped traits in the response variable to investigate phenotypic divergence in functionally correlated traits. TL and gut length were each fit as a univariate response; the four defence traits (plate number, DS1, DS2 and PS) and the four gill raker traits (GRL2, GRL3, GRW, GRN) were fit as multivariate response traits, respectively. This resulted in a total of 12 models (three models with different random effects structures for each of the four responses), all fitted with sex as a fixed factor. Models for gut length, defence traits, and gill raker traits included TL as a covariate. Fitting the interaction between sex and TL did not improve model fits, so we present results from models with sex and TL fit as single term effects. Owing to varying levels of replication for each sex at each site (Table 1) we cannot estimate or exclude sex differences in the patterns of divergence.

TABLE 1 Overview of sample size, ecological variables, and population genetic parameters for threespine stickleback sampled across 12 sites and five habitat types in Lake Mývatn

Site	N_{ind}	m	f	Habitat type	Temp (°C)	Depth (m)	CPUE	zPC2	zPC3	zPC4	Bird density (n/m^2)	H_o	H_e	F
23	13	6	7	Cladophorales	12.2	3.24	34	0.689	0.507	−0.492	0.0004	0.262 (0.007)	0.268 (0.027)	0.021 (0.027)
27	9	7	2	Cladophorales	11.9	2.52	68	−1.026	0.231	−0.898	0.0004	0.266 (0.005)	0.268 (0.018)	0.008 (0.018)
44	15	15	0	Cladophorales	12.1	2.95	214	1.074	−1.764	−0.053	0.0007	0.319 (0.043)	0.268 (0.061)	−0.191 (0.061)
124	37	31	6	Mined	12.6	4.75	885	−0.708	−0.27	−0.069	0.0006	0.298 (0.027)	0.268 (0.001)	−0.111 (0.001)
128	20	20	0	Pondweed	13	1.62	825	−0.559	0.256	0.007	0.0006	0.280 (0.021)	0.268 (0.078)	−0.041 (0.078)
135	8	3	5	Pondweed	11.6	2	14	1.793	−1.324	−1.059	0.0005	0.261 (0.006)	0.268 (0.023)	0.026 (0.023)
CS	40	8	32	Rocky shore	12.7	1.3	177	−0.546	1.189	−1.1	0.0003	0.281 (0.022)	0.268 (0.082)	−0.041 (0.082)
DN	10	8	2	Pondweed	13	1.18	920	−0.993	−0.078	−0.569	0.0006	0.286 (0.016)	0.268 (0.059)	−0.061 (0.059)
HS2	33	20	13	Warm	23.4	0.15	1342	−0.559	0.256	0.007	0.0018	0.292 (0.025)	0.268 (0.093)	−0.091 (0.093)

Note: The columns indicate site, total sample size (N_{ind}), number of males (m) and females (f) sampled per site, habitat type, average temperature (Temp, °C), water depth (depth, metres), catch per unit effort (CPUE), invertebrate principal component scores zPC2, zPC3, and zPC4, density of piscivorous birds (Bird density) and expected heterozygosity (H_e), observed heterozygosity (H_o) and inbreeding coefficient (F). Population genetic parameters were measured using VCFTOOLS and averaged across all individual, with standard deviations presented in parentheses.

The multivariate models fit full variance–covariance matrices for random effects, allowing us to estimate phenotypic covariances at both individual (residual) and habitat/site levels (Chenoweth et al., 2010). Note that because of the different sample sizes for each group of traits (see below), it was not possible to run a single multivariate model with all traits to measure the full covariance matrix. Fish from sites were considered phenotypically divergent if 95% credible intervals of the posterior distributions of predicted trait values did not overlap.

Association with ecological parameters

We next ran a suite of univariate, linear mixed effects models that investigated the effect of ecological predictors on each phenotype independently. All models were fitted with TL and sex as fixed effects and site as a random effect (except for model on total length, which only had sex as a fixed factor). Because many of our eight ecological variables were highly correlated (Table 1), we ran one model per ecological predictor per phenotype (total = 7 models per phenotypic trait) and compared each to a null model without any ecological variables using DIC (as above). In these models, all predictor variables were standardized to have a mean of 0 and standard deviation of 1 to improve comparability between models. We present the mode and 95% credible intervals of the posterior distribution for the linear coefficients for each ecological predictor, unless ΔDIC to the null model was >2 because this indicated that the null model was not improved by fitting the ecological variable.

2.5.2 | Genomic divergence

To identify the extent of genetic divergence among sites, we used two approaches: (1) Principal component analysis (PCA) was used to explore genetic clustering of Mývatn stickleback, and (2) a model-based admixture analysis was used to determine population genetic structure by calculating the proportion of an individual's genome that originates from different hypothetical ancestral gene pools (i.e., admixture coefficients). PCA was conducted in ADEGENET package (Jombart & Ahmed, 2011), and as suggested by Jombart et al. (2010) 100% of the initial PCs were retained when identifying the number of clusters. Admixture analyses were run using SNMF, which estimates admixture coefficients using non-negative matrix algorithms (Frichot et al., 2014) and makes no assumptions about drift or Hardy–Weinberg equilibrium (HWE). SNMF was run using the LEA package in R (Frichot & François, 2015) for number of ancestral populations (*K*) 1–10. Pairwise genome-wide Nei's F_{STs} were calculated between all sites and habitats using VCFTOOLS. Contemporary effective population size (N_e) was estimated using the LD-based method, as implemented in NeEstimator version 2 (Do et al., 2014). To remove physical linkage (as required in this method), we first thinned the SNPs across 10 kb windows using VCFTOOLS. To provide confidence intervals of estimates of N_e, we calculated N_e across each chromosome independently.

2.5.3 | Genome-wide-association analyses

To estimate SNP-based heritability for each trait, we ran Bayesian mixture models for each trait independently using the BAYESR software (Moser et al., 2015). We selected this method as it has been found to be more accurate in estimating additive genetic variance explained by SNPs than alternative methods (e.g., BSLMM or LMM; Moser et al., 2015). All models included TL and sex as covariates (except when modelling TL explicitly, which included only sex as a factor), and all traits were standardized to a mean of 0 and standard deviation of 1. We also aimed to estimate pairwise genetic correlations between traits using bivariate models in BAYESR. However, none of these models converged, probably owing to the large sample sizes usually required to estimate genetic covariances, and hence are not reported further.

To identify SNPs underlying phenotypic variation, we performed a GWA using a linear mixed effects model approach with the program GEMMA (Zhou & Stephens, 2012). This program fits models that control for relatedness among samples and/or population stratification, which reduced false positives even with small sample sizes. GEMMA was selected because it implements generalized linear mixed models for traits with non-normal distributions, and therefore robustly handles data on different scales. These models estimate the linear coefficient for the relationship between each SNP in turn and a given trait. When identifying whether a SNP was putatively associated with trait variance, Wald tests were used to assess significance (*p*-value cutoff of -log10(*P*) = 5). This value was chosen to minimize the number of expected false positives given the number of loci in our data set (12 false positives are expected). All models included sex as a fixed factor and length as a covariate, except for the model with total length as a response variable which just fix sex. The number of SNPs used in each model was dependent on the number of individuals available for each trait, because GEMMA only models SNPs with <5% missingness. We then identified whether there was overlap between regions that were associated with trait variation in our data, and quantitative trait loci (QTLs) previously mapped on the stickleback genome (Peichel & Marques, 2017), using LIFTOVER and custom R scripts.

2.5.4 | Genome-environment association analyses

We investigated gene–environment associations using latent-factor mixed models (LFMM) in the R package LEA (Frichot & François, 2015), which models loci against environmental variables while controlling for unobserved latent variables (i.e., spatial structure or autocorrelation). Models were run for 100,000 iterations, with 10,000 burnin cycles and five replicates. *z*-scores were combined from five runs, and we used adjusted *p*-values using the genomic control method (a recalibration procedure which decreases the false discovery rate (Frichot & François, 2015)). Following the guidance of Frichot and François (2015), the resulting *p*-values for the outlier tests were adjusted for multiple testing

to Q-values using the false-discovery rate method (Benjamini & Hochberg, 1995) as implemented in the "qvalue" 2.6.0 package. A Q-value for SNPs of <0.05 was considered significant. We compared DIC of models that included one ecological variable at the time to try to identify which ecological variable best predicted the genomic data. To identify whether gene–environment associations were linked to phenotypic variation, we then investigated whether any of the candidate SNPs identified in any of the LFMM analyses were linked (within 5 kb) to SNPs identified in GWA analyses. We used 5 kb windows as a proxy for linkage on the genome, which is a common window size used when physical linkage across the genome is unknown (Artemov et al., 2017, Kingman et al., 2021). To identify the extent to which we would expect an overlap between SNPs identified in LFMM and GWA by chance, we ran permutation tests using custom R scripts. These kept the number of SNPs identified in each analysis and the genomic location of the GWA SNPs constant but randomized the genomic location of environmentally associated SNPs. This was done by randomly selecting SNPs from the global set of loci (N = 1,205,604 SNPs). We ran 1000 permutations and the probability of an overlap occurring by chance was calculated as the proportion of times an overlap occurred in the permutations.

To explore the molecular function of genomic regions that showed signatures of selection, we first analysed candidate genes for enrichment of molecular functions. To do this, we identified genes that the candidate SNPs were within 5 kb of and compared these candidate genes with the reference set of 20,805 genes across the stickleback genome ("gene universe"). Gene ontology (GO) information was obtained from the stickleback reference genome on ENSEMBL using the R package BIOMART (Durinck et al., 2009), and functional enrichment was investigated using the package TOPGO 2.42 (Alexa & Rahnenfuhrer, 2020) and the Fisher's exact test (at $p < .01$). To reduce false positives, we pruned the GO hierarchy by requiring that each GO term had at least 10 annotated genes in our reference list ("nodeSize = 10"). Second, physical overlap on the genome between candidate SNPs identified in environment-association analyses and GWA SNPs (as identified above) indicate regions of the genome under selection. For regions of the genome containing both environmentally associated candidate SNPs and GWA SNPs, we therefore identified (1) the genes in this region (within 5 kb), (2) whether haplotypes on that gene in our data set are predicted to cause variation in protein translation, and (3) the function of the gene. We used the program "snpEff" (Cingolani et al., 2012) to detect whether SNPs fall on coding regions changing amino acid sequence.

3 | RESULTS

Out of 200 individuals originally sent for sequencing, 14 samples did not pass the quality control and were therefore not sequenced, resulting in a total of 186 sequenced individuals. Quality filtering after variant discovery resulted in a data set of 1,205,604 SNPs. This resulted in an average of one SNP per 270 bp across the stickleback genome. During dissections, some samples were broken resulting in slightly different sample sizes for each trait; total length: N = 186, gut length: N = 106, plate number: N = 160, DS1 and DS2: N = 159, PS: N = 158, GRN: N = 133, GRW: N = 161, GRL2 and GRL3: N = 158 and 159.

3.1 | Phenotypic divergence

3.1.1 | Total length

Model comparisons showed that TL varied across sites rather than habitats (Table 2). This pattern was predominantly driven by stickleback from HS2 site being shorter than stickleback from other sites (Figure 1). TL was negatively correlated with both temperature (Table 3) and bird density (Table 3), but the model with temperature as a predictor fit the data better (i.e., had a lower DIC; Table 3). All traits, except plate number and GRN, were positively correlated with TL (Table 2), and all results presented hereafter refer to effects on size-corrected traits.

Defence traits

There were no sex differences in the relative length of either dorsal or pelvic spines, but males had more armour plates than females (Table 2). Model comparisons suggested that defence traits tended to vary according to habitat rather than site, although there was only very weak statistical support for this effect (Table 2, Figure 1). Whilst none of the ecological variables predicted relative length of DS1 and armour plate number (Table 3), relative length of DS2 increased as the density of piscivorous birds increased (Table 3) and relative length of PS increased in deeper water (Table 3, Figure 2). However, statistical support for these environmental associations was weak (the ΔDIC to the null model was within 2, and the lower credible interval of the posterior distribution of the linear coefficient was only just above zero; Table 3).

Individuals with relatively longer DS1 had correspondingly longer DS2 and PS (pairwise phenotypic covariances [CoV], posterior mode and 95%$_{CI}$: DS1:DS2 = $0.224_{[0.165,\ 0.289]}$; DS1:PS = $0.133_{[0.077,\ 0.190]}$; DS2:PS = $0.122_{[0.070,\ 0.190]}$), indicating that spine traits covaried at the individual level. However, relative length of spines did not seem to covary with armour plate number (see Table S3). There was no evidence for phenotypic covariance across the habitats between any of the defence traits, suggesting that spatial divergence in defence traits was not correlated at the habitat level (see Table S3). However, to increase statistical power for detecting divergence in phenotypic covariances, greater level of replication is likely needed.

Trophic traits

Males had relatively longer GRL2 and GRL3, and relatively more gill rakers (GRN), than females (Table 2), but there were no sex differences in GRW (Table 2). Model comparisons showed that gill raker traits varied

TABLE 2 Results of linear mixed effects models used to investigate the extent of spatial divergence of threespine stickleback phenotypes across Lake Mývatn

	ΔDIC			β		V		
Trait	**Null**	**Site**	**Habitat**	**Length**	**Sex M**	**Site**	**Habitat**	**Residual**
Length	68.4	0	8.05	–	$-0.512_{(-0.782, 0.279)}$	$0.495_{(0.130, 1.023)}$	–	$0.562_{(0.451, 0.612)}$
Defence traits								
Plate number	0	2.21	1.68	$0.012_{(-0.010, 0.031)}$	$0.339_{(0.009, 0.762)}$	–	$3.919_{(0.094, 12.241)}$	$0.988_{(0.766, 1.195)}$
DS1				$0.089_{(0.077, 0.100)}$	$0.186_{(-0.039, 0.387)}$	–	$4.634_{(0.076, 9.501)}$	$0.309_{(0.249, 0.388)}$
DS2				$0.091_{(0.081, 0.102)}$	$0.076_{(-0.136, 0.297)}$	–	$4.295_{(0.059, 11.727)}$	$0.299_{(0.231, 0.369)}$
PS				$0.076_{(0.064, 0.088)}$	$-0.046(_{-0.271, 0.159)}$	–	$3.281_{(0.080, 9.915)}$	$0.337_{(0.267, 0.410)}$
Trophic traits								
GRL2	41.26	0	2.8	$0.069_{(0.050, 0.089)}$	$0.543_{(0.222, 0.870)}$	$0.519_{(0.092, 1.333)}$	–	$0.551_{(0.418, 0.700)}$
GRL3				$0.074_{(0.056, 0.091)}$	$0.559_{(0.259, 0.882)}$	$0.564_{(0.089, 1.521)}$	–	$0.492_{(0.372, 0.616)}$
GRW				$0.075_{(0.056, 0.095)}$	$0.088_{(-0.265, 0.424)}$	$0.430_{(0.063,1.090)}$	–	$0.620_{(0.478, 0.792)}$
GRN				$0.011_{(-0.013, 0.033)}$	$0.449_{(0.051, 0.843)}$	$0.945_{(0.127, 2.318)}$	–	$0.772_{(0.592, 0.982)}$
Gut length	0.39	0	4.42	$0.072_{(0.057, 0.085)}$	$-0.263_{(-0.497, -0.042)}$	$0.274_{(0.059, 0.655)}$	–	$0.210_{(0.151, 0.271)}$

Note: Models with different random effects structure (no random effect ["Null"], site ["Site"] or habitat type ["Habitat"]) were compared using DIC, and results from the model with the lowest DIC (at ΔDIC = 2) are shown. (For details of analyses and model structure, see material and methods). The results are shown for the following traits: total length (Length), number of lateral plates (Plate number), lengths of two dorsal spines (DS1 and DS2), the pelvic spine (PS) and two gill rakers (GRL2 and GRL3), as well as gill raker gap width (GRW), number of gill rakers (GRN) and gut length. β reflects the linear coefficient estimate; V is variance estimates of random effects. Posterior means for all parameter estimates are presented, with 95% credible intervals in subscript parentheses. The models included sex and, where appropriate, length as a covariate and therefore all results reflect effect on size corrected traits.

according to site rather than habitat (Table 2), but this variation was not associated statistically with any of the ecological variables (Table 3). Specifically, stickleback from the CS site had relatively longer and fewer gill rakers than stickleback from other sites, and stickleback from site CS and DN had fewer gill rakers than stickleback from all other sites (Figure 1). There was evidence for phenotypic covariances at the residual (i.e., individual) level for some gill raker traits. For instance, individuals with relatively longer GRL2 had correspondingly longer GRL3 (CoV, posterior mode and 95%$_{CI}$: GRL2:GRL3 = $0.288_{(0.188,\ 0.403)}$). Furthermore, individuals with more gill rakers also had narrower GRW (CoV, posterior mode and 95%$_{CI}$: GRW:GRN = $-0.184_{(-0.311,\ -0.052)}$). There was no evidence for phenotypic covariances at the site level between any of the gill raker traits, suggesting that spatial divergence in gill raker characteristics was not correlated (see Table S3).

Gut length

Males had relatively shorter guts than females (Table 2). Model comparisons suggested that relative gut length varied among sites (Figure 1, Table 2), but this variation was not associated to any of the ecological variables (Table 3).

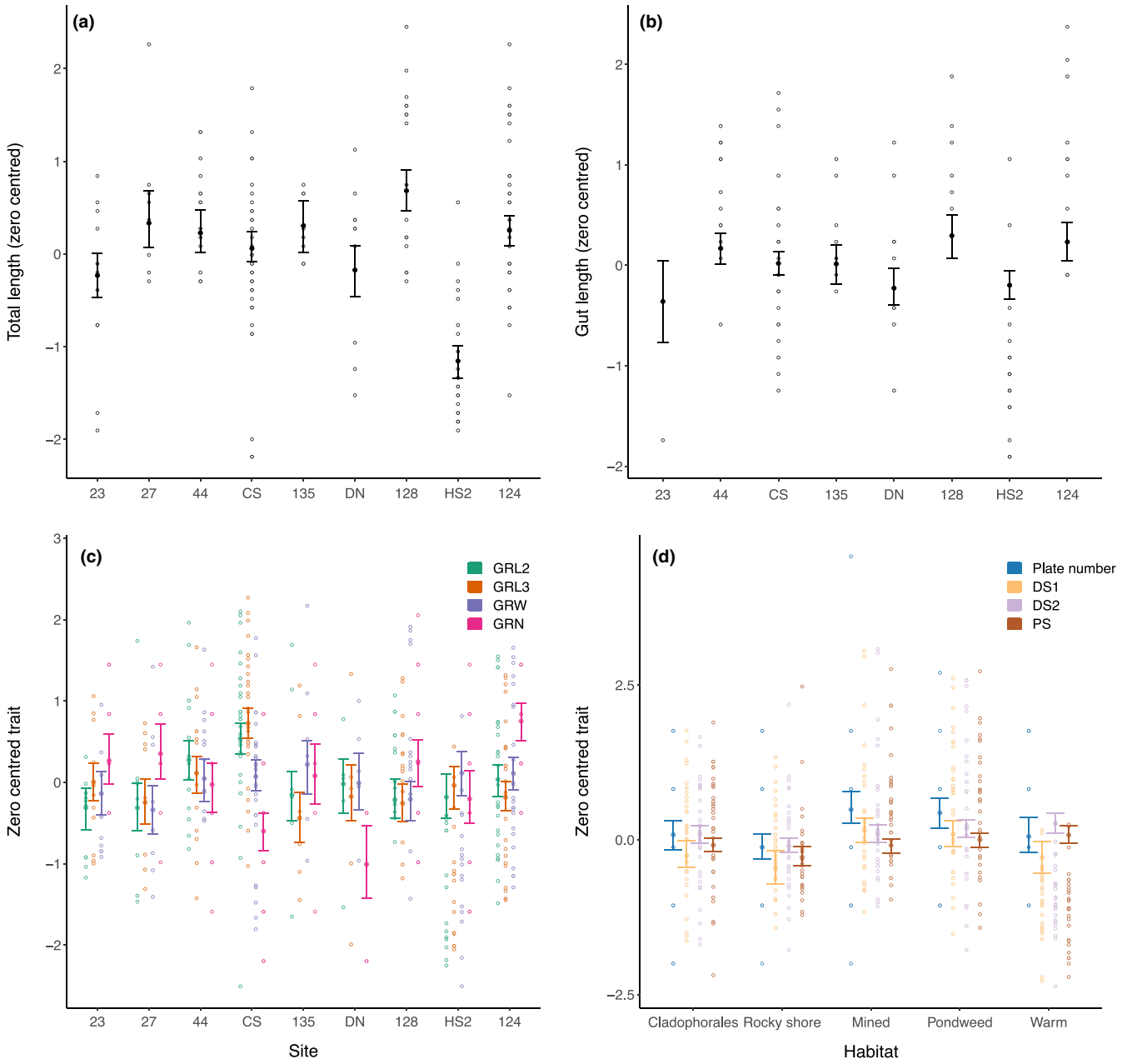

FIGURE 1 Phenotypic divergence of threespine stickleback, for total length (a), relative gut length (b), gill rakers (c) and defence traits (d). Empty circles are raw data and solid points represent the predicted mean phenotype after correcting for length (b–d) and sex (a–d) at each site (a–c) or habitat (d). Bars show the 95% credible intervals of the prediction derived from the model. All traits are standardized to have zero mean and standard deviation of one, such that zero represents the average phenotype in the data set

TABLE 3 Results of linear mixed effects models used to investigate the effect of ecological variables on trait variation across Lake Mývatn

	Temperature		Depth		Bird density		CPUE		zPC2		zPC3		zPC4	
Phenotype	ΔDIC	β	ΔDIC	β	ΔDIC	β	ΔDIC	β	ΔDIC	β	ΔDIC	β	ΔDIC	β
Length	**−69.114**	**−0.506 (−0.707, −0.319)**	−68.311	0.298 (−0.179, 0.848)	**−66.078**	**−0.478 (−0.82, −0.138)**	−68.217	−0.262 (−0.659, 0.158)	−68.068	0.053 (−0.322, 0.432)	−68.222	−0.12 (−0.565, 0.356)	−68.138	−0.109 (−0.616, 0.417)
Plate number	1.657	−0.051 (−0.27, 0.189)	0.033	0.123 (−0.068, 0.29)	1.855	−0.007 (−0.231, 0.214)	0.739	0.095 (−0.085, 0.279)	1.672	−0.037 (−0.203, 0.142)	0.519	−0.106 (−0.289, 0.093)	0.378	0.109 (−0.072, 0.287)
DS1	0.004	0.07 (−0.057, 0.196)	1.415	−0.022 (−0.149, 0.1)	−1.619	0.101 (−0.016, 0.211)	0.183	0.063 (−0.054, 0.177)	1.752	0.003 (−0.101, 0.104)	−0.554	−0.078 (−0.191, 0.018)	−0.768	0.082 (−0.028, 0.186)
DS2	−1.945	0.118 (0, 0.232)	0.514	−0.033 (−0.197, 0.149)	**−3.729**	**0.139 (0.028, 0.243)**	−0.583	0.086 (−0.044, 0.197)	0.389	−0.022 (−0.142, 0.1)	0.352	−0.044 (−0.178, 0.109)	−2.422	0.116 (−0.008, 0.22)
PS	1.754	−0.025 (−0.142, 0.095)	**−1.983**	**0.104** $_{(0.007,\ 0.214)}$	1.641	0.021 (−0.106, 0.145)	0.810	0.053 (−0.052, 0.159)	1.901	0.009 (−0.09, 0.1)	−3.711	−0.117 (−0.220, −0.026)	−5.105	0.136 (−0.04, 0.241)
GRL2	1.900	−0.011 (−0.176, 0.155)	1.468	0.049 (−0.116, 0.195)	1.841	−0.022 (−0.182, 0.14)	1.894	−0.016 (−0.161, 0.143)	1.920	−0.016 (−0.142, 0.11)	1.931	0.013 (−0.128, 0.164)	1.886	−0.014 (−0.16, 0.151)
GRL3	0.109	0.031 (−0.22, 0.28)	0.203	−0.017 (−0.263, 0.25)	0.710	−0.019 (−0.246, 0.283)	0.664	−0.066 (−0.261, 0.13)	0.280	−0.027 (−0.205, 0.141)	−1.124	0.143 (0, 0.317)	0.881	−0.059 (−0.266, 0.144)
GRW	1.776	0.046 (−0.141, 0.197)	1.182	0.07 (−0.09, 0.22)	1.549	0.05 (−0.113, 0.209)	0.924	0.071 (−0.093, 0.197)	2.032	0.004 (−0.131, 0.125)	1.388	−0.061 (−0.200, 0.076)	1.365	0.06 (−0.102, 0.192)
GRN	0.178	−0.093 (−0.628, 0.518)	3.800	–	0.192	−0.025 (−0.598, 0.578)	0.052	−0.098 (−0.654, 0.417)	0.179	0.047 (−0.350, 0.484)	0.203	−0.073 (−0.617, 0.404)	0.615	0.215 (−0.369, 0.713)
Gut length	0.192	−0.067 (−0.181, 0.037)	−1.698	0.129 (−0.023, 0.264)	0.926	−0.053 (−0.175, 0.046)	0.996	−0.045 (−0.141, 0.061)	1.600	0.023 (−0.064, 0.109)	1.685	−0.024 (−0.113, 0.076)	1.693	0.026 (−0.085, 0.147)

Note: Models were run with one ecological variable fit as a fixed effect at a time, with site as a random effect. All models included sex and, where appropriate, length as a covariate and therefore all results reflect effect on size corrected traits. All models were compared to a null model without any ecological variables, and ΔDIC here reflects difference in DIC to the null model (ΔDIC < 0 indicates a better fit than the null model). The ecological variables included are average water temperature, mean depth, piscivorous bird density, catch per unit effort (CPUE) of stickleback, and three multivariate descriptors of invertebrate community (zPC2, zPC3, zPC4). Posterior means of the posterior distribution for linear coefficients estimates (β) are presented, with 95% credible intervals in subscript parentheses. β estimates not presented when ΔDIC to the null model > 2. Boldface indicates that the posterior distribution of the linear coefficient (β) estimates does not overlap with zero.

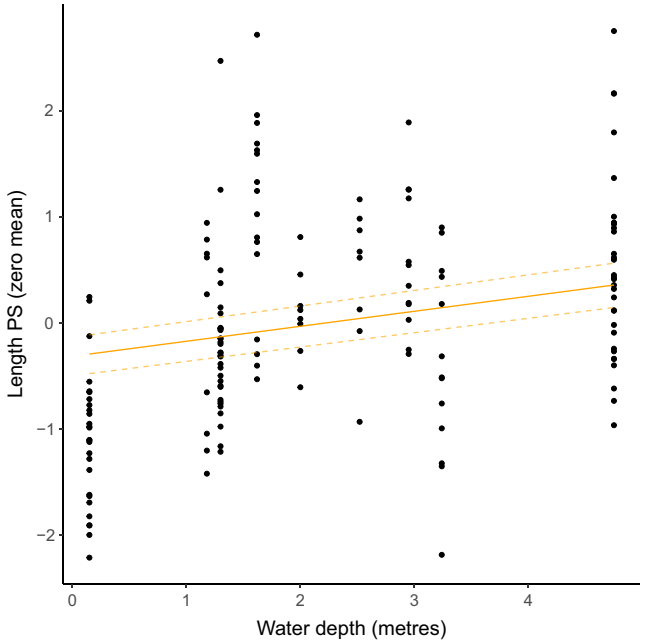

FIGURE 2 Effect of water depth on relative pelvic spine length (PS) of threespine stickleback in Lake Mývatn. The points are raw data and the line is the predicted effect of water depth on pelvic spine length, after correcting for the effect of standard length. *Y*-axis is standardized to have zero mean and standard deviation of one, such that zero represents the average trait in the data set

3.2 | Genomic divergence

Using 1,205,604 SNP markers across 186 individuals, principal component analysis (PCA), pairwise F_{ST} and admixture analyses all revealed little genome-wide differentiation. First, the first two principal components (PC1 and PC2) explained only 0.009% and 0.008% of variance, respectively (see Table S1). There was some clustering of individuals along PC1 (Figure S4), which could be indicative of clustering based on genotype (associated with, for example, spatial structure, sex or phenotype), on areas of low recombination or, alternatively, arise as a statistical artefact. Our PCA did not cluster individuals according to any a priori hypotheses for which we had data (i.e., according to basin, habitat type, site, sex, phenotype or tapeworm presence). Second, pairwise Nei's F_{ST} were very low at both habitat and site level (pairwise F_{ST} between sites or habitats ranged from 0.0004 to 0.005, Table S4). Third, admixture analyses using SNMF revealed that the model with $K = 1$ had the lowest cross-entropy score, suggesting that $K = 1$ best predicted the population genetic structure in our data set (Figure 3). This suggests that Mývatn stickleback form one panmictic population. The effective population size (N_e) for the population was estimated as 1976 (±640).

3.3 | Genome-wide-association analyses

Additive genetic variance (V_A) ranged from 0.10 for relative gut length to 0.62 for GRN and 0.69 for plate number (Table 4). SNP based heritability ranged from 0.42 (for relative gut length) to 0.62 (for plate number) and 0.65 (for GRL2) (Table 4). We found between five and 100 putatively causal SNPs associated with variation in each trait (Table 4). In nine cases, pairs of SNPs that were associated with trait variation were also closely linked (within 1 kb) on the genome. All of these were between functionally similar traits. Specifically, one 1 kb region (on chr VII) had SNPs associated with DS1 and DS2, seven regions (on chr I, IV, XIV and XVIII) had SNPs associated with GRL2 and GRL3, and one region (chr VI) had SNPs associated with both GRL3 and GRW. Genomic regions associated with the relative lengths of GRL2, GRL3 and the gut were linked to the same 10 QTLs for various feeding traits, and the relative length of GRL3 was linked to a further 28 QTLs for feeding traits, including gill raker length, spacing and number. Genomic regions associated with relative GRW overlapped with all those for GRL2 and GRL3, as well as a further 68 QTLs for feeding traits, including for lateral, medial and middle raker spacing. Regions of the genome associated with lateral plate number in our analysis were physically linked to 17 regions with QTLs previously identified for armour plating phenotypes, nine of which were for lateral plate number. Most notably, the major peak on the Manhattan plot for lateral plate number in our analyses (see Figure S5) fell on the major effect *EDA* gene on chr IV, which is known to control the recurrent plate loss in freshwater stickleback (Archambeault et al., 2020; O'Brown et al., 2015). All trait related SNPs were linked to regions on the genome with previously mapped QTLs for landmark positions of body shape (Albert et al., 2008; Table S5).

3.4 | Genome - environment association analyses

The number of candidate SNPs where allele frequencies significantly correlated with each ecological variable ranged between 14,160 and 16,778 (Table 5), on 2104–2440 genes. Many of the candidate SNPs were the same across models, likely due to the highly correlated nature of the ecological variables (see Table 1 and Table S2). More specifically, the total number of unique SNPs identified across all LFMM analyses was 70,772, and the total number of unique genes identified in LFMM analyses was 7299. There was considerable overlap between genes that were linked to each ecological variable. For instance, of the genes found to be linked to one ecological variable, an average of 45% of those were also linked to another ecological variable. Furthermore, although the candidate SNPs were distributed across the whole genome, there was considerable linkage between SNPs with clear peaks at specific genomic locations (see Figure S6 for Manhattan plots). The model for zPC2 (which described the negative covariance of *Rotifers* and *Alona* sp. with other cladocerans and copepods) had the lowest DIC, suggesting that genomic variation was best predicted by invertebrate composition, followed by water depth and temperature (Table 5).

We found between 18 and 34 enriched molecular functions per ecological variable (Table 5 and Table S7). The biological process occurring most commonly in enriched terms across all

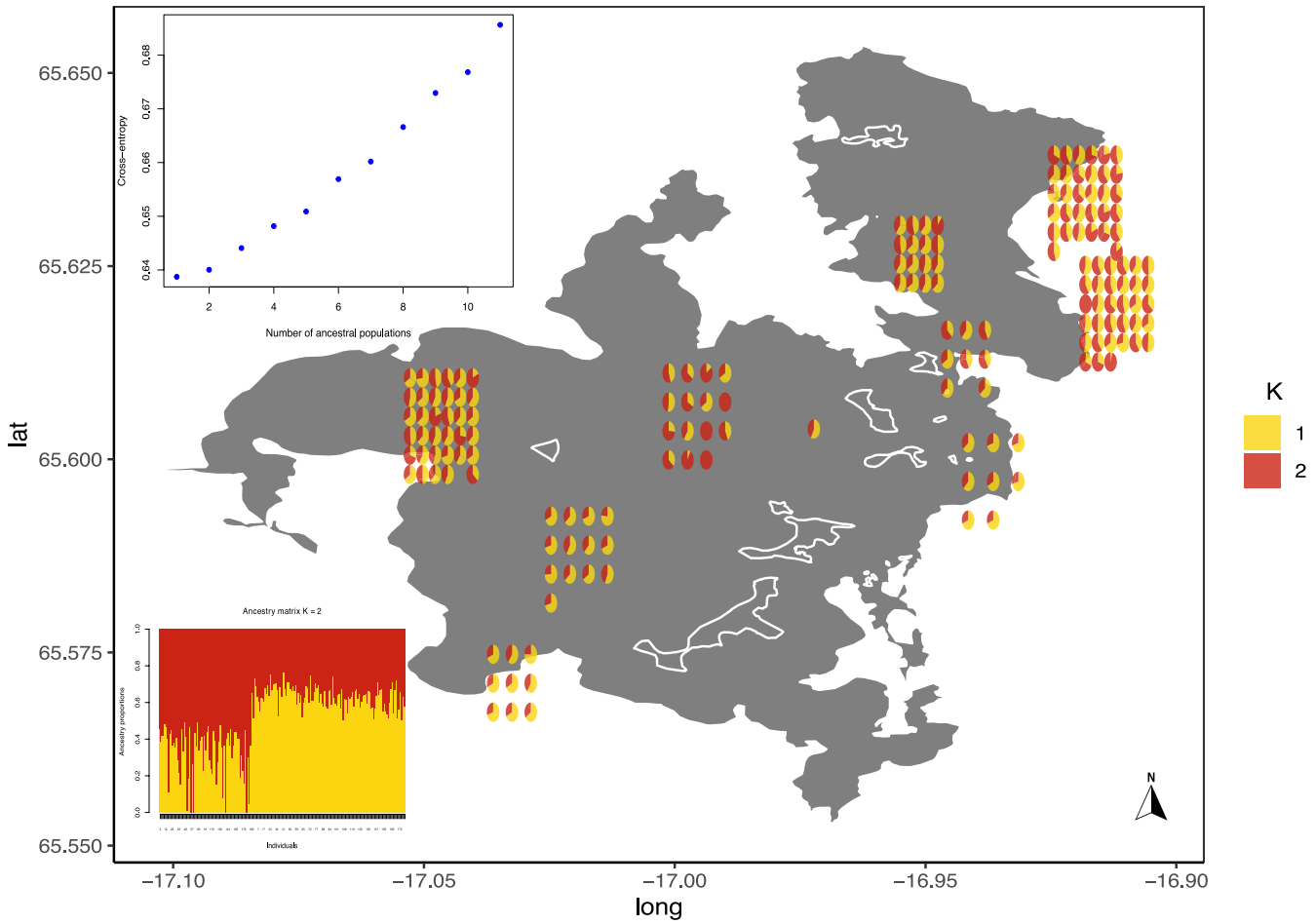

FIGURE 3 Plot shows the results of admixture analyses (conducted using SNMF, see Methods) used to assess the population genetic structure of threespine stickleback in Lake Mývatn. Model outputs showing cross entropy score for models with *K* 1–10 (top left), where lowest score reflects likely *K*. For illustrative purposes only (i.e., to illustrate admixture throughout the lake), the plot insert (bottom left) shows admixture proportions for $K = 2$, and colours in pies plotted at each site (per individual) reflect predicted admixture proportions estimated for $K = 2$

environments was the development of the central nervous system, followed by other developmental processes (i.e., the development of circulatory system, kidneys, tissue, embryo development, fin development, and ossification) and metabolic processes (most commonly involving protein metabolism). For temperature, protein phosphorylation was the most significantly enriched; for depth, it was ion transmembrane transport; for bird density and zPC3, it was the cell surface receptor signalling pathway category (i.e., signal transduction); for CPUE, it was cell adhesion; for zPC2 it was neuron development; and for zPC4 it was nervous system development (Table 5). Interestingly, we found that terms associated with sensory systems (including response to abiotic stimulus and perception of sound) were enriched for zPC3 and zPC4, which each reflect different components of the community structure of potential prey species. Loci associated with aspects of kidney function, including renal filtration, were enriched for temperature, depth and bird density, and terms associated with immune system and function were enriched in relation to temperature, depth and zPC4.

We found that candidate SNPs identified in LFMM analyses were linked on the genome to putative QTLs (as identified in GWA analyses) for DS1 (for all environments), DS2 (for water depth, zPC2, zPC3 and zPC4), and PS (for water depth, zPC2, zPC3 and zPC4). Our permutation tests using the maximum number of SNPs identified in LFMM results ($N = 16,778$) indicated that an overlap between these two sets of SNPs was extremely unlikely to occur by chance (N permutations where overlap occurred = 0, $p = 0$). There was a total of 23 genes on regions of the genome where there were both candidate SNPs identified in environmental association analyses, and QTLs associated with phenotypic variance (see Table S6). Of those genes, *ULK2* and *CCNB1* genes had haplotypes causing changes in amino acid sequence.

4 | DISCUSSION

Inferring the genomic basis of adaptive trait variation, including the interplay between adaptive divergence and gene flow, remains

Trait	V_A	V_E	h^2	GEMMA sample size	SNPs
Length	0.544	0.453	0.545	862,988	9
Defence traits					
Plate number	0.699	0.455	0.606	862,161	38
DS1	0.169	0.175	0.492	817,043	11
DS2	0.146	0.181	0.447	817,043	5
PS	0.168	0.216	0.438	819,714	11
Trophic morphology					
GRL2	0.448	0.241	0.650	819,546	33
GRL3	0.288	0.384	0.429	817,619	48
GRW	0.390	0.319	0.550	856,710	100
GRN	0.625	0.560	0.527	830,020	8
Gut length	0.104	0.146	0.415	849,418	20

Note: Posterior means for estimates of additive genetic (V_A) and residual (V_E) variance, and SNP-based heritability (h^2) are shown for all measured traits. "SNPs" refers to the number of SNPs significantly associated with a given trait (identified in GWA analyses run in GEMMA). All analyses (apart from length) included total length as a fixed effect covariate, and therefore results presented here reflect effects on size corrected (i.e., relative) trait values.

TABLE 4 Results of genome-wide-association analyses on size-corrected traits (total length, defence traits and trophic morphology) used to estimate single nucleotide polymorphism (SNP) based heritability and number of putatively causative SNPs in stickleback of Lake Mývatn.

a central endeavour in evolutionary biology. To increase rigour, data on both selective agents and organisms' multivariate phenotypes are needed when identifying regions of the genome potentially implicated in adaptation (Jones et al., 2013; Stinchcombe & Hoekstra, 2008). We show that there was phenotypic divergence in several traits of threespine stickleback from Mývatn, Iceland, but little evidence for genome wide population genomic structure, suggesting extensive gene flow. However, a combination of GWA and landscape genomics approaches allowed us to isolate genomic regions associated with both environmental and phenotypic variation, suggesting genomic divergence in response to natural selection in the face of gene flow.

4.1 | Phenotypic divergence in the face of gene flow

Mývatn stickleback showed spatial divergence in several traits, some of which was associated with ecological variation. Notably, stickleback were smaller at the warm shore site, had more armour plates and relatively longer first dorsal spines in the North than in the South basin, and relatively longer, but fewer, gill rakers in the rocky shore site than in the rest of the lake. These results align partially with previous findings from the same population sampled in 2009 (Millet et al., 2013), which found spatial phenotypic divergence in body size (N basin: larger), spine length (N basin: longer spines) and gill raker gap width, but no divergence in lateral plate number. The number of armour plates and length of spines are important predator defence traits in stickleback (Reimchen, 1992, 2000), with increased predation pressure by birds or fish selecting for more armour plates and/or longer spines (Vamosi & Schluter, 2004). Mývatn stickleback represent a low plated morph (range 3–10) typical of freshwater environments (Reid et al., 2021), but the higher plate number and longer spines in the N than the S basin indicates stronger predator induced selection in the N basin. This was further supported by phenotype-environment association analyses that showed that Mývatn stickleback had longer second dorsal spines where piscivorous waterfowl density was higher, and relatively longer pelvic spines in deeper water. The latter may reflect variation in predation pressure by altering the occurrence and visibility to visual predators (e.g., salmonids; Ålund et al., 2022; Rypel et al., 2007; Veen et al., 2017).

Lake fish, including stickleback, often diverge along the benthic-limnetic axis, whereby fish that specialize on a benthic diet have fewer and shorter gill rakers than those on a limnetic diet (McGee et al., 2013; Schluter & McPhail, 1992). Interestingly, we show that gill raker divergence (i.e., shorter and more vs. longer and less gill rakers) did not follow a typical benthic-limnetic divergence. Whilst we found some spatial divergence in gill raker number and length, gill raker morphology was not associated with any of our environmental measures (including prey abundances) suggesting that gill rakers may be responding along an unmeasured axis of divergence. Moreover, our findings contrast to some extent with Millet et al. (2013) who found divergence between Mined and Warm habitats in gill raker number and gap width rather than gill raker number and length. This discrepancy might suggest that spatiotemporal variation in availability of stickleback prey in Mývatn (Einarsson et al., 2004; Ives et al., 2008; Örnólfsdóttir & Einarsson, 2004), may induce fluctuating selection (Bell, 2010) – an interesting target for future research.

Despite observed phenotypic divergence, we found no evidence for genome-wide divergence, suggesting a single panmictic population and phenotypic divergence in the face of gene flow. Although this aligns with results of Millet et al. (2013), they contrast with those of Ólafsdóttir et al. (2007) who found genetic divergence between two sites and suggested the presence of two morphs "Lava" and "Mud". This discrepancy in genetic divergence may reflect temporal

TABLE 5 Summary of analyses used to identify gene–environment associations on threespine stickleback in Lake Mývatn

Ecological variable	Candidate SNPs	ΔDIC	QTL	N_{SNPS}	Genes	Number of enriched GO terms	Top enriched GO term
Water depth	14,337	9.898	DS1	11	2299		
			DS2	2		22	Protein phosphorylation
			PS	11			
Temperature	15,719	11.001	DS1	10	2486	27	Ion transmembrane transport
CPUE	15,677	57.321	DS1	9	2349	24	Cell adhesion
Bird density	15,533	54.034	DS1	9	2420	22	Cell surface receptor signalling pathway
zPC2	16,263	0	DS1	11	2440	21	Neuron development
			DS2	5			
			PS	2			
zPC3	14,160	179.136	DS1	11	2104	18	Cell surface receptor signalling pathway
			DS2	5			
			PS	7			
zPC4	16,778	83.462	DS1	11	2257	34	Nervous system development
			DS2	5			
			PS	2			

Note: The number of candidate SNPs are given, as identified in environment-association analyses to be associated with each ecological variable ("Candidate SNPs"); ΔDIC refers to difference in the deviance information criterion used to compare the models which fit ecological variables separately; traits for which their QTL (as determined in GWA analyses) overlaps with candidate SNPs on the genome ("QTL"); number of candidate SNPs that overlap with that QTL ("N_{SNPS}"); number of genes the candidate SNPs overlap with ("Genes"); number of enriched molecular functions identified in gene ontology analyses ("Number of enriched GO terms"); and, the most enriched molecular function identified in GO analyses ("Top enriched GO term").

changes in the extent of spatial genetic divergence and the loss of previous ecotypes, potentially as a result of strong population fluctuations in stickleback population size (Phillips et al., 2023). Notably, population demographic analyses indicate that the high-density North basin periodically subsidizes the low-density South basin through dispersal (Phillips et al., 2023), which could result in periodic gene flow. However, it is also possible that differences in the resolution of both spatial sampling and sequencing may have resulted in the discrepancies between studies.

4.2 | Gene-phenotype-environment associations: Evidence for a role of natural selection?

Gene flow is thought to constrain divergence by swamping locally adapted alleles (but see Bal et al., 2021; Räsänen & Hendry, 2008), and while phenotype-environment associations can indicate responses to natural selection (Endler, 1995), they may also reflect phenotypic plasticity and nonrandom dispersal (Westneat et al., 2019). Evidence for selection is therefore strengthened when observed trait divergence has a genomic component (i.e., "gene-phenotype-environment" associations). Importantly, all traits we measured had substantial additive genetic variation, suggesting high levels of potential for traits to respond to selection. This was further reflected at the genomic level, where we were able to successfully recover similar genomic architecture as previously reported for this species. Using the mapped genomic architecture, we show that while divergence of most traits was not linked to a genomic component, relative spine lengths was. Specifically, genomic regions that were associated with pelvic spine length (that were longer in deeper waters), were divergent across water depth strengthening the conclusion that the observed divergence in pelvic spine length is a result of divergent natural selection – potentially due to fish predation. It should be noted, however, that our GWA analyses may have been limited in statistical power to identify loci of small effect. Therefore, our analyses linking genome, phenotype and environment are probably quite conservative as we may have missed relationships with these small effect loci.

Mapping the causative pathways from genes to phenotypes is a notoriously challenging task (Brodie et al., 2016), but identifying the genes on regions involved in gene-phenotype-environment associations provide insight into the molecular mechanisms underlying adaptation. On the genomic regions associated with the pelvic spine length - depth relationship, we found two genes (ULK2 and CCNB1) with haplotypes coding for alternate amino acids. These are functionally interesting as ULK2 is a serine/threonine kinase which, along with its homologue ULK1, interacts with the master regulator of metabolism (mTOR) and regulates apoptosis in response to starvation, and *CCNB1* codes for cyclin B1, a major kinase regulator

which activates mitosis and regulates the dynamics of the cell cycle. Whilst these findings suggest candidate molecular functions underlying responses to natural selection, explicit follow up studies (e.g., CRISPR or gene expression studies) would be needed to get at the causal relationships with pelvic spine length variation.

Many of the regions of the genome that were correlated with environmental variation in our study were not linked to observed trait divergence. This is not surprising given that we only measured a selected subset of traits within specific functional categories. An understanding of what biological functions these regions are associated with can therefore provide hypotheses about targets of divergent natural selection for future studies. In our study, the biological processes implicated in genomic regions included developmental processes, such as development of nervous and sensory systems. Notably, visual response to abiotic stimulus and perception of sound were enriched biological functions that were associated with the community structure of invertebrates. This is particularly interesting for Mývatn stickleback due to potential for influencing ability to respond to prey stimulus, and because the sensory drive hypothesis posits that an organisms' communication system should be especially sensitive to ecological variation (Endler, 1992; Endler et al., 1993).

Although adaptation can occur across exceptionally short time frames (Kingman et al., 2022), selection often acts multifariously and environments are rarely stable temporally, resulting in fluctuating selection pressures (Bell, 2010). Mývatn is a highly dynamic ecosystem, where multiple dimensions of its ecology and, importantly, stickleback population size fluctuate substantially through time (Phillips et al., 2023; Örnólfsdóttir & Einarsson, 2004; Ives et al., 2008). Our environmental, phenotypic and genomic data were collected at a single point in time, and may therefore not accurately reflect the selective environment experienced by Mývatn stickleback within or across generations. Whilst using ecological data collected at the same (single) time point as genomic and phenotypic data is common practice in studies that investigate selection in wild populations (Bolnick & Ballare, 2020; Magalhaes et al., 2021), tracking gene-phenotype-environment associations through time would allow inferences on how patterns of phenotypic and genomic variation withstand, or respond to, temporal fluctuations in selective pressures. In context of Mývatn stickleback, this would also facilitate the exploration of the spatiotemporal balance between adaptive divergence and gene flow.

5 | CONCLUSIONS

Our results provide evidence for substantial genetic variation underlying functionally relevant traits, and suggest adaptive divergence in face of gene flow in a large, panmictic population inhabiting an environmentally heterogeneous lake. In particular, gene-phenotype-environment association analyses allowed us to identify genomic signatures of selection by testing which phenotypic traits and genomic variants are associated with putative selective agents. Whilst we found evidence for genome-phenotype-environment correlations for spine length, we also found evidence for phenotypic divergence in body size (total length) and trophic morphology (gut length, gill raker length and gill raker number) without apparent genomic divergence – despite substantial additive genetic variation in these traits. The lack of genomic trait divergence across environments could reflect a combination of phenotypic plasticity and/or habitat choice (Crispo, 2008; Edelaar et al., 2017; Garant et al., 2007; Westneat et al., 2019), both of which can constrain or accelerate adaptive divergence (Crispo, 2008; Levis & Pfennig, 2020; Schoener, 1974; Wund, 2012). Whilst drawing definitive conclusions about selective processes causing genome-phenotype-environment correlations (as opposed to drift or plasticity) would require data on phenotype-fitness associations, our study sets the stage for a holistic understanding of patterns of divergence and the maintenance of genomic and phenomic variation in spatiotemporally varying wild populations.

AUTHOR CONTRIBUTIONS

Kasha Strickland conceptualized, designed and led the research, collected and analysed data, and led the writing of the manuscript; Katja Räsänen contributed to conceptual development; Bjarni Kristofer Kristjánsson contributed to generation of phenotype data; Joseph S. Phillips contributed to designing phenotypic analyses; Arni Einarsson collected field data; Ragna G. Snorradóttir generated the phenotype data; Mireia Bartrons contributed ecological data; Zophonías Oddur Jónsson contributed to conceptualisation and all molecular work. All authors contributed to editing the manuscript and approved the final version.

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CONFLICT OF INTEREST

Authors have no conflicts of interest to declare.

DATA AVAILABILITY AND BENEFIT-SHARING STATEMENT

All raw reads generated and analysed in this study have been archived at the European Nucleotide Archive (ENA), accession number PRJEB58765. Phenotypic and ecological data needed to replicate analyses presented in this study have been deposited in the Dryad repository at https://doi.org/10.5061/dryad.mkkwh7147 (Strickland et al., 2023).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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