Testing the predictability of morphological evolution in naturally warmed stickleback populations

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ABSTRACT

In light of climate change, the ability to predict evolutionary responses to temperature changes is of central importance for conservation efforts. Studying parallel evolution in natural populations inhabiting contrasting thermal environments presents a powerful approach for understanding and predicting responses to increasing temperatures. In this study, we used a natural experiment in Iceland, where freshwater populations of threespine sticklebacks are found in waters warmed by geothermal activity, adjacent to populations in ambient-temperature water bodies. We used three sympatric and three allopatric warm-cold population pairs to test for morphological divergence in relation to thermal habitat. We found that thermal habitat explained over 50% of body shape variation: fish from warm habitats had a deeper mid-body, a shorter jaw, smaller eyes, and longer dorsal spines. Population age did not influence the magnitude or direction of morphological divergence between warm and cold habitats. However, the absence of gene flow seemed to facilitate parallel evolution across thermal habitats: all three allopatric population pairs were on a common evolutionary trajectory, but this was not the case for sympatric population pairs. Our findings therefore suggest that morphological evolution in response to rising temperatures can be predictable to some extent but only if there is restricted gene flow.

INTRODUCTION

Understanding whether populations evolve predictably when exposed to similar environmental conditions is crucial for understanding adaptation. Studies on a wide range of taxa (insects: Nosil et al. 2002; fishes: Bernatchez et al. 2010; birds: Mundy 2005; mammals: Hoekstra 2006) have shown that different populations in similar environments can evolve similar phenotypes (Losos 2011). This pattern of parallel evolution can arise due to natural selection, developmental bias, or their interaction, and it suggests that adaptive evolutionary change can be predictable and repeatable (Schluter and Nagel 1995, Brakefield 2006, Oke et al. 2017, Uller et al. 2018).

Studying parallel evolution can be especially valuable in light of global environmental change (de Amorim et al. 2017), as the ability to predict population responses to anthropogenic change is of central importance for management and conservation efforts. In the coming decades, climate change will arguably be the most significant threat to biodiversity; rising temperatures are already altering abiotic and biotic environmental conditions and imposing novel selection pressures on organisms (Crozier & Hutchings 2014). Ectotherms, such as fishes and reptiles, are particularly vulnerable because of their high sensitivity to temperature changes (Zuo et al. 2012). Consequently, there is now a pressing need to understand the scope for populations to adapt to climate change. Although studies on plastic (within-generation) responses to temperature are rapidly accumulating, studies on the long-term evolutionary responses to climate change are still lacking (Crozier & Hutchings 2014). Studying parallel evolution in natural populations inhabiting contrasting thermal environments provides a powerful approach for better understanding and predicting population responses to increasing temperatures.

We used a 'natural experiment' in Iceland, where freshwater populations of threespine sticklebacks (*Gasterosteus aculeatus*) are found in waters warmed by geothermal activity (warm habitats), adjacent to populations in ambient-temperature water bodies (cold habitats). This unique study system provides repeated and independent examples of populations experiencing long-term contrasting thermal environments over a small geographic scale, thereby avoiding the confounding factors associated with latitudinal or elevational comparisons. Another notable attribute of this study system is that while most of these warm and cold habitats are in separate water bodies (allopatric), some are found in different parts of the same water body (sympatric). Movement, and thus gene flow, is possible between sympatric but not allopatric populations. This allows us to examine whether gene flow influences the magnitude and/or direction of divergence between warm and cold habitats (hereafter referred to as thermal divergence). Lastly, the age of these warm habitats, and hence the maximum time these populations have experienced elevated temperatures, ranges from decades to thousands of years (Table 1). These different timescales make it possible to examine whether populations exposed to higher temperatures for a relatively short time have diverged to the same extent as much older populations.

Here, we focus on temperature-driven parallel evolution in morphology. Morphology can determine fitness by influencing reproduction, foraging ability and feeding behaviour, and swimming performance (e.g., manoeuvrability, stability, and agility; Rowinski et al. 2015). Morphological variation is also related to sexual selection and reproductive isolation and can therefore contribute to population divergence and speciation (e.g., Head et al. 2013). Earlier work has shown that morphology often exhibits similar patterns of adaptive divergence across populations in response to common environmental conditions (Jastrebski and Robinson 2004, Cooper et al. 2010).

Rearing temperature, in particular, has been shown to directly influence the development of body shape in fishes (e.g., Sfakianakis et al. 2011, Ramler et al. 2014, Rowinski et al. 2015). Yet, it is still unknown whether evolutionary responses to changes in temperature are repeatable and thus predictable.

Our study addresses this gap in our knowledge by testing whether there is morphological divergence between sticklebacks from six warm-cold population pairs, and if so, whether it follows parallel patterns. Evidence for parallelism would suggest that responses to elevated temperature are predictable. Nevertheless, even if our six warm-cold population pairs share evolutionary trajectories, they may not necessarily show the same degree of divergence. Hence, we also investigated whether the magnitude of thermal divergence differs across population pairs, as a function of population age or habitat connectivity (i.e., potential for gene flow). By addressing these issues, the present study can advance our ability to predict evolutionary responses to elevated temperatures in light of global climate change.

METHODS

Specimen collection

We used unbaited minnow traps to collect adult threespine sticklebacks from six warm-cold population pairs in Iceland in May–June 2016 (Table 1, Figure S1). Three of these population pairs were allopatric (designated A1-3), meaning that the warm and cold habitats were in separate water bodies with no potential for gene flow (Table 1). The other three population pairs were sympatric (designated S1-3), meaning that the warm and cold habitats were found in the same water body with no physical barriers between them (Table 1).

The cold habitats have all existed since the last glacial period about 10,000 years ago (Einarsson et al. 2004), but there is some variation in the age of the warm habitats (Table 1). The A1, S2, and S3 warm habitats originated 50–100 years ago and are fed by excess hot water runoff from nearby residences that use geothermal heating. The remaining warm habitats have been naturally heated by geothermal vents for over 1000 years (Hight 1965, Einarsson et al. 2004).

Specimen preparation

Fish were euthanised using an overdose of phenoxyethanol and preserved in 10% buffered formalin. Specimens were bleached and cleared to remove skin pigmentation and make the body translucent (Potthoff 1984). They were then stained with Alizarin Red S to emphasize bone morphology and were stored in 75% glycerol until excess stain was removed. Individual specimens (n=331) were photographed on their left side with a Canon EOS 1100D digital camera (Canon Inc, Tokyo, Japan). All photographs included a scale and were taken from a fixed distance and angle using a copy stand.

Linear measurements

The photographs were used to measure morphological traits related to swimming (pectoral fin length) and defence (first and second dorsal spine lengths). Using the software program tpsDig (Rohlf 2008), we placed landmarks on the base and tip of the longest fin ray (pectoral fin length) and on the base and tip of each spine (dorsal spine lengths). We then calculated interlandmark distances using CoordGen (Zelditch et al. 2012) and regressed each length measurement against centroid size to correct for body size and allometric effects. These landmarks were only used for obtaining linear distances and were not included in the body shape analysis.

Body shape variation

To measure body shape, we placed 22 anatomical landmarks and 10 sliding semilandmarks (Bookstein 1997) on each image to quantify variation in the lateral view using a geometric morphometric approach (Figure S2). Sliding semilandmarks are points along curves that measure variation that cannot be captured by anatomical landmarks. We placed 10 equally spaced sliding landmarks between the anterior tip of the upper jaw and the posterior tip of the frontal bone to measure variation in head curvature (Figure S2). The sliding procedure was conducted based on chord distance using the tpsRelw32 software (Rohlf 2008).

To reduce the effects of size and orientation across individuals, we performed a Generalized Procrustes Analysis using CoordGen8 (Rohlf and Slice 1990). This process superimposes landmark configurations to minimise the sum of squared distances between corresponding landmark configurations by scaling, rotating, and translating specimens in relation to their geometric centre. To minimise the potential effects of allometry on the data, we used Standard6 (Zelditch et al. 2012) to perform a multiple regression of shape on geometric centroid size to generate residuals. We then performed a thin-plate spline (TPS) procedure to generate partial warp scores for further statistical analysis. This procedure models the form of an infinitely thin metal plate that is constrained at some combination of points (i.e., landmarks) but is otherwise free to adopt a target form in a way that minimizes bending energy. In morphometrics, this interpolation is applied to a Cartesian coordinate system in which deformation grids are constructed from two landmark configurations (Bookstein 1991). The total deformation of the thin-plate spline (including uniform components) can be decomposed into geometrically orthogonal components (partial warps) based on scale (Rohlf and Marcus

1993). TPS and the generation of partial warps scores was performed using PCAgen (Zelditch et al. 2012).

Data analysis

Analyses were run using R version 3.5.1 (R Core Team 2017) unless otherwise noted. We used the ggplot2 package (Wickham 2009) for generating figures.

Linear measurements—For each individual, we obtained linear distances for three morphological traits: pectoral fin length, first dorsal spine length, and second dorsal spine length. After checking for homogeneity of slopes, we regressed each linear distance against centroid size and used the residuals from these regressions in separate ANOVA models for each trait. The explanatory variables in these models were population pair, thermal habitat, and the interaction between population pair and thermal habitat. The main effect of population pair summarises properties unique to different replicates (Bolnick et al. 2018). The main effect of thermal habitat measures the extent to which thermal divergence is shared across replicate locations and thus measures parallel evolution (Bolnick et al. 2018). The population pair × thermal habitat interaction indicates how the direction or magnitude of thermal divergence varies among population pairs, implying nonparallel evolution. To determine the partial variance explained by each factor and interaction, we used the heplots package to calculate partial eta squared (η^2) values (Fox et al. 2007).

Body shape variation—To test whether temperature affects body shape, we performed a discriminant function analysis (DFA) for each population pair using thermal habitat as a grouping variable to explain variation in partial warp scores (i.e., body shape).

This analysis allowed us to characterize the potentially divergent effects of temperature within each population pair. We then performed another DFA which included all population pairs together to examine the overall effect of temperature on body shape.

Next, we used a MANOVA model that included partial warp scores from all populations as the response variable. This model allowed us to separate the independent and interactive effects of thermal habitat (warm vs cold) and population pair on body shape. As mentioned above, a significant effect of thermal habitat would imply parallel evolution, whereas a significant effect of the thermal habitat × population pair interaction would imply nonparallel evolution. We again calculated partial eta squared (η^2) values to determine the partial variance explained by each factor or interaction in this MANOVA model. Our MANOVA and DFA approaches modelled variation in body shape but did not allow us to separate the effects of direction and magnitude of thermal divergence, so we adopted two additional approaches.

Direction of thermal divergence in body shape—We compared the scale-free vector of divergence for each population pair using the canonical scores derived from the DFA. To derive the vector of divergence, we regressed the Procrustes-superimposed landmark data from each population pair on its corresponding canonical axis. The angle between vectors for all pairwise comparisons of populations was then calculated as the arccosine. We ran 900 bootstraps with replacement for each population pair independently and calculated 95th percentiles of the range of angles obtained by resampling. To carry out the bootstrapping procedure, the two thermal groups were merged into a common pool, and two groups with the same sample size as the original data sets were drawn with replacement from the common pool.

The observed angle between two population pairs was compared against the angle within each pair to determine whether it differed from random processes (Zelditch et al. 2012). If the between-population-pair angle exceeds both of the within-population-pair angles, this means that the population pairs were evolving along different evolutionary trajectories (Parsons et al. 2011, Zelditch et al. 2012, Parsons et al. 2016). These procedures were performed using the tool VecCompare in the software Regress8 (Zelditch et al. 2012).

Magnitude of thermal divergence in body shape—To assess the magnitude of shape divergence between warm and cold habitats, we used a Procrustes distance-based approach, which allowed us to compare our six population pairs in a common shape space (i.e., a common scale). To this end, we calculated the Procrustes mean for each of the warm and cold groups in each population pair. Following the determination of observed distances in Procrustes means based on an F-value, we performed 900 bootstraps to determine the probability for it to have been produced by chance. As before, we carried out the bootstrapping procedure by merging the two thermal groups into a common pool; two groups with the same sample size as the original data sets were then drawn with replacement from the common pool. This analysis was carried out using the IMP TwoGroup8 software (Zelditch et al. 2012).

RESULTS

Linear measurements

Sticklebacks from warm habitats had longer second dorsal spines in most (but not all) population pairs, as indicated by a statistically significant interaction between thermal habitat and population pair (Table 2, Figure S3). In contrast, first dorsal spine length did not vary according to thermal habitat or population pair (Table 2). Lastly, the effect of temperature on pectoral fin length varied across population pairs, as indicated by the interaction between thermal habitat and population pair (Table 2, Figure S3). Sticklebacks from warm habitats had longer pectoral fins in some pairs (A2, S1) but shorter pectoral fins in other pairs (A1, S3).

Body shape variation

The discriminant function analysis showed strong groupings based on thermal habitat in all six of our population pairs (97% correct classification for the A3 population pair and 100% correct classification for all other pairs), indicating that fish could be reliably assigned to warm or cold populations on the basis of their body shape (Figure 1). When all six population pairs were included together in the DFA, there was 82% correct classification (Figure 2). Overall, sticklebacks from warm habitats tended to have smaller eyes, a shorter jaw, and a deeper mid-body tapering to a narrower caudal peduncle (Figure 2).

The MANOVA model provided further evidence that differences in temperature have led to a divergence in body shape: thermal habitat had a significant effect on body shape across population pairs, explaining 53% of the observed variation in shape (Table 3). Body shape was also influenced by the interaction between thermal habitat and population pair, indicating that changes in body shape due to temperature were not equally extreme in all population pairs (Table 3).

Direction of thermal divergence in body shape

We found mixed evidence for parallelism in the evolutionary trajectories of our warm-cold population pairs (Table 4). The trajectories of thermal divergence were parallel for all comparisons between allopatric population pairs (A1–A2, A1–A3, and A2–A3) but none of the comparisons between sympatric population pairs (S1–S2, S1–S3, S2–S3). For example, in all three allopatric population pairs, warm-habitat fish have smaller eyes and shorter jaws than cold-habitat fish (Figure 1). In contrast, in sympatric population pairs, eye size and jaw length do not seem to vary consistently based on thermal habitat (Figure 1). There was also evidence for parallelism between some allopatric and sympatric population pairs (Table 4).

Magnitude of thermal divergence in body shape

Consistent with the DFA and MANOVA results, the distance-between-means test provided strong evidence for divergence in body shape between sticklebacks from warm and cold habitats (Table 5). The magnitude of thermal divergence varied across population pairs but was not related to population age or connectivity between warm and cold habitats (Table 5).

DISCUSSION

In this study, we tested whether there is morphological divergence between sticklebacks from warm (geothermal) and cold (ambient-temperature) habitats, and whether such divergence follows parallel patterns across population pairs. We found clear differences in body shape between sticklebacks from warm and cold habitats, with temperature explaining just over half of the variation in body shape. Population age did not influence the magnitude or direction of thermal divergence, but connectivity between thermal habitats did influence the direction of thermal divergence: allopatric population pairs had parallel evolutionary trajectories, whereas sympatric population pairs appeared to be on different trajectories.

Effects of temperature on morphological evolution

It is well established that water temperature can influence body shape development in fishes (Marcil et al. 2006, Sfakianakis et al. 2011, Ramler et al. 2014, Rowinski et al. 2015). The effects of temperature on body shape can be either direct or indirect (i.e., mediated by changes in other ecological conditions). Generally, higher temperatures lead to an increase in body depth (Marcil et al. 2006, Sfakianakis et al. 2011, Rowinski et al. 2015). Consistent with this, we found that in most population pairs, sticklebacks from warm habitats were more deep-bodied with a narrower caudal peduncle than those from cold habitats. They also tended to have smaller eyes, a shorter lower jaw, and a longer second dorsal spine.

Although our study presents clear evidence for morphological differences between sticklebacks from different thermal habitats, it is unclear whether these differences are adaptive, and if so, what the underlying causes are. For example, changes in jaw length and body depth may be driven by differences in food availability (Rowinski et al. 2015) or diet composition (Hjelm et al. 2001). Greater body depth is also thought to improve predator escape performance through increased manoeuvrability or predator gape limitation (Reimchen 1991, Walker 1997). Similarly, dorsal spines are an antipredator defence and are generally longer in populations that experience elevated predation pressure (e.g., Blouw and Hagen 1984).

Thus, one potential explanation for our findings is that sticklebacks in warm habitats have evolved deeper bodies and longer dorsal spines in response to a higher risk of predation. Indeed, we expect bird predation to be higher in warm habitats, due to the lack of ice cover during the winter and the fact that birds tend to be attracted to warmer areas (Rowiniski et al. 2015). On the other hand, sticklebacks in warm habitats likely experience a lower risk of predation from piscivorous fish, which may be unable to cope with high temperatures (Eliason et al. 2011). Further work is needed to investigate the functional significance of the morphological differences we found.

Magnitude of thermal divergence in body shape

The magnitude of divergence between individuals in warm and cold habitats may be influenced by various factors, such as connectivity between these contrasting thermal habitats. For example, some researchers have argued that gene flow will constrain divergence between ecotypes when there is a potential for physical dispersal (Slatkin 1985, Lenormand 2002, Hendry and Taylor 2004). Under this scenario, we would expect sympatric population pairs to be less divergent than allopatric population pairs (Hendry and Taylor 2004, Pinho and Hey 2010). However, sympatric pairs could instead be more divergent because of character displacement, whereby differences between ecotypes are more pronounced in areas where the ecotypes co-occur and minimised in areas where their distributions do not overlap. This pattern results from trait evolution driven by competition among ecotypes, or closely related species, for a limited resource (Brown and Wilson 1956, Losos 2011).

In our study, the presence or absence of geographical barriers did not seem to influence the magnitude of thermal divergence in body shape. It is interesting to note that despite the potential for gene flow in sympatric population pairs, we found no evidence for intermediate phenotypes (Figure 1). This was particularly surprising for sympatric pairs S2 and S3, where the warm and cold habitats are only 30 and 100 meters apart, respectively. In fact, the only population pair where we observed some overlap in the phenotypic distribution of body shape was an allopatric pair. There are several plausible explanations for the absence of intermediate phenotypes in sympatric population pairs, including strong performance trade-offs, assortative mating, and hybrid inviability (Maynard Smith 1966, Schluter 2009).

The magnitude of thermal divergence may also vary depending on population age. In populations that have been diverging for longer, there is more scope for natural selection and genetic drift to introduce adaptive or stochastic phenotypic differences (Ord and Summers 2015). In our study system, we would thus expect relatively young population pairs (<100 years old) to be less divergent than old populations pairs (>2,000 years old). Nevertheless, we found no evidence that the magnitude of thermal divergence in body shape was related to population age: populations in warm habitats that have only existed for a few decades were equally divergent from their corresponding cold populations as populations in warm habitats that have existed for thousands of years. This suggests that elevated temperature may drive rapid morphological changes, which are then relatively stable over an evolutionary timescale.

Direction of thermal divergence in body shape

Habitat connectivity may influence not only the magnitude of divergence but also the degree of parallelism between replicate populations (Bolnick et al. 2018). Gene flow between different habitat types is thought to constrain local adaptation within each habitat, so if there is variation in the extent of gene flow among replicate populations, migration-selection balance will act differently contributing to nonparallel evolution (Hendry and Taylor 2004, Moore et al. 2007, Stuart et al. 2017). As a result, we may expect allopatric population pairs (no gene flow) and sympatric population pairs (potential for gene flow) to differ in their degree of parallelism. We indeed found that the absence of gene flow facilitates parallel evolution in warm-cold population pairs of sticklebacks: allopatric population pairs have all evolved along parallel trajectories, whereas none of the sympatric population pairs share evolutionary trajectories.

Previous theoretical and empirical work suggests that the degree of parallelism between replicate populations may also depend on the duration of evolutionary divergence (Lucek et al. 2014, Ord and Summers 2015). For example, population pairs that have been diverging for longer have more scope for natural selection and genetic drift to alter their evolutionary trajectories, resulting in a lower degree of parallelism in older populations (Bolnick et al. 2018). Yet, if evolution is limited by mutation rate, older populations will have had more time to accumulate similar adaptive mutations that produce a similar phenotypic solution in response to a particular environment (Orr 2005, Whitlock and Gomulkiewicz 2005). Under this scenario, older populations would have a higher degree of parallelism. Our findings did not support either of these outcomes, since the extent of parallel evolution did not differ between young and old population pairs.

Lastly, we note that even though we focused on the effects of habitat connectivity and population age in this study, several other factors can influence the magnitude and direction of divergence in natural populations. These include ancestry and evolutionary history (Langerhans and DeWitt 2004), initial and ongoing effective population sizes (Szendro et al. 2013), variation in sexual selection (Bonduriansky 2011, Maan and Seehausen 2011), and many-to-one mapping, which refers to multiple phenotypic solutions to the same functional problem (Gould and Lewontin 1979, Wainwright et al. 2005). Further work is needed to examine these additional factors.

Predictability of evolution and adaptation to climate change

As discussed above, all the allopatric population pairs in our study share a similar evolutionary trajectory in terms of their thermal divergence in body shape. This parallelism could be due to natural selection, developmental bias, or their interaction (Losos 2011, Brakefield 2006, Uller et al. 2018). It is not possible to separate the effects of these processes based on our findings.

Regardless of the underlying process, our results suggest that the absence of gene flow facilitates parallel evolution between warm and cold populations. Thus, morphological evolution in response to increasing temperatures may be predictable to some extent for fish populations where there is no gene flow from other thermal habitats. Under these conditions, we may expect fish to evolve smaller eyes, shorter jaws, and/or a deeper mid-body after being exposed to elevated temperatures over multiple generations. On the other hand, migration between different thermal habitats or microhabitats will exaggerate nonparallel evolution (Oke et al. 2017, Bolnick et al. 2018) and reduce our ability to predict evolutionary responses to changes in temperature.

CONCLUSION

Studying parallel evolution in natural populations inhabiting contrasting thermal environments presents a powerful approach for understanding and predicting population responses to increasing temperatures. Here, we have taken advantage of a unique study system that provides repeated and independent examples of populations found in different thermal environments in the absence of latitudinal or elevational variation. We show that it may be possible to predict morphological evolution in response to elevated temperatures, but only for populations where there is no gene flow from other thermal habitats. Our findings provide novel insights into how gene flow can influence temperature-driven parallel evolution and how fish populations may adapt to a warming world.

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TABLES

Table 1. Sampling locations and sample sizes of warm- and cold-habitat sticklebacks collected in May–June 2016. All cold habitats have existed since the last glacial period and are therefore approximately 10,000 years old, whereas warm habitats can be classified as either young (<100 years old) or old (>1,000 years old). Distance refers to how far apart the warm-habitat and cold-habitat sample sites are for each population pair. The water temperature listed is the average temperature recorded at each sampling location during the summer.

Population pair	Connectivity	Age of warm habitat	Distance (km)	Water body	Thermal habitat	Water temperature (°C)	Number of specimens
A1	Allopatric	Young	0.03	Unnamed	Warm	22.4	29
				Unnamed	Cold	14.0	31
A2	Allopatric	Old	21.04	Grettislaug	Warm	24.9	29
				Garðsvatn	Cold	14.6	30
A3	Allopatric	Old	6.20	Unnamed	Warm	28.6	14
				Unnamed	Cold	13.0	18
S1	Sympatric	Old	3.18	Mývatn	Warm	22.8	30
					Cold	11.5	30
S2	Sympatric	Young	0.03	Áshildarholtsvatn	Warm	24.1	30
					Cold	12.2	30
S3	Sympatric	Young	0.10	Húseyjarkvísl	Warm	23.9	28
					Cold	10.3	32

Table 2. Results of ANOVA models testing the effects of thermal habitat (warm vs cold), population pair, and the interaction between thermal habitat and population pair on first dorsal spine length, second dorsal spine length, and pectoral fin length. Df denotes degrees of freedom. Partial eta-squared (η^2) values provide an estimate for partial variance explained by each factor or interaction. Statistically significant P-values are indicated in bold.

	First dorsal spine length				Second dorsal spine length				Pectoral fin length			
	Df	*F*	*P*	Partial variance explained	Df	*F*	*P*	Partial variance explained	Df	*F*	*P*	Partial variance explained
Thermal habitat	1	0.16	0.69	<0.1%	1	24.8	**<0.001**	7.0%	1	0.06	0.80	<0.1%
Population pair	5	1.24	0.29	1.9%	5	2.46	**0.033**	3.9%	5	8.59	**<0.001**	12.4%
Thermal habitat × population pair	5	1.06	0.38	1.6%	5	9.65	**<0.001**	13.0%	5	3.44	**0.005**	5.4%
Error	319				319				319			

Table 3. Results of MANOVA model testing the effects of thermal habitat, population pair, and their interaction on body shape (i.e., partial warp scores). Df denotes degrees of freedom. Statistically significant P-values are indicated in bold.

Explanatory variable	Df	Wilk's λ	*F*	*P*	Partial variance explained (η^2)
Thermal habitat	1	0.471	4.96	**<0.0001**	53%
Population pair	5	0.012	6.28	**<0.0001**	56%
Thermal habitat × population pair	5	0.033	4.38	**<0.0001**	47%
Error	319				

Table 4. Vector analysis comparing all population pairs to each other. We present data on the angle between each set of population pairs, as well as the 95th percentile of the range of angles (obtained by resampling) within each population pair. If the angle between two population pairs exceeds both of the angles within each population pair, we can conclude that those population pairs are evolving along different evolutionary trajectories. The results in bold indicate evidence for parallelism in thermal divergence between the two population pairs.

Population pairs	Angle between population pairs	Angle within population pair 1	Angle within population pair 2
A1–A2	**67°**	**42°**	**69°**
A1–A3	**62°**	**84°**	**59°**
A2–A3	**81°**	**101°**	**60°**
A1–S1	127°	41°	32°
A1–S2	93°	42°	64°
A1–S3	72°	35°	41°
A2–S1	112°	70°	31°
A2–S2	110°	69°	65°
A2–S3	**50°**	**36°**	**70°**
A3–S2	**73°**	**58°**	**94°**
A3–S3	**81°**	**115°**	**59°**
A3–S1	122°	60°	61°
S1–S2	92°	31°	64°
S1–S3	116°	35°	32°
S2–S3	117°	35°	63°

Table 5. Distances-between-means test comparing the magnitude of divergence in body shape between warm- and cold-habitat sticklebacks in each population pair. "Young" or "old" refers to the age of the warm habitat in that population pair. Statistically significant P-values are indicated in bold.

Population pair	*F*	*P*	Distance between means
A1 (young)	6.49	**0.001**	0.022
A2 (old)	1.90	0.082	0.011
A3 (old)	2.71	**0.039**	0.024
S1 (old)	12.57	**0.001**	0.032
S2 (young)	2.55	**0.030**	0.014
S3 (young)	9.48	**0.001**	0.028

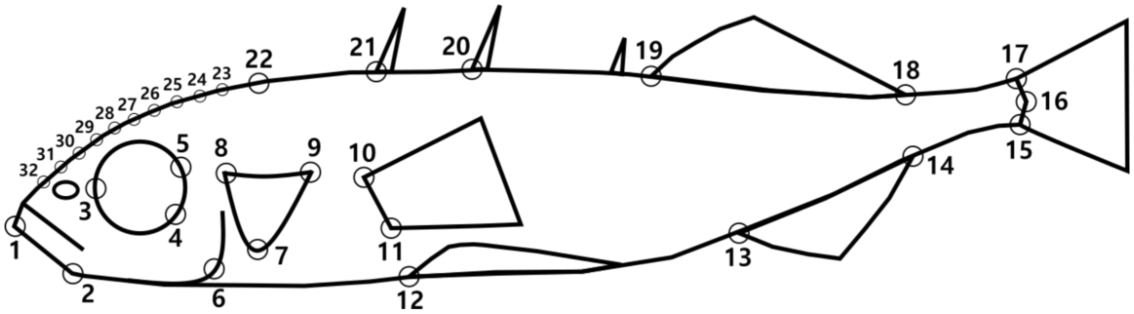

Figure 1. Depiction of the anatomical landmarks (large open circles) and sliding landmarks (small open circles) used to compare the body shape of sticklebacks from cold and warm habitats. The anatomical landmarks were placed on the lower jaw (1, 2), eye orbit (3–5), preopercle (6), opercle (7–9), pectoral fin insertion points (10, 11), anterior tip of pelvic spine (12), anal fin insertion points (13, 14), caudal fin insertion points (15, 17), caudal border of hypural plate at lateral midline (16), dorsal fin insertion points (18, 19), dorsal spine insertion points (20, 21), and the posterior tip of the frontal bone (22). The sliding landmarks were placed between the tip of the upper jaw and the posterior tip of the frontal bone (23–32) to examine head curvature.

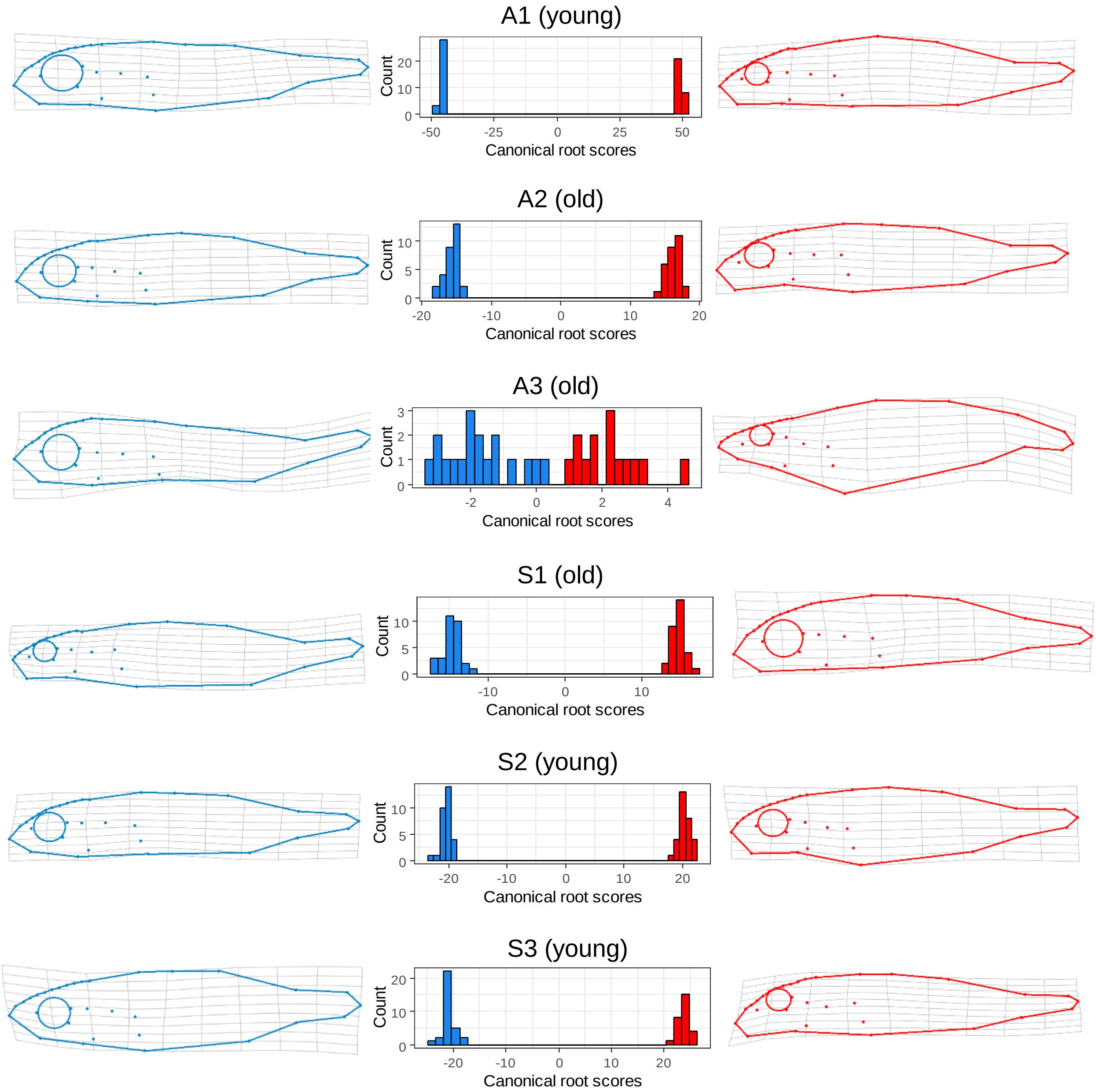

Figure 2. Thin plate spline deformations (with 3× magnification) showing the observed extremes in each population pair and frequency histograms of linear discriminant (LD1) scores from the DFA run on partial warp scores. The deformation grids were generated using tpsRegr (22). Specimens from cold and warm habitats are indicated in blue and red, respectively. “Young” or “old” refers to the age of the warm habitat in that population pair.

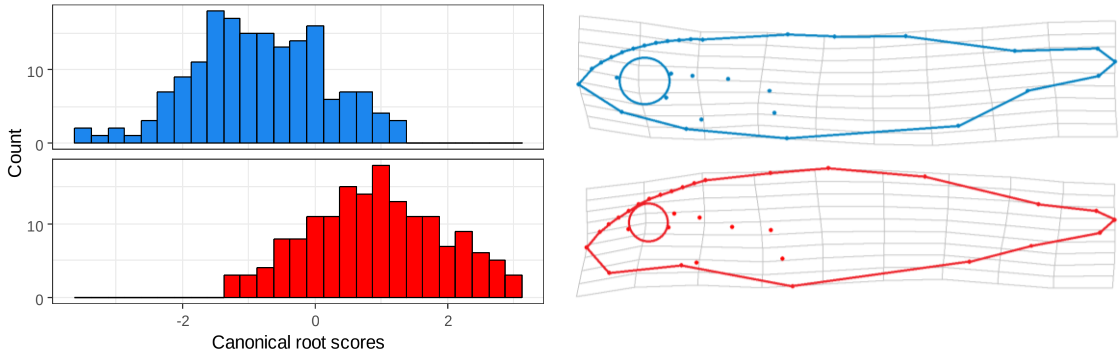

Figure 3. Frequency histograms of linear discriminant (LD1) scores from the DFA run on partial warp scores and thin plate spline deformations (with 3× magnification) showing the observed extremes across all population pairs (A1, A2, A3, S1, S2, and S3). The deformation grids were generated using tpsRegr (22). Specimens from cold and warm habitats are indicated in blue and red, respectively.