Divergent evolution of head morphology between marine and freshwater sticklebacks

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Abstract

Intraspecific phenotypic differentiation is of common place occurrence, but the degree to which it reflects phenotypic plasticity or local adaptation remains often unclear. To be considered as adaptive, the differentiation must be genetically based and exceed what could be expected by neutral processes only. Using laboratory reared full-sib family data from replicate nine-spined stickleback (*Pungitius pungitius*) populations, we show that freshwater and marine fish display genetically based adaptive differentiation in head size and shape. Utilising identity-by-descent relationships among full-sibs as estimated with the aid of molecular markers, we further show that the studied traits are also highly heritable in all populations indicating and that they can respond to future episodes of natural selection. The head shape and size of pond fish suggests that observed adaptive differentiation has been driven by selection favoring limnetic feeding strategy among the pond fish. Analyses of gill-raker morphology were less conclusive: genetic differentiation was found in gill-raker length (pond > marine) and number, but the degree of divergence in these traits did not exceed neutral expectations. Yet, the direction of divergence in gill raker traits are suggestive of the limnetic feeding mode of pond fish, aligning with the inference from the head morphology analyses.

Keywords: Geometric morphometrics, adaptation, heritability, Q_{ST}, quantitative genetics, *Pungitius pungitius*

Introduction

Phenotypic differentiation associated with different habitat characteristics is of commonplace occurrence in both animal (Endler 1978; Foster 1999) and plant (Linhart and Grant 1996; Bossdorf et al. 2005) kingdoms. Likewise, phenotypic responses to changing environmental conditions have been frequently reported (Walther et al. 2002; Parmesan 2006; Sheridan & Bickford 2011), but many studies struggle to disentangle phenotypic plasticity and genetically based differentiation as the underlying cause for observed differentiation (Gienapp et al. 2008; Merilä 2012; Merilä & Hendry 2014; Stamp & Hadfield 2020). Moreover, even if evidence for genetically based differentiation is found, additional evidence is required to prove that the observed differentiation is adaptive.

Common garden studies combined with neutrality tests (e.g., Lande 1976; Rogers 1986; Spitze 1993; Merilä & Crnokrak 2001) provide a fairly straightforward way to differentiate adaptive *vs.* non-adaptive causes of phenotypic differentiation (Whitlock 2008; Leinonen et al. 2013; Savolainen et al. 2013). In particular, the neutrality tests implemented in the program 'driftsel' (Karhunen et al. 2013, 2014) provide a statistically powerful approach able to identify footprints of natural selection even in a fairly small sample of populations (Ovaskainen et al. 2011). Nevertheless, the driftsel-approach has not become widely used in studies of population differentiation, possibly because the the field has moved to use genomic data to identify footprints of natural selection (e.g. Storz 2005; Nosil et al. 2009; Narum & Hess 2011), and because common garden studies are logistically demanding. While genomic studies of natural selection appear to provide less laborious paths towards identifying adaptive differentiation than common garden experiments, they are still grappling with the problem of false positive tests (Mallick et al. 2009; Bierne et al. 2013; Hoban et al. 2016), as well as with the difficulty of linking the loci under selection to their phenotypic targets especially in the case of polygenic traits (McKay & Latta 2002; De Kovel 2006; Le Corre &

Kremer 2012). In addition, genome scan approaches become very inefficient in picking up signals of selection in systems having high background levels of differentiation, such as in highly subdivided populations experiencing strong drift (Hoban et al. 2016).

Variation in fish head shape and jaw structures have been extensively studied as they are important trophic traits influencing fitness (e.g., Roy et al. 2010; McGee et al. 2013) and even believed to have spearheaded adaptive radiations (Brouwers 2011; Sallan & Friedman 2012). While population differences in them have been sometimes shown to be genetically based (Kimmel et al. 2005; Albert et al. 2008; McGee & Wainwright 2013), there is also evidence that there is a strong plastic component to variation in head shape and feeding structures (Troy et al. 1994; Dingemanse et al. 2009). Hence, while there are many reports of head and jaw shape differentiation among fish populations (e.g., Walker and Bell 2000; Tobler et al. 2011 Østbye et al. 2016), few studies have firmly established an adaptive (contra plastic) basis for this differentiation.

Gill-raker morphology constitutes another set of traits that have been extensively studied in the context of fish foraging ecology (e.g., Berner et al. 2008; Wund et al. 2012; Hosoki et al. 2019). Fish species have adapted to various habitats by evolving unique gill-raker characteristics that correspond to their specific feeding strategies. As for the case of three-spined sticklebacks (*Gasterosteus aculeatus*), marine populations typically have more, closely spaced and longer gill rakers than freshwater populations (Gross and Anderson. 1984; Leaver et al. 2012; Glazer et al. 2014; Magalhaes et al. 2021), reflecting their limnetic feeding strategy in marine ecosystems and the benthic feeding strategy in freshwater ecosystems (Ravinet et al. 2014). However, formal tests of the adaptive basis of this differentiation are few (but see: Raeymaekers et al. 2007; Seymour et al. 2019).

The aim of this study was to test (1) whether pond and marine populations of nine-spined sticklebacks (*Pungitius pungitius*) differ in head and gill raker morphology and (2) whether this differentiation exceeds neutral expectations meaning that observed divergence has been driven by natural selection. To do this, we conducted a common garden experiment and raised fish from four pond and four marine populations to control for environmental effects on phenotypes, and subjected the data to driftsel analyses to identify footprints of selection. The results revealed genetically based phenotypic differentiation in head and gill-raker traits between marine and pond sticklebacks, and that the differentiation in head traits has been driven by divergent natural selection.

Methods

Study populations and sampling

The parental fish used in this study were collected May-June 2018 from four pond (Rytilampi 66.38482°N, 29.31561°E; Pyöreälampi 66.26226°N, 29.42916°E; Kirkasvetinenlampi 66.43673°N, 29.13568°E; Bynastjärnen 64.45416°N, 19.44075°E) and four marine (Tvärminne 59.83333°N, 23.24900°E; Pori 61.59111°N, 21.47295°E; Raahe 64.68818°N, 24.46189°E; Bölesviken 63.66110°N, 20.20940°E) populations from Fennoscandia using minnow traps or beach seine (Fig. 1a). The captured adults were transported to aquaculture facilities of University of Helsinki, and maintained in 1 m^3 flow-through freshwater aquaria (one per population) until used in artificial fertilisations.

Common garden experiments and genotype data

Artificial fertilizations among randomly selected adult fish in breeding condition were conducted between May 24th and July 4th 2018. Standard *in vitro* fertilization techniques and egg rearing methods were applied following Arnott and Barber (2000) and as described in

105 detail in Fraimout *et al.* (2022). In brief, eggs from gravid females were obtained by gently
106 squeezing their abdomens over a petri dish. Sperm was retrieved from males after they were
107 over-anesthetized with tricaine methanesulfonate (MS-222) by dissecting the testes and
108 subsequently mincing them in the same petri dish containing the eggs. Eggs and sperm were
109 mixed to ensure fertilization and kept in water until hatching. To avoid fungal infections,
110 water in the petri dishes was changed twice daily and clutches were inspected for signs of
111 fungal infections or death - dead and infected eggs were removed. At hatching each clutch
112 was split in two replicate 11 x 10 x 10 cm plastic containers filled with filtered freshwater in
113 which they were maintained for a four week period during which yolk resorption took place.
114 The larvae were fed *ad libitum* with live brine shrimp (*Artemia sp*. nauplii). After this, all
115 replicated families were transferred to Allentown Zebrafish Rack Systems (Aquaneering Inc.,
116 San Diego, USA). These racks had a closed water circulation system with physical, chemical,
117 biological and UV filters. The fish in racks were fed for the first four weeks with a mixture of
118 *Artemi*a nauplii and chopped chironomid larvae, and after this, with chopped chironomid
119 larvae. Part of the water in the rack systems was changed every fortnight to maintain good
120 water quality. Fish were reared in family groups (maximum five individuals per aquarium) in
121 racks for a period of ca. 1 year (mean age: 316.4 days) after which the fish were euthanized
122 with MS-222. Temperature and light conditions were kept constant throughout the
123 experiment (12:12 LD; 15°C) except during an overwintering period of 3 months (November
124 – January) when water temperature was lowered to 10°C and light cycle to 5:19 LD.

125 We used data on molecular variation among populations to estimate a neutral baseline for the
126 tests of selection (see below). Specifically, we obtained genome-wide SNP data for all
127 individuals using a restriction site-associated DNA (RAD) sequencing technique (2b-RAD;
128 Wang et al., 2012). Genomic DNA was extracted from fin clips using a standard salting out
129 protocol (LoperaBarrero et al. 2008) and 2b-RAD libraries were built following Momigliano

et al (2018) using a slightly modified version of the protocol available online at https://github.com/z0on/2bRAD_GATK/blob/master/2bRAD_protocol_may15_2017_nnrw.pdf). Briefly, high molecular weight DNA was digested using the *BcgI* enzyme (New England Biolabs), adaptors were ligated and the fragments amplified via PCR following the protocol provided in Momigliano *et al.* (2018). Libraries were pooled and the target fragments were isolated using a BluePippin size selector (Sage Science). Libraries were sequenced using Illumina technology (HiSeq 4000) at the Beijing Genomic Institute (BGI; Hong-Kong). Raw reads were demultiplexed and mapped to the *P. pungitius* genome (v.6; Varadharajan *et al.* 2019) using bowtie2 (Langmead and Salzberg, 2012). We used samtools (v.1.10; Li *et al.* 2009) to convert SAM files to BAM files.

We also used SNP-data to identify sex of all F_1 offspring, as they were measured before reaching sexual maturity. From the genotype data file, we used the *snpRelate* R package (Zheng *et al.* 2012) to perform a Principal Component Analysis (PCA) based on all markers located on the sex chromosome (Linkage group 12; Natri *et al.* 2019) and assigned sex to the F_1 individuals according to their clustering with the parental individuals of known sex. Finally, we pruned the dataset from markers in high linkage disequilibrium (LD) using the *snpgdsLDpruning* function of the *snpRelate* package and retained only markers with LD < 0.8 and a minimum allele frequency (MAF) > 0.05 and removed all sex-linked SNPs. This resulted in a total of 2,660 informative SNPs.

Analyses of head size and shape

We quantified variation in head size and shape among the study populations following a landmark-based geometric morphometrics approach (Bookstein 1991). A total of 32 landmarks (see Fig. 1b for details) and semi-landmarks were digitized from the left side of

individuals from digital images using the tpsDig2.1 software (Rohlf 2006). Specifically, 4 fixed landmarks were placed as in Yang *et al.* (2016) at: the posterior extent of the supraoccipital (landmark 1; Fig. 1b), the anterior insertion of the premaxilla (landmark 2; Fig. 1b), the anterior-ventral extent of the preopercular (landmark 3; Fig. 1b) and the ventral extent of the preopercular bone (landmark 4; Fig. 1b). Semilandmarks were placed by resampling a total of 30 points along an outline starting from landmark 1 and ending on landmark 4 describing head shape using the 'draw curves' mode in tpsDig. To avoid redundancy the first and last semi landmarks (overlapping with fixed landmarks 1 and 4) were removed prior to analyses. All subsequent morphometric analyses from the obtained landmark configuration were performed in R using the *geomorph* package (v.4.0.1; Adams *et al*. 2013). First, we used the plotOutliers function in geomorph to identify outlier individuals based on their Procrustes distance from the mean head shape of the samples. We removed 16 individuals outside the upper quartile range and retained a total of 425 individuals for subsequent head shape analyses. Landmarks were superimposed following the Procrustes generalized least square superimposition (Dryden & Maria 1992) and semi-landmarks were slid to the mean shape configuration and their position optimized using Procrustes distance by setting the *ProcD* option of the *gpagen* function to "TRUE" in geomorph. Head centroid size obtained from the Procrustes alignment was used as measurement for head size in all subsequent analyses. For head shape, we performed a PCA on the procrustes coordinates using the *gm.prcomp* function. The non-null principal components (PC) describing shape variation were used as shape data in the subsequent analyses and standardized for allometric variation (Rolshausen *et al.* 2015). Specifically, we followed the allometric approach of Lleonart *et al.* (2000) where the standardized trait measurement PC_S is defined such that:

$$PC_S = PC_0 (HS_m/HS_0)^b \quad (1)$$

where PC_0 is the original (i.e., unstandardized) trait measurement, HS_M is the mean head centroid size per population; HS_0 is the head centroid size of each individual and b is the population-specific coefficient of within-slope regression of head centroid size on the focal PC.

We tested for population and sex differences for head size and shape using procrustes analyses of variance as implemented in the *geomorph* package. Differences in head centroid sizes were tested using the *procD.lm* function and fitting a model including body length, sex, habitat and population as explanatory variables. Similarly, we tested for differences in head shape by fitting a model including log-transformed head centroid size, sex, habitat and population as explanatory variables. We further tested for differentiation in head shape data using multivariate analysis of variance (MANOVA) based on the standardized PC described above.

Analyses of gill raker number and size

Gill raker traits were measured from a total of 126 individuals from 64 families across eight populations — four pond (n=51) and four marine (n=75) populations. These samples included 62 wild-caught individuals and 64 of their laboratory-raised F_1 generation offspring, comprising 32 females, 92 males, and two individuals of unknown sex.

Four gill raker traits were measured: number of gill rakers, length, width, and spacing between rakers. The number of gill rakers were counted on the left side of the first gill arch unless the left gill rakers were damaged, in which case the right side counts were used instead. As the dorsal arch is prone to damage during dissection, only gill rakers on the ventral arch were counted. We measured the length and width of the longest gill raker along the arch, and

defined spacing as the center-to-center spacing between the base of a pair of adjacent rakers, specifically between the longest raker and the adjacent one on the upper side (Fig. 1c).

To ensure high repeatability of the measurements, we followed the laboratory protocol of Nicholas and Craig (2016). In summary, we first dissected the first branchial gill arch, digested it in a 10% Potassium Hydroxide (KOH) solution for two hours, and stained it with 0.008% Alizarin Red S in 1% KOH in water for at least two hours, as the staining time varies depending on the gill arch size. We then rinsed the raker to remove excess stain, placed the specimen in 89% glycerol, and flat-mounted the skeleton on bridged cover slips. Measurements were obtained using digital images taken with a fluorescent stereomicroscope equipped with an Olympus SZX16 or a Nikon FI3 camera. Length-related measurements were accurate to the nearest 0.01 mm using ImageJ software v. 1.51 (Schneider et al. 2012). To validate the measurement data, we independently measured the same set of traits for 20% of randomly selected samples and calculated the repeatability (Nakagawa and Schielzeth, 2010). The repeatability of these traits from two independent measurements ranged from 0.855 to 0.967 ($p < 0.001$), indicating a high level of precision in the measured traits.

As body size (standard length) varied between habitats and sexes, we size-standardized all linear gill raker traits prior to statistical analysis. This was done by constructing linear mixed effect models, incorporating habitat and sex as fixed factors and population and pedigree information as random factors, to determine if marine and pond populations differed in mean values of gill raker traits and whether these traits displayed sexual dimorphism. This analysis was performed using the 'mmer' function in the 'sommer' R package (Giovanny, 2016) using R version 4.2.2 (R Core Team, 2022).

Tests of natural selection

To test whether observed population differentiation in morphology exceeded neutral expectations, the approach of Ovaskainen et al. (2011) as implemented in the R packages driftsel and rafm (Karhunen *et al.* 2013) was adopted. We followed the workflow from Karhunen *et al.* (2013) and first estimated the matrix of population-level coancestry from genomic data. Due to the computational burden of the MCMC-based algorithm, we sampled 2000 random markers from the pruned SNP dataset (Li *et al.* 2019) which were used as input for the *do.all* function of the rafm package. We ran the model for 15,000 MCMC iterations with a burn-in period of 5000 and sampling every 10th iteration. The resulting coancestry matrix was then used as a neutral baseline for the driftsel analysis. We ran the *MH* function of the driftsel package using the posterior samples of the coancestry matrix as prior information and used either head centroid size or the three first size-standardized-PC most representative of habitat shape variation as input traits. Sex of the individuals was used as covariate and body length was added as covariate for the model using head centroid size only. We subsequently used the *S.test* and *H.test* functions to calculate the *S* and *H* statistics, respectively, as indicators of signal of selection. The rationale behind each statistic is similar to that of classical Q_{ST}-F_{ST} comparisons with values of *S* or *H* > 0.95 indicating signal of divergent selection, values equal to 0.5 indicates a scenario compatible with neutral evolution and values < 0.05 imply stabilizing selection. Contrary to the *S.test*, the *H.test* allows to incorporate habitat information into the driftsel framework to test whether habitat similarity correlates with phenotypic similarity among populations (Karhunen *et al.* 2014).

To verify the robustness of the selection test results, we also applied the method of Martin *et al.* (2008) to our genetic and phenotypic data. This method is a multivariate approach to the Q_{ST}-F_{ST} comparison and tests for proportionality between the genetic (co)variance matrix (**G**) and the among-population divergence (**D**). Under a scenario of purely neutral evolution, the proportionality between **G** and **D** is defined as:

$$\mathbf{D} = 2F_{ST}/(1-F_{ST})\mathbf{G} \quad (2)$$

where F_{ST} is estimated from putatively neutral molecular markers (Lande 1979, Lofsvold 1988, Marroig and Cheverud, 2004, McGuiguan et al 2005, Martin et al. 2008, Berner et al 2010). We applied the framework of Martin *et al*. (2008) to calculate the coefficient of proportionality ρ_{ST} between **D** and **G**, and compared it to the value of $2F_{ST}/(1-F_{ST})$ obtained from the pruned SNP dataset. The rationale behind this test is that values of $\rho_{ST} > 2F_{ST}/(1-F_{ST})$ would indicate a signal divergent selection. We used the *pairwise.prop* R function of the *neutrality* package from Martin et al. (2008) to calculate ρ_{ST} and the *hierfstat* R package (v.0.5.11; Goudet & Jombart 2022) to get bootstrapped estimates of F_{ST} from neutral loci. Statistical difference between ρ and $2F_{ST}/(1-F_{ST})$ was assessed based on the overlapping of confidence intervals around the two estimates.

Heritability of head morphology

We estimated the heritability of head shape variation using animal models under a Bayesian framework using the MCMCglmm R package (Hadfield 2010). We partitioned the phenotypic variance V_P of head shape into its additive V_A genetic component and estimated heritability as $h^2=V_A/V_P$. For each population, we fitted a model including the first PC (PC1) describing habitat variation in head as a response variable and added head centroid size as covariate and sex of the individuals as fixed effect. We used the same approach to estimate h^2 of head centroid size using sex of the individuals as a fixed effect. For both models, to account for actual relatedness among individuals in each population, we appended the Genomic Relationship Matrix (GRM) estimated from SNP data as a random effect in the model. The GRM was estimated from the pruned SNP data using the *G.matrix* function of the *snpReady* R package (Granatto *et al*. 2018). Each model was run with 303000 MCMC

iteration with a burn-in period of 3000 and sampling every 100th iteration. Model checking was performed by visually inspecting the trace plots of the MCMC chains and by inspecting the effect sizes of variance components using the *effecSize* function of the *coda* R package (Plummer *et al*. 2006). All h^2 values are reported as the median of the posterior samples along with their 95% Highest Posterior Density (HPD) intervals.

Results

Population differentiation in head morphology

We found significant effects of sex, population of origin and habitat on head centroid size (Table 1). Freshwater fish had larger heads than marine fish (Fig. 2a) and head centroid size varied to a greater extent among pond than among marine populations (Fig. S1). Comparison between sexes revealed sexual dimorphism in head centroid size, males having bigger relative head centroid size than females (Fig. 2a). We also found differences in head shape among habitats with the first PC of shape variation discriminating between pond and marine individuals (Fig. 2b). This difference was reflected by a significant habitat effect in the procrustes Anova on head shape (p <0.001, Table 2).

Population differentiation in gill raker traits

Individuals from pond populations were larger than those from marine populations (X_{pond} = 53.39 ± 2.39 [S.E.] mm, X_{marine} = 42.78 ± 1.12 mm; $F_{1,124}$ = 27.75, p < 0.001), and females were bigger than males ($X_{females}$ = 57.16 ± 2.52 mm; X_{males} = 43.20 ± 1.23 mm; $F_{1,123}$ = 51.79, p < 0.001). Marine and pond populations differed significantly in both the number (X_{pond} =

297 9.94 ± 0.13; X_{marine} = 8.59 ± 0.08; $t_{3.02}$ = 5.572, p = 0.01) and length (X_{pond} = 1.45 ± 0.04 mm;
298 X_{marine} = 1.09 ± 0.02 mm; $t_{5.91}$ = 2.630, p = 0.039) of gill rakers, as did the sexes ($X_{females}$ =
299 9.11 ± 0.20; X_{males} = 9.14 ± 0.10; $t_{122.79}$ = 2.794, p = 0.006) for number and ($X_{females}$ = 1.31 ±
300 0.06 mm; X_{males} = 1.21 ± 0.03 mm; $t_{119.68}$ = 4.549, p < 0.001) for length. Sticklebacks from
301 pond populations had more and longer rakers (Fig. 3), and males possessed shorter rakers for
302 their size relative to females. However, no significant differences were observed in the width
303 or spacing of rakers across habitats or sexes (p > 0.05 in all tests).

304

305 ***Tests of selection***

306 The three first PCs from the PCA of size-standardized-PC of shape variation were used as
307 input for driftsel which revealed a signal of divergent selection (S = 0.953 and H = 0.993; Fig.
308 4). All populations from both habitats were indicated to have diverged more than expected by
309 chance from their common ancestor (Fig. 4). The direction of divergence differed
310 consistently for marine and pond populations, with the exception one pond population (KRK;
311 Fig. 4b). Individuals from this population had an intermediate head morphology compared to
312 other populations and were not significantly different from the reconstructed ancestral
313 population (Fig. 4). We found similar results when analyzing head centroid size differentiation
314 with S = 0.978 and H = 1.

315

316 The multivariate Q_{ST}-F_{ST} approach indicated that the proportionality coefficient ρ was higher
317 than $2F_{ST}$ /(1-F_{ST}) obtained from neutral markers (ρ_{ST} = 1.8 [1.1; 5.3]; $2F_{ST}$ /(1-F_{ST}) = 0.828
318 [0.766; 0.890]), thus indicating a signal of divergent selection for head shape. Neither X^2 or
319 Bartlett-corrected X^2 test rejected the null hypothesis of proportionality between **D** and **G** (p
320 = 0.07; p=0.159, respectively) thus indicating that both matrices are proportional.

321

We did not find evidence for diversifying natural selection for any gill raker trait (S = 0.58, H = 0.86 for the univariate model for raker number; S = 0.69, H = 0.89 for length; S = 0.49, H = 0.62 for width; and S = 0.54, H = 0.74 for gap), suggesting that pond and marine populations have not evolved significantly further away from the ancestral mean than would be expected under neutrality. The same conclusion was reached combining all four gill raker traits in a multivariate analysis (S = 0.55; H = 0.91). This was also confirmed by the proportionality coefficient (ρ_{ST} = 0.33 [0.21; 0.80]) being not significantly higher than $2F_{ST}/(1-F_{ST})$ of 0.828 [0.766; 0.890]).

Heritability of head size and shape

We found moderate to high h^2 values for head shape and size in all populations (Table 3). Estimates for different populations were not significantly different as indicated by the overlapping 95% HPD intervals (Table 3).

Discussion

The results revealed consistent differences in head and gill-raker morphology between marine and pond populations of nine-spined sticklebacks reared in common garden conditions. This together with the results of the selection tests provide firm evidence that the genetically based differentiation in head size and shape (but not in gill-raker morphology) has been driven by divergent natural selection favouring different head morphology in marine and pond habitats. Since the studied pond populations have evolved independently in total isolation since their colonisation after the last glaciation (Herczeg et al. 2009; Shikano et al. 2010), the results also provide strong evidence for parallel phenotypic evolution in head morphology: random processes are not expected to result in evolution of similar morphologies in different

346 localities (Schluter 2000). However, even if the heritabilities of head traits were similar in
347 different populations, the question whether the genetic underpinnings of the similar head
348 morphology in different pond populations are underlied by the same or different genetic loci
349 remains to be investigated. Earlier studies of other traits in these populations suggest that the
350 similar phenotypic outcomes have been the result of recruitment of different genetic loci to
351 underlie similar phenotypic adaptations (Kemppainen et al. 2021; Fraimout et al. 2022; Yi et
352 al. 2023). In the following sections, we discuss how the results advance our understanding of
353 local adaptation in general, and that of sticklebacks in particular.

354 The driftsel approach circumvents many shortcomings associated with traditional Q_{ST}-F_{ST}
355 approaches aiming to test whether trait differentiation among populations exceeds that to be
356 expected due to stochastic processes alone (Karhunen et al. 2013). Here, it provided evidence
357 for adaptive nature of head size and shape differentiation among pond and marine stickleback
358 populations. As such, the results align with earlier evidence for adaptive differentiation
359 among the studied pond and marine populations (e.g. Herczeg et al. 2009; Ab Ghani et al.
360 2013; Karhunen et al. 2014; Fraimout et al. 2022). Particularly noteworthy is that despite the
361 high degree of background differentiation (mean F_{ST} = 0.286 across all populations) in our
362 data, driftsel was able to pick up a signal of divergent natural selection. The approach of
363 Martin *et al.* (2008) also suggested that differentiation in head shape was greater than the
364 neutral expectation but that the magnitude of divergence was moderate. Nevertheless, it is
365 conceivable that attempts to achieve the same with any F_{ST} outlier tests in data with this high
366 neutral background level of differentiation would likely fail (e.g., Hoban et al. 2016; Liet al.
367 2019). Besides, as pointed out by Bierne *et al.* (2013), outlier tests are not expected to work
368 well when genetic architecture of local adaptation has a polygenic basis, as is likely to be the
369 case for most quantitative traits of ecological interest.

While the differences in head size and shape were very clear and consistent between marine and freshwater populations, the driftsel analysis revealed one anomaly: head traits in one of the pond populations (KRK) seemed to diverge in an orthogonal direction from marine and pond populations. While it might be tempting to ascribe such a deviation to random genetic drift from the hypothetical ancestor, it is also possible that there might be a biological explanation for this. Namely, in earlier analyses of behavioural differences between the same marine and freshwater populations, the KRK population showed a similar deviation in response to predation risk by piscine predators (Fraimout et al. 2021). What differentiates the KRK population from other studied pond populations is that it has a history of artificial introduction of brown trout (*Salmo trutta*) to this locality (Herczeg et al. 2009, 2010). Hence, while sticklebacks from the three other pond populations have evolved in absence of piscine predators since the ponds became isolated, the fish in KRK have been faced with recent brown trout predation. Hence, it is conceivable that this might have selected their behaviour and head morphology to converge back towards that of marine ancestors. Nonetheless, no piscine predators were observed in the field at the time of sampling and further studies should investigate the link between predation pressure and head shape morphology in *P. pungitius.*

While the evidence for adaptive differentiation in head size and shape was clear-cut, the results regarding the gill-raker morphology were not. We observed significant genetically based differentiation in gill-raker number and length between pond and marine fish, this differentiation did not exceed neutral expectations. This result does not exclude the possibility that natural selection could be behind the observed differentiation, only that statistical evidence for this is lacking. The consistent results for head gill-raker traits confirmed that the pond fish have evolved to become better suited for feeding on planktonic prey (longer snouts, upturned jaws, more and longer rakers) and marine fish better at feeding benthic prey with a blunt head, short snouts, fewer and shorter rakers. While this might at the

first sight seem counterintuitive and go against expectations grounded on the work done in the related three-spined stickleback in which freshwater fish have benthic like heads and gill-rakers and marine fish limnetic like heads and gill-rakers (Rundle et al. 2003; Reimchen and Nosil, 2006; Raeymaekers et al. 2007), one has to keep in mind that there are subtle differences in ecologies of the three- and nine-spined stickleback species (Merila 2013). Specifically, the lack of piscine predators in isolated ponds opens up the pelagic niche for nine-spined sticklebacks (Karlsson & Byström 2005) and could favor development of more numerous and longer gill-rakers. Conversely, marine nine-spined sticklebacks (and those living in large lakes with predatory fish) living with piscine predators differ from three-spined sticklebacks in that they are not as gregarious as three-spined sticklebacks and tend to hide and feed in protection vegetation. Hence, the lack of marine three-spined stickleback-like gill-raker morphology in marine nine-spined sticklebacks makes sense if they rely less on planktonic diet and feed more benthically compared to syntopic three-spined sticklebacks.

What would be the selective advantage of the large head with strongly upward protruding jaw for pond sticklebacks? In absence of data on diet composition and/or trophic positioning data derived from stable isotopes from the study populations, we can only speculate to this effect. In the absence of native predatory fish, sticklebacks colonising freshwater habitats tend to evolve large heads with strongly upward protruding jaws (Walker & Bell 2000; Bell & Aguirre 2013; but see: Voje et al. 2013; Østbye et al. 2016). Such head morphology is thought be an adaptation to feed on limnetic diets and improved feeding performance on zooplankton (Lavin & McPhail 1986; Walker & Bell 2000). On the other hand, the head shapes of freshwater three-spined sticklebacks feeding primarily either on a limnetic or benthic diet (Wootton 1984; Gow et al. 2007) resemble closely those of pond and marine nine-spined sticklebacks, respectively. This could indicate that pond nine-spined sticklebacks released from predation pressure from piscine predators have shifted their diet towards

feeding more zooplankton. Data from one of our study ponds (Rytilampi) indicates that although sticklebacks in this pond tend to feed mostly benthic prey, they do also consume pelagic zooplankton (Merilä & Eloranta 2017). Furthermore, data from Swedish lakes with predatory fish show that large nine-spined sticklebacks tend to be more planktivorous than their smaller conspecifics as they experience reduced predation risk in the pelagic open-water areas (Karlsson & Byström, 2005). The results of the analyses of gill-raker morphology align with these inferences: pond sticklebacks had long gill-rakers typical for fish adapted on feeding zooplankton, whereas marine ones had gill-rakers typical for benthivorous fish (Ingram et al. 2012). Hence, while the information gleaned from literature gill-rakers suggests that the observed divergence - clearly driven by divergent natural selection - is likely associated with differences in food acquisition, further studies looking into diets and feeding modes are needed to establish this firmly. Nevertheless, what is clear is that the observed divergence in head morphology cannot be explained by neutral process or phenotypic plasticity, and that the observed differences are not subtle, but apparent also for the naked eye (Fig.1b) and corresponding to substantial differences with large effect sizes.

The results further confirm that not only are freshwater stickleback populations adapted to their respective habitats, but also possess ample heritable variation in both relative head size and shape. Heritability estimates were fairly similar for marine and pond populations, suggesting that the latter have not suffered from massive loss of adaptive variation in spite of having lost significant amounts of neutral genetic variation due to population size bottlenecks and drift (Shikano et al. 2010; Kivikoski et al. 2023). Although these heritability estimates derive from full-sib analyses and hence potentially include maternal effect and dominance variance, earlier analyses of body size variation from two of these populations suggest that maternal effects dissipate quickly (Shimada et al. 2011) and there are no quantifiable dominance effects (Ab Ghani et al. 2012; Fraimout et al. 2021). Hence, we conclude that

445 head size and shape are heritable, and hence able to respond to natural selection in all studied
446 populations also in future.

447 Finally, although the mean head size and shape differed between marine and pond
448 populations, one should note that there was also some degree of overlap among pond and
449 marine populations, and heterogeneity in mean values of these variables as for instance
450 reflected in variance in the degree that the different ponds were indicated to have diverged
451 from the reconstructed ancestral form. This is not surprising given that freshwater habitats are
452 heterogenous ecosystems in terms of their biotic and abiotic conditions and therefore, the
453 direction and strength of natural selection on phenotypic traits are unlikely to be equal in all
454 ponds. In fact, this kind of heterogeneity in mean trait values in replicate freshwater
455 populations of sticklebacks has been seen also in earlier studies (e.g., Kaeuffer et al. 2012;
456 Østbye et al. 2016).

457 In line with earlier results (Herczeg et al. 2010), we observed strong sexual dimorphism in
458 head size and shape, males having larger differently shaped heads than females. While this
459 might be indicative of niche partitioning among the sexes, it is also possible that sex
460 differences are related to different sex roles. Nine-spined sticklebacks males use their jaws to
461 build nests into which females lay their eggs, and males also defend their nests and hatchling
462 offspring with their mouths (Wootton 1984). The degree of divergence among the two sexes
463 was less compared to that among habitats - this may suggest that strength of habitat
464 associated selection exceeds that of sex-specific selection.

465

466 ***Conclusions***

467 To sum up, the results provide evidence for adaptive divergence in head size and shape
468 between pond and marine nine-spined sticklebacks, as well as genetically based divergence in

gill-raker length and number. However, we failed to find evidence to indicate that the latter differentiation has been driven by natural selection. We further show the male and female sticklebacks display pronounced sexual dimorphism in head size and shape, and that further studies would be needed to understand the functional significance of this dimorphism. The result further suggests moderate to high heritability of relative head size and head shape suggesting that these traits have high potential to evolve in response to natural selection also in future.

Ethical statement

All experiments were conducted under a permit from the Animal Experiment Board in Finland (permit reference ESAVI/4979/2018). Permission to collect fish from PYO, KRK and RYT ponds was obtained from Metsähallitus (license # MH794/2018). Authorisation to catch fish from the marine populations was provided with personal National Fishing Licenses.

Data Availability

The raw data underlying this article including pictures, TPS files and genotype data will be made available in the Dryad Digital Repository. R scripts to replicate all analyses are publicly available at: https://github.com/afraimout/

Author contributions

J.M. and A.F. conceived and designed the research. A.F. conducted field work and collected field samples. A.F. and Y.C. performed wet lab and data analyses. J.M. and A.F. led the writing effort, K.R. and Y.C. provided input and reviewed the manuscript.

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References

Ab Ghani, N.I., G. Herczeg, and J. Merilä. 2012. Body size divergence in nine-spined sticklebacks: disentangling additive genetic and maternal effects. Biological Journal of the Linnean Society 107(3):521-528.

Ab Ghani, I.N., G. Herczeg, T. Leinonen, and J. Merilä. 2013. Evidence for genetic differentiation in timing of maturation among nine-spined stickleback populations. Journal of Evolutionary Biology 26:775-782.

Adams, D. C., and E.Otárola□Castillo, 2013. geomorph: an R package for the collection and analysis of geometric morphometric shape data. *Methods in ecology and evolution*, *4*(4): 393-399.

Albert, A.Y., S. Sawaya, T.H. Vines, A.K. Knecht, C.T. Miller, B.R. Summers, S. Balabhadra, D.M. Kingsley, and D. Schluter. 2008. The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. Evolution 62(1):76-85.

512 Arnott, S., and I. Barber. 2000. Split-clutch IVF: a technique to examine indirect fitness
513 consequences of mate preferences in sticklebacks. Behaviour 137(7-8):1129-1140.

514 Bates, D., Maechler, M., Bolker, B., & Walker, S. 2015. Fitting linear mixed-effects models
515 using lme4. *J. Stat. Softw*, *67*(1): 1-48.

516 Bell, M.A. and W.E. Aguirre. 2013. Contemporary evolution, allelic recycling, and adaptive
517 radiation of the threespine stickleback. Evolutionary Ecology Research 15(4):377-411.

518 Berner, D., D.C. Adams, A.C. Grandchamp, and A.P. Hendry. 2008. Natural selection drives
519 patterns of lake–stream divergence in stickleback foraging morphology. Journal of
520 Evolutionary Biology 21(6):1653-1665.

521 Bierne, N., D. Roze, and J.J. Welch. 2013. Pervasive selection or is it…? Why are F_{ST}
522 outliers sometimes so frequent? Molecular Ecology 22:2061-2064.

523 Bookstein, F.L. 1991. Morphometric Tools for Landmark Data: Geometry and Biology. New
524 York: Cambridge University Press.

525 Bossdorf, O., H. Auge, L. Lafuma, W.E. Rogers, E. Siemann, and D. Prati. 2005. Phenotypic
526 and genetic differentiation between native and introduced plant populations. Oecologia
527 144(1):1-11.

528 Brouwers, L. 2011. Heads before tails: Ancient fish evolved head-first. Scientific
529 American.https://blogs.scientificamerican.com/thoughtomics/heads-before-tails-ancient-fish-
530 evolved-head-first/

531 Cohen, J. 1988. Statistical power analysis for the behavioral sciences. 2nd edn. Mahwah, NJ:
532 Lawrence Erlbaum Associates.

533 Covarrubias-Pazaran, G. 2016. Genome-assisted prediction of quantitative traits using the R
534 package sommer. PloS One 11(6):e0156744.

535 Day, T., J. Pritchard, and D. Schluter. 1994. A comparison of two sticklebacks. Evolution
536 48(5):1723-1734.

537 DeFaveri, J., T. Shikano, and J. Merilä. 2014. Geographic variation in age structure and
538 longevity in the nine-spined stickleback (*Pungitius pungitius*). PLoS One 9(7):e102660.

539 De Kovel, C.G.F. 2006. The power of allele frequency comparisons to detect the footprint of
540 selection in natural and experimental situations. Genetics Selection and Evolution 38:3-23.

541 Dingemanse, N.J., C. Oosterhof, F. Van Der Plas, and I. Barber. 2009. Variation in
542 stickleback head morphology associated with parasite infection. Biological Journal of the
543 Linnean Society 96(4):759-768.

544 Dryden, I.L., and K.V. Mardia. 1992. Size and shape analysis of landmark data. Biometrika
545 79(1):57-68.

546 Ellis, N.A., and C.T. Miller. 2016. Dissection and flat-mounting of the threespine stickleback
547 branchial skeleton. JoVE (Journal of Visualized Experiments) 111:e54056.

548 Endler. 1978. Geographic Variation, Speciation and Clines. Yale Univ Press.

549 Foster, S.A. 1999. The geography of behaviour: an evolutionary perspective. Trends in
550 Ecology and Evolution 14(5):190-195.

551 Fraimout, A., Z. Li, M.J. Sillanpää, P. Rastas, and J. Merilä. 2021. Dissecting the genetic
552 architecture of quantitative traits using genome-wide identity-by-descent sharing among full-
553 sibs. bioRxiv. https://doi.org/10.1101/2021.03.01.432833

554 Glazer, A.M., P.A. Cleves, P.A. Erickson, A.Y. Lam, and C.T. Miller. 2014. Parallel
555 developmental genetic features underlie stickleback gill raker evolution. Evodevo 5(1):1-16.

556 Gienapp, P., C. Teplitsky, J.S. Alho, J.A. Mills, and J. Merilä. 2008. Climate change and
557 evolution: disentangling environmental and genetic responses. Molecular Ecology 17(1):167-
558 178.

559 Gow, J.L., C.L. Peichel, and E.B. Taylor. 2007. Ecological selection against hybrids in
560 natural populations of sympatric threespine sticklebacks. Journal of Evolutionary Biology
561 20:2173-2180.

562 Gross, H.P., and J.M. Anderson. 1984. Geographic variation in the gillrakers and diet of
563 European threespine sticklebacks, *Gasterosteus aculeatus*. Copeia 1984(1):87-97.

564 Gunz, P., and P. Mitteroecker. 2013. Semilandmarks: a method for quantifying curves and
565 surfaces. Hystrix, the Italian Journal of Mammalogy 24(1):103-109.

566 Herczeg, G., A. Gonda, and J. Merilä. 2009. Evolution of gigantism in nine□spined
567 sticklebacks. Evolution 63(12):3190-3200.

568 Herczeg, G., M. Turtiainen, and J. Merilä. 2010. Morphological divergence of North-
569 European nine-spined sticklebacks (*Pungitius pungitius*): signatures of parallel evolution.
570 Biological Journal of the Linnean Society 101(2):403-416.

571 Hoban, S., J.L. Kelley, K.E. Lotterhos, M.F. Antolin, G. Bradburd, D.B. Lowry, M.L. Poss,
572 L.K. Reed, A. Storfer, and M.C. Whitlock. 2016. Finding the genomic basis of local
573 adaptation: pitfalls, practical solutions, and future directions. American Naturalist
574 188(4):379-397.

575 Hosoki, T., S. Mori, S. Nishida, M. Kume, T. Sumi, and J. Kitano. 2019. Diversity of gill
576 raker number and diets among stickleback populations in novel habitats created by the 2011
577 Tōhoku earthquake and tsunami. Evolutionary Ecology Research 20(2):213-230.

578 Ingram, T., R. Svanbäck, N.J. Kraft, P. Kratina, L. Southcott, and D. Schluter. 2012.
579 Intraguild predation drives evolutionary niche shift in threespine stickleback. Evolution
580 66(6):1819-1832.

581 Kaeuffer, R., C.L. Peichel, D.I. Bolnick, and A.P. Hendry. 2012. Parallel and nonparallel
582 aspects of ecological, phenotypic, and genetic divergence across replicate population pairs of
583 lake and stream stickleback. Evolution 66(2):402-418.

584 Karhunen, M., J. Merilä, T. Leinonen, J.M. Cano, and O. Ovaskainen. 2013. driftsel: an R
585 package for detecting signals of natural selection in quantitative traits. Molecular Ecology
586 Resources 13(4):746-754.

587 Karhunen, M., O. Ovaskainen, G. Herczeg, and J. Merilä. 2014. Bringing habitat information
588 into statistical tests of local adaptation in quantitative traits: A case study of nine□spined
589 sticklebacks. Evolution 68(2):559-568.

590 Karlsson, J., and P. Byström. 2005. Littoral energy mobilization dominates energy supply for
591 top consumers in subarctic lakes. Limnology and Oceanography 50:538-543.

592 Kimmel, C.B., B. Ullmann, C. Walker, C. Wilson, M. Currey, P.C. Phillips, M.A. Bell, J.H.
593 Postlethwait, and W.A. Cresko. 2005. Evolution and development of facial bone morphology
594 in threespine sticklebacks. Proc. Natl. Acad. Sci. USA 102:5791-5796.

595 Kivikoski, M., X. Feng, A. Löytynoja, P. Momigliano, and J. Merilä. Determinants of genetic
596 diversity in sticklebacks. https://doi.org/10.1101/2023.03.17.533073

597 Lande, R. 1976. Natural selection and random genetic drift in phenotypic evolution.
598 Evolution 30(2): 314-334.

599 Langmead, B., and Salzberg, S. L. 2012. Fast gapped-read alignment with Bowtie 2. Nature
600 methods 9(4): 357-359.

601 Lavin, P.A. and J.D. McPhail. 1986. Adaptive divergence of trophic phenotype among
602 freshwater populations of the three-spine stickleback (*Gasterosteus aculeatus*). Can. J. Fish.
603 Aquat. Sci. 43:2455-2463.

604 Le Corre, V. and A. Kremer. 2012. The genetic differentiation at quantitative trait loci under
605 local adaptation. Molecular Ecology 21(7):1548-1566.

606 Leaver, S.D. and T.E. Reimchen. 2012. Abrupt changes in defence and trophic morphology
607 of the giant threespine stickleback (*Gasterosteus sp*.) following colonization of a vacant
608 habitat. Biological Journal of the Linnean Society 107(3):494-509.

609 Leinonen, T., R.J. McCairns, R.B. O'hara, and J. Merilä. 2013. QST–FST comparisons:
610 evolutionary and ecological insights from genomic heterogeneity. Nature Reviews Genetics
611 14(3):179-190.

612 Linhart, Y.B. and M.C. Grant. 1996. Evolutionary significance of local genetic differentiation
613 in plants. Annual Review of Ecology and Systematics 27(1):237-277.

614 Lopera-Barrero, N.M., Povh, J.A., Ribeiro, R.P., Gomes, P.C., Jacometo, C.B., and Lopes,
615 T.D.S. 2008. Comparison of DNA extraction protocols of fish fin and larvae samples:
616 Modified salt (NaCl) extraction. Ciencia e Investigación Agraria 35(1): 77-86.

617 Mallick, S., Gnerre, S., Muller, P., and Reich, D. 2009. The difficulty of avoiding false
618 positives in genome scans for natural selection. Genome Research 19(5): 922-933.

619 Magalhaes, I.S., Whiting, J.R., D'Agostino, D., Hohenlohe, P.A., Mahmud, M., Bell, M.A.,
620 Skúlason, S., and MacColl, A.D. 2021. Intercontinental genomic parallelism in multiple
621 three-spined stickleback adaptive radiations. Nature Ecology and Evolution 5(2): 251-261.

622 McGee, M.D., and Wainwright, P.C. 2013. Convergent evolution as a generator of
623 phenotypic diversity in threespine stickleback. Evolution 67: 1204-1208.

McGee, M.D., D. Schluter, and P.C. Wainwright. 2013. Functional basis of ecological divergence in sympatric stickleback. BMC Evolutionary Biology 13(1):1-10.

McKay, J.K. and R.G. Latta. 2002. Adaptive population divergence: markers, QTL and traits. Trends in Ecology and Evolution 17:285-291.

Merilä, J. 2012. Evolution in response to climate change: in pursuit of the missing evidence. BioEssays 34(9):811-818.

Merilä, J. 2013. Nine□spined stickleback (*Pungitius pungitius*): an emerging model for evolutionary biology research. Annals of the New York Academy of Sciences 1289(1):18-35.

Merilä, J. and P. Crnokrak. 2001. Comparison of marker gene and quantitative genetic differentiation among populations. Journal of Evolutionary Biology 14:892-903.

Merilä, J. and A.P. Eloranta. 2017. Cannibalism facilitates gigantism in a nine□spined stickleback (*Pungitius pungitius*) population. Ecology of Freshwater Fish 26(4):686-694.

Merilä, J. and A.P. Hendry. 2014. Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. Evolutionary Applications 7(1):1-14.

Momigliano, P., A.B. Florin, and J. Merilä. 2021. Biases in demographic modeling affect our understanding of recent divergence. Molecular Biology and Evolution 38(7):2967-2985.

Nakagawa, S. and H. Schielzeth. 2010. Repeatability for Gaussian and non□Gaussian data: a practical guide for biologists. Biological Reviews 85(4):935-956.

Narum, S.R. and J.E. Hess. 2011. Comparison of FST outlier tests for SNP loci under selection. Molecular Ecology Resources 11:184-194.

Nosil, P., D.J. Funk, and D.A.N.I.E.L. Ortiz□Barrientos. 2009. Divergent selection and heterogeneous genomic divergence. Molecular Ecology 18(3):375-402.

646 Ovaskainen, O., M. Karhunen, C. Zheng, J.M.C. Arias, and J. Merilä. 2011. A new method to
647 uncover signatures of divergent and stabilizing selection in quantitative traits. Genetics
648 189(2):621-632.

649 Parmesan, C. 2006. Ecological and evolutionary responses to recent climate change. Annu.
650 Rev. Ecol. Evol. Syst. 37:637-669.

651 R Core Team. 2022. R: A language and environment for statistical computing. R Foundation
652 for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

653 Raeymaekers, J.A., J.K. Van Houdt, M.H. Larmuseau, S. Geldof, and F.A. Volckaert. 2007.
654 Divergent selection as revealed by PST and QTL□based FST in three□spined stickleback
655 (*Gasterosteus aculeatus*) populations along a coastal□inland gradient. Molecular Ecology
656 16(4):891-905.

657 Rastas, P., F.C. Calboli, B. Guo, T. Shikano, and J. Merilä. 2016. Construction of ultradense
658 linkage maps with Lep-MAP2: stickleback F2 recombinant crosses as an example. Genome
659 Biology and Evolution 8(1):78-93.

660 Ravinet, M., N. Takeuchi, M. Kume, S. Mori, and J. Kitano. 2014. Comparative analysis of
661 Japanese three-spined stickleback clades reveals the Pacific Ocean lineage has adapted to
662 freshwater environments while the Japan Sea has not. PLoS One 9(12):e112404.

663 Rogers, A.R. 1986. Population differences in quantitative characters as opposed to gene
664 frequencies. American Naturalist 127(5):729-730.

665 Rohlf, F. J. 2006. tpsDig 2.10. Stony Brook, NY: Department of Ecology and Evolution,
666 State University of New York.

667 Roy, D., K. Lucek, E. Bühler, and O. Seehausen. 2010. Correlating shape variation with
668 feeding performance to test for adaptive divergence in recently invading stickleback

669 populations from Swiss peri-alpine environments. In Morphometrics for
670 Nonmorphometricians (pp. 233-257). Springer, Berlin, Heidelberg.

671 Rundle, H.D., S.M. Vamosi, and D. Schluter. 2003. Experimental test of predation's effect on
672 divergent selection during character displacement in sticklebacks. Proceedings of the
673 National Academy of Sciences 100(25):14943-14948.

674 Savolainen, O., M. Lascoux, and J. Merilä. 2013. Ecological genomics of local adaptation.
675 Nature Reviews Genetics 14(11):807-820.

676 Schneider, C.A., W.S. Rasband, and K.W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of
677 image analysis. Nature Methods 9(7):671-675.

678 Schluter, D. 2000. Ecological character displacement in adaptive radiation. American
679 Naturalist 156:S4-S16.

680 Seymour, M., K. Räsänen, and B.K. Kristjánsson. 2019. Drift versus selection as drivers of
681 phenotypic divergence at small spatial scales: The case of Belgjarskógur threespine
682 stickleback. Ecology and Evolution 9(14):8133-8145.

683 Sheridan, J.A. and D. Bickford. 2011. Shrinking body size as an ecological response to
684 climate change. Nature Climate Change 1(8):401-406.

685 Shimada, Y., T. Shikano, A. Kuparinen, A. Gonda, T. Leinonen, and J. Merilä. 2011.
686 Quantitative genetics of body size and timing of maturation in two nine-spined stickleback
687 (*Pungitius pungitius*) populations. PLoS One 6(12):e28859.

688 Spitze, K. 1993. Population structure in *Daphnia obtusa*: quantitative genetic and allozymic
689 variation. Genetics 135(2):367-374.

690 Stamp, M.A. and J.D. Hadfield. 2020. The relative importance of plasticity versus genetic
691 differentiation in explaining between population differences; a meta□analysis. Ecology
692 Letters 23(10):1432-1441.

693 Storz, J.F. 2005. Using genome scans of DNA polymorphism to infer adaptive population
694 divergence. Molecular Ecology 14(3):671-688.

695 Tobler, M., M. Palacios, L.J. Chapman, I. Mitrofanov, D. Bierbach, M. Plath, L.
696 Arias□Rodriguez, F.J. Garcia de Leon, and M. Mateos. 2011. Evolution in extreme
697 environments: replicated phenotypic differentiation in livebearing fish inhabiting sulfidic
698 springs. Evolution 65(8):2213-2228.

699 Østbye, K., C. Harrod, F. Gregersen, T. Klepaker, M. Schulz, D. Schluter, and L.A. Vøllestad.
700 2016. The temporal window of ecological adaptation in postglacial lakes: a comparison of
701 head morphology, trophic position and habitat use in Norwegian threespine stickleback
702 populations. BMC Evolutionary Biology 16(1):1-16.

703 Voje, K.L., A.B. Mazzarella, T.F. Hansen, K. Østbye, T. Klepaker, A. Bass, A. Herland, K.M.
704 Bærum, F. Gregersen, and L.A. Vøllestad. 2013. Adaptation and constraint in a stickleback
705 radiation. Journal of Evolutionary Biology 26(11):2396-2414.

706 Walker, J.A. and M.A. Bell. 2000. Net evolutionary trajectories of body shape evolution
707 within a microgeographic radiation of threespine sticklebacks (*Gasterosteus aculeatus*).
708 Journal of Zoology 252:293-302.

709 Walther, G.R., E. Post, P. Convey, A. Menzel, C. Parmesan, T.J. Beebee, J.M. Fromentin, O.
710 Hoegh-Guldberg, and F. Bairlein. 2002. Ecological responses to recent climate change.
711 Nature 416(6879):389-395.

712 Whitlock, M.C. 2008. Evolutionary inference from Q_{ST}. Molecular Ecology 17(8):1885-1896.

713 Wootton, R.J. 1984. A functional biology of sticklebacks. Croom & Helm, London.

714 Wund, M.A., S. Valena, S. Wood, and J.A. Baker. 2012. Ancestral plasticity and allometry in
715 threespine stickleback reveal phenotypes associated with derived, freshwater ecotypes.
716 Biological Journal of the Linnean Society 105(3):573-583.

717 Yi, X., Kemppainen, P., Reid, K., Chen, Y., Rastas, P., Fraimout, A., & Merilä, J. 2023.
718 Heterogeneous genomic architecture of skeletal armour traits in sticklebacks. bioRxiv,
719 https://doi.org/10.1101/2023.05.28.542672.

TABLES AND FIGURES

Table 1. Results from the Procrustes ANOVA on head centroid size. The analysis of variance (ANOVA) table is shown for the model testing for differences in head centroid size. Effect: the fixed effect included in the models. Df: degrees of freedom. SS: sums of squares; MS: mean squares; Rsq: correlation coefficient (R^2); F: values from the F-distribution; Z: effect sizes. *p*: *p-value* with bold font indicating statistical significance ($p < 0.05$).

Effect	Df	SS	MS	Rsq	*F*	*Z*	*p*
Body length	1	14.735	14.735	0.716	3227.578	12.631	**0.001**
Habitat	1	2.966	2.966	0.144	649.773	9.425	**0.001**
Population	6	0.157	0.026	0.007	5.737	3.975	**0.001**
Sex	1	1.424	1.424	0.055	250.226	7.018	**0.001**
Residuals	348	1.589	0.005	0.077			

727 **Table 2. Results from the Procrustes ANOVA on head shape.** The analysis of variance
728 (ANOVA) table is shown for the model testing for differences in head shape. Effect: the fixed
729 effect included in the models with semi-columns indicating interaction. Df: degrees of
730 freedom. SS: sums of squares; MS: mean squares; Rsq: correlation coefficient (R^2); *F*: values
731 from the F-distribution; *Z*: effect sizes. *p*: *p-value* with bold font indicating statistical
732 significance ($p < 0.05$).

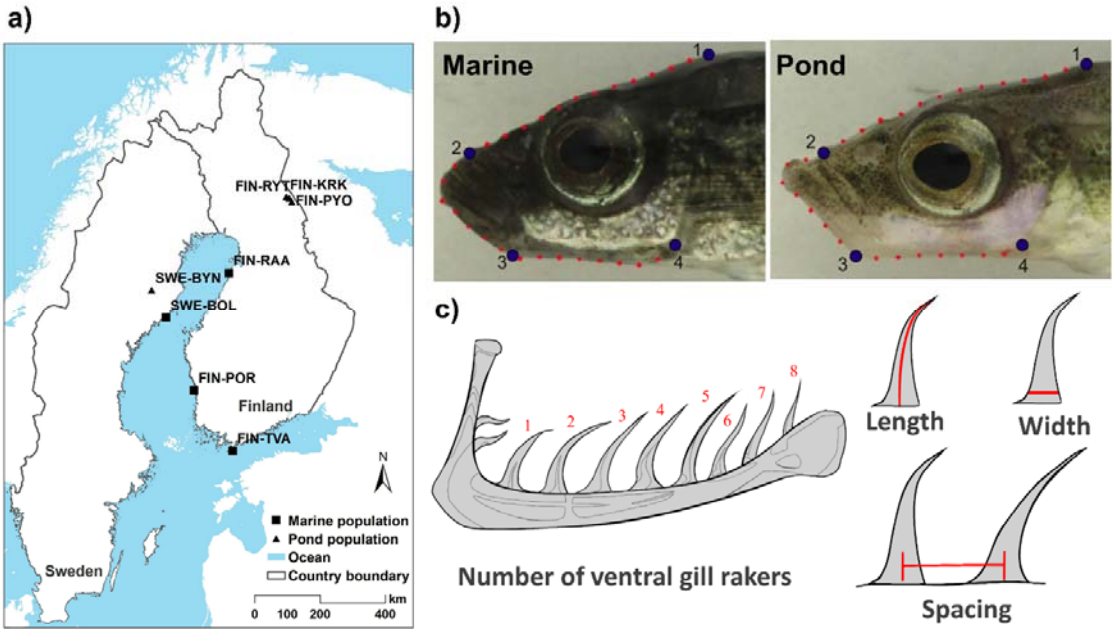
Effect	Df	SS	MS	Rsq	F	*Z*	*p*
Log(Csize)	1	0.072	0.072	0.123	69.743	7.479	**0.001**
Habitat	1	0.085	0.085	0.144	81.928	7.176	**0.001**
Population	6	0.059	0.009	0.100	9.156	7.307	**0.001**
Sex	1	0.012	0.012	0.019	11.302	4.508	**0.001**
Log(Csize):Habitat	1	0.001	0.001	0.002	1.284	0.724	0.241
Residuals	347	0.359	0.001	0.610			

733

734 **Table 3. Estimates of heritability for head centroid size and head shape.** For each
735 population, the posterior median of h^2 is shown for head centroid size (h^2_{size}) and the PC1 of
736 head shape variation (h^2_{shape}) along with their 95% highest posterior density intervals.

Population	h^2_{size}	h^2_{shape}
SWE-BYN	0.466 [0.223; 0.766]	0.566 [0.393; 0.718]
FIN-KRK	0.509 [0.168; 0.826]	0.519 [0.286; 0.778]
FIN-PYO	0.449 [0.222; 0.723]	0.543 [0.363; 0.721]
FIN-RYT	0.389 [0.125; 0.701]	0.674 [0.538; 0.785]
SWE-BOL	0.464 [0.221; 0.732]	0.529 [0.387; 0.655]
FIN-RAA	0.424 [0.201; 0.682]	0.630 [0.489; 0.741]
FIN-TVA	0.504 [0.250; 0.270]	0.562 [0.439; 0.700]
FIN-POR	0.499 [0.221; 0.798]	0.559 [0.415; 0.710]

737



738

739 **Fig. 1.** a) Map of the study area displaying the eight sampling locations of nine-spined
740 stickleback populations, which include four marine (Tvärminne, TVA; Pori, POR; Raahe,
741 RAA; Bölesviken, BOL) and four pond populations (Rytilampi, RYT; Pyöreälampi, PYO;
742 Kirkasvetinenlampi, KRK; Bynastjärnen, BYN) in Finland and Sweden. b) Landmark
743 positions. Positions of the four fixed landmarks (blue filled circles) and semilandmarks (red
744 filled circles) used to quantify head shape variation in marine (left panel) and pond (right
745 panel) populations. Numbering of fixed landmarks are as in *Methods*. c) Diagram illustrating
746 the four gill raker trait measurements: number, length, width, and spacing between rakers.

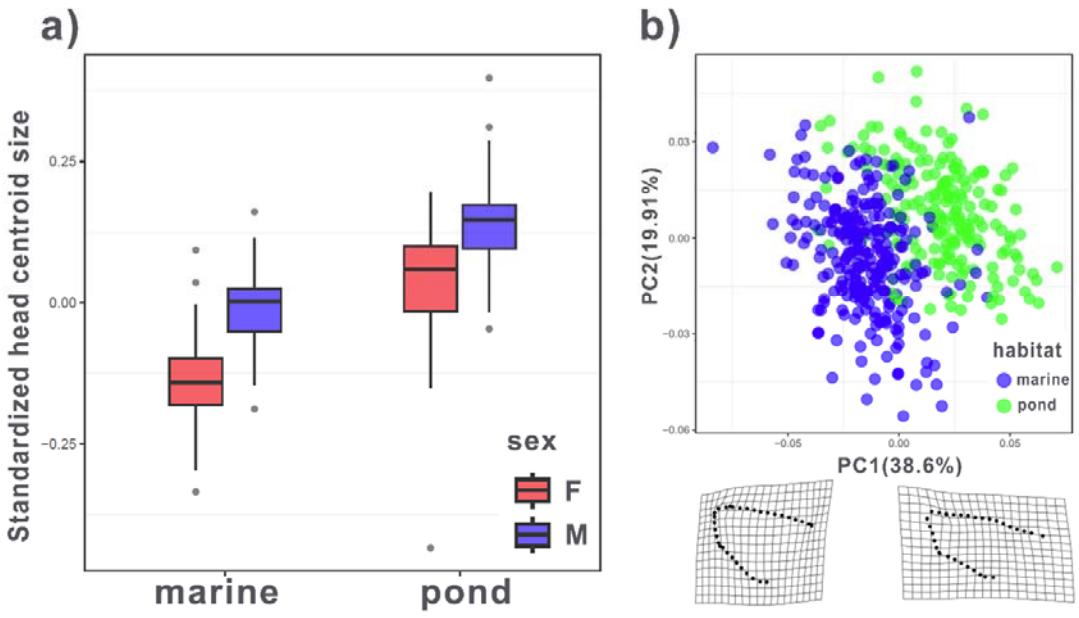
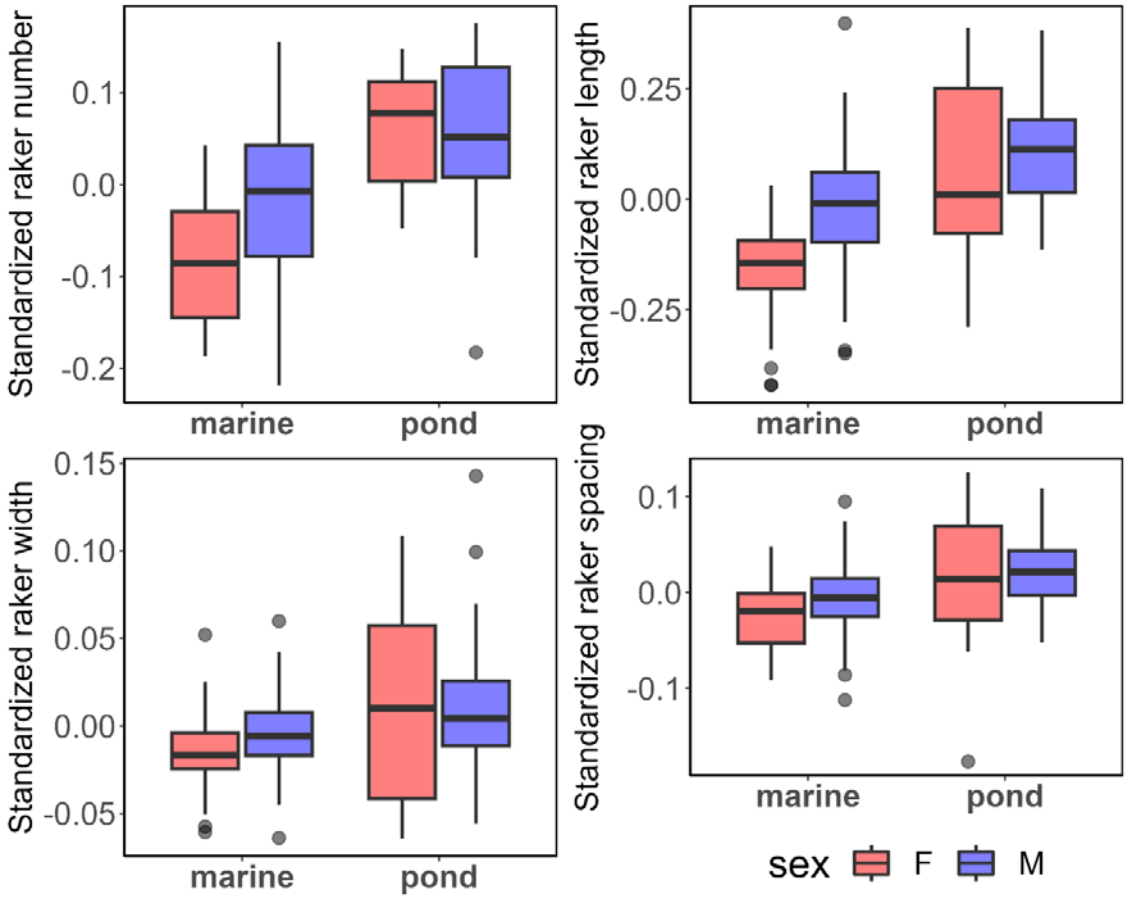


Fig. 2. Head centroid size and shape differentiation. a) The residuals from a regression of head centroid size on body length were used to describe standardized head size and are plotted for each habitat (marine or pond) and the two sexes separately (males = M, females = F). b) Principal component (PC) analysis of head shape variation. Individuals from marine (blue filled circles) and pond (green filled circles) habitats are plotted along the two first PC axes. Deformation grids depict the shape change associated with PC1 of head shape variation.



754

755 **Fig. 3.** Differences in mean size standardized values of four raker traits in marine and pond

756 environments, as well as in two sexes (females = F, males = M).

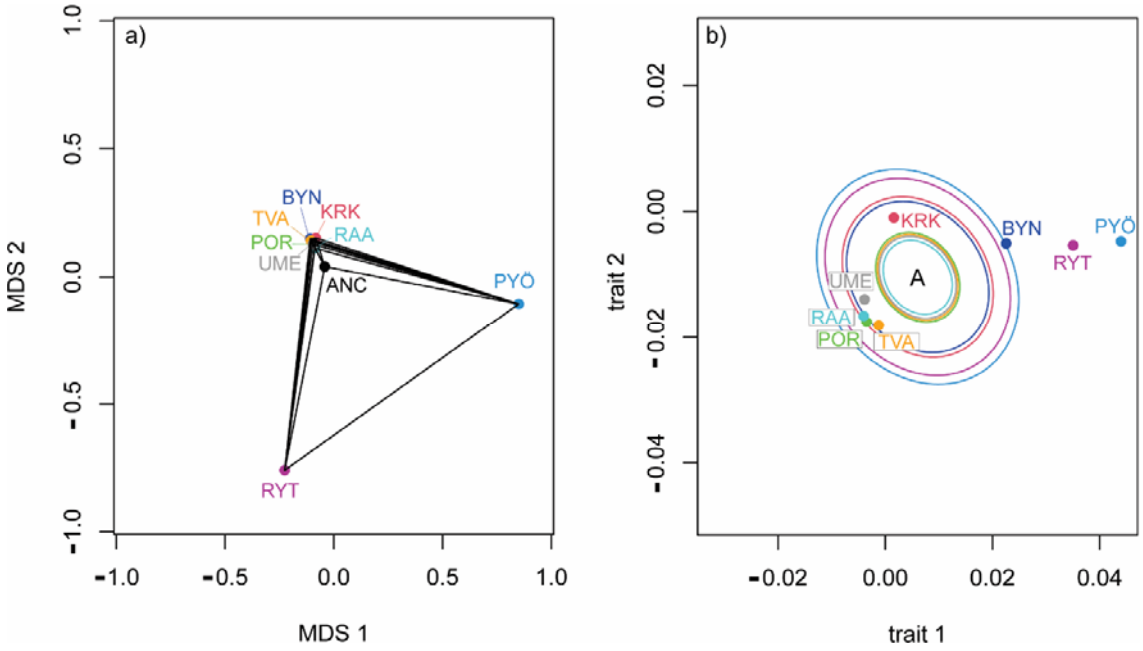


Fig. 4. Results of the driftsel analysis. a) Multidimensional scaling (MDS) patterns of neutral genetic differentiation estimated from SNP data obtained from the *viz.theta* function in program driftsel. b) Population means in the trait space from the *viz.trait* function. Ellipses represent the expected median distance from the inferred ancestral population (ANC) under neutral evolution.