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Constraints on speciation suggested by comparing lake-stream stickleback divergence across two continents

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Abstract

Adaptation to ecologically distinct environments can coincide with the emergence of reproductive barriers. The outcome of this process is highly variable and can range along a continuum from weak population differentiation all the way to complete, genome-wide divergence. The factors determining how far diverging taxa will move along this continuum remain poorly understood but are most profitably investigated in taxa under replicate divergence. Here, we explore determinants of progress towards speciation by comparing phenotypic and molecular divergence within young (<150 years) lake-stream stickleback pairs from Central Europe to divergence in older (thousands of years) archetypal lake-stream pairs from Vancouver Island, Canada. We generally find relatively weak divergence in most aspects of foraging morphology (gill raker number, body shape) in the European pairs, although substantial adaptive divergence is seen in gill raker length. Combined with striking overall phenotypic differences between the continents, this argues for genetic and time constraints on adaptive divergence in the European pairs. The European lake-stream pairs also do not display the strong habitat-related differentiation in neutral (microsatellite) markers seen in the Canadian watersheds. This indicates either the lack of strong reproductive barriers owing to weak adaptive divergence, or alternatively that neutral markers are poorly suited for detecting reproductive barriers if these emerge rapidly. Overall, our comparative approach suggests constraints on speciation due to genetic architecture and limited time for divergence. The relative importance of these factors remains to be quantified by future investigation.

Keywords: biological invasion, ecological speciation, *Ectodysplasin*, *Gasterosteus aculeatus*, reproductive isolation, trophic morphology

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Introduction

A major achievement of recent theoretical and empirical speciation research is the appreciation of natural selection's role in driving reproductive barriers (Rice & Hostert 1993; Schilthuizen 2000; Schluter 2000; Kirkpatrick & Ravigné 2002; Coyne & Orr 2004; Rundle & Nosil 2005; Via 2009; Sobel *et al.* 2010). Populations occupying ecologically distinct habitats often experience divergent selection, which in turn drives adaptive divergence in functionally important traits. This divergence (or associated divergence in genetically linked traits) will often generate barriers to gene flow between habitats. The underlying mechanisms are diverse and include factors such as selection against dispersal and hybridization across distinct habitats owing to performance trade-offs, or divergence in reproductive behaviour.

Adaptive divergence and associated reproductive barriers will often build up despite initially substantial gene flow across habitat boundaries (i.e., in parapatry; Endler 1977; Barton & Hewitt 1985; Rice & Hostert 1993; Jiggins & Mallet 2000; Gavrilets *et al.* 2000;

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Gavrilets 2004; Gavrilets & Losos 2009). This has led to the perspective of speciation as a continuum in the extent of genomic differentiation (Wu 2001; Gavrilets & Vose 2005; Mallet 2008; Hendry *et al.* 2009; Via 2009). At the lower end of this continuum, divergent selection is just potent enough to maintain differentiation in a few genomic regions of strong ecological significance (quantitative trait loci, QTLs), while the remainder of the genome is homogenized by gene flow. Full-blown species figure at the other end of the continuum. Here, adaptive divergence between habitats has led to the emergence of strong and generalized reproductive barriers, thus permitting differentiation at both QTLs and ecologically neutral loci.

A major current challenge is to understand the factors that determine how far ecologically diverging populations will progress along this continuum (Hendry *et al.* 2009; Nosil *et al.* 2009; Thibert-Plante & Hendry 2009). A promising approach to addressing this challenge empirically is through the comparison of natural populations that display adaptive divergence in multiple independent localities (replicates), and that differ in their progress along the speciation continuum. Research along these lines indicates that the strength and dimensionality of divergent selection, as well as the geographic arrangement and relative size of diverging populations, influence progress towards complete speciation (Nosil 2007; Seehausen *et al.* 2008; Berner *et al.* 2009). Additional factors, however, are likely to play a role as determinants of progress. For instance, genetic variants that promote strong phenotypic divergence in some places might be lacking in other places, thereby also limiting the potential for the emergence of reproductive barriers associated with adaptation (Schluter 2000; Kirkpatrick & Ravigné 2002; Gavrilets & Vose 2005). Furthermore, progress towards complete speciation might be limited by time (Coyne & Orr 2004; Gavrilets & Vose 2005; Gavrilets *et al.* 2007; Hendry *et al.* 2007). Both genetic and time constraints to speciation remain largely unexplored empirically. We here use stickleback fish occurring in lake and stream habitats to initiate such an investigation.

Threespine stickleback (*Gasterosteus aculeatus*) reside in contiguous lake and stream habitats in many watersheds that were colonized independently by marine ancestors (Reimchen *et al.* 1985; Lavin & McPhail 1993; Thompson *et al.* 1997; Reusch *et al.* 2001; Hendry & Taylor 2004; Berner *et al.* 2008, 2009). Work on lake-stream populations from Vancouver Island, British Columbia, Canada has shown that these habitats typically differ in predominant prey resources: lakes provide opportunities for exploiting limnetic prey (zooplankton in the open water), whereas streams provide mainly benthic resources (macroinvertebrates on the substrate) (Berner *et al.* 2008, 2009). Lake-stream transitions thus represent ecotones that generate divergent selection driving adaptive divergence in stickleback foraging traits. Although ongoing gene flow does sometimes hamper divergence, strong differences in foraging morphology are frequently maintained over distances of a few hundred metres, even in the absence of physical dispersal barriers. Moreover, morphological shifts are often associated with divergence in neutral marker frequencies, indicating that adaptive divergence across lake-stream transitions has produced generalized reproductive barriers (Berner *et al.* 2009).

Lake and stream stickleback in multiple Canadian watersheds have thus progressed substantially towards complete speciation. We here explore genetic and time constraints by using this archetypal phenotypic and molecular divergence as a baseline for comparison with recently established lake-stream stickleback pairs from Central Europe (Fig. 1). This comparative approach is appropriate because, first, the genetic raw material available for adaptive lake-stream divergence might differ between these regions due to historical contingency. Second, the lake-stream pairs on Vancouver Island are typically thousands of years old (Caldera & Bolnick 2008; Berner *et al.* 2009; see also Bell & Foster 1994). A comparison with lake-stream pairs of known recent origin (<150 years old) has the potential to inform on how rapidly speciation can progress.

Fig. 1 Origin of the lake-stream stickleback populations considered in our study, Vancouver Island (Canada) and Central Europe, indicated by yellow circles in the map. The photographs display exemplary lake (bottom) and stream (top) specimens (all reproductive males) from the Robert's watershed (left; Canada) and the Lake Constance watershed (right; Europe).

Table 1 Description of the six European stickleback samples, taken from the Lake Constance and Lake Geneva watersheds. For stream sites, the approximate swimming distance from the lake is given. Sample sizes used for morphological analysis are total, and males and females in parentheses

Lake-stream pair (code)	Locality (code)	Habitat	Distance [km]	Latitude (N)	Longitude (E)	Year	Sample size
Constance West (COW)	Romanshorn (ROM)	Lake		47°33′22.5″	9°22′48.25″	2008/2009	30 (15/15)
	Niederaach (NID)	Stream	8.6	47°33′29.25″	9°16′42.38″	2008/2009	30 (15/15)
Constance South (COS)	Fussach (FUS)	Lake		47°29′29.7″	9°39′40.37″	2008	19 (4/15)
	Rankweil (RAN)	Stream	27.7	47°16′19.28″	9°35′32.72″	2008	30 (15/15)
Geneva (GEN)	Saint Sulpice (SAS)	Lake		46°31′2.89″	6°34′41.70″	2009	30 (15/15)
	Chessel (CHE)	Stream	6.1	46°20′52.18″	6°54′37.43″	2009	29 (16/13)

Materials and methods

Study populations and sampling

The new data for our study come from stickleback that recently colonized lake and stream habitats in the Lake Constance and the Lake Geneva watersheds in Central Europe (Switzerland and Austria). We sampled two streams draining into Lake Constance, and the lake next to each stream's mouth (for details on the localities see Table 1 and supporting online Fig. S1). These two lake-stream pairs are hereafter referred to as 'Constance West' (COW) and 'Constance South' (COS). Stickleback are not native to the Lake Constance watershed, as indicated by old distribution records (Fatio 1882) and recent phylogeographic information (Lucek *et al.* 2010). The species was introduced from a probably North German source around 1850 and was abundant in both the lake and tributaries a few years later (Heller 1871). In addition to these two lake-stream pairs from the Lake Constance watershed, we also sampled stickleback from Lake Geneva and from a stream draining into it [hereafter referred to as the 'Geneva' (GEN) sample pair (Table 1)]. Stickleback in the Lake Geneva watershed also derive from an introduction that occurred in 1872 (and perhaps again around 1900; Bertin 1925) from a source population from France (Fatio 1882; see also Lucek *et al.* 2010). The species was then found to be abundant in the lake and some tributaries in the early twentieth century (Vouga 1921; Blanc 1922).

The above localities were sampled with unbaited minnow traps [except electrofishing at the Rankweil site, see Table 1] in the spring of 2008 and/or 2009, with sample sizes of 15 individuals per sex for most localities (Table 1). No among-year differences in morphology were detected for the sites sampled in both years (analysis not presented), so that we ignore sample year in all analyses. The fish were euthanized with an overdose of MS-222, photographed immediately as described in Berner *et al.* (2009) for later body shape analysis, and then transferred to 95% ethanol.

To compare habitat-related divergence in the above stickleback pairs to divergence in well-characterized Canadian populations, we re-analysed morphological and genetic data from five lake-stream pairs occurring in independently colonized watersheds on Vancouver Island. These are hereafter referred to as the 'Canadian', as opposed to the 'European', lake-stream pairs. In both cases, we acknowledge that the chosen pairs are not necessarily representative of stickleback from each continent in general. The Canadian population pairs are a subset of the eight pairs examined in Berner *et al.* (2008) and were chosen to represent full-blown adaptive lake-stream divergence. Specifically, we excluded two population pairs where stomach content analysis indicated spatially variable (rather than consistently divergent) selection on trophic morphology (McCreight and Morton watersheds, see Table 1 in Berner *et al.* 2008), and one pair where excessive gene flow strongly constrains adaptive divergence between the habitats (Misty watershed; Moore *et al.* 2007; Berner *et al.* 2009). The Canadian samples thus include those from the Beaver, Boot, Joe's, Pye, and Robert's watersheds. The swimming distance between the lake and stream site in all Canadian watersheds was 4.3 km or less (for the European pairs see Table 1). Sample size per locality was 20 individuals (further detail on the Canadian samples is provided in Berner *et al.* 2008).

Morphology

Our main interest was in morphological traits relevant to adaptation to limnetic (open water) versus benthic (substrate) foraging environments. The traits included the number and length of gill rakers on the first branchial arch, key elements of the gill raker apparatus used for filtration of suspended prey (Gerking 1994; Sanderson *et al.* 2001). Gill raker morphology influences prey capture and handling performance in stickleback (Bentzen & McPhail 1984; Lavin & McPhail 1986; Schluter 1993, 1995; Robinson 2000) and typically displays divergence between limnetic and benthic-foraging

individuals and populations (Schluter & McPhail 1992; Lavin & McPhail 1993; Robinson 2000; Araujo *et al.* 2008; Berner *et al.* 2008, 2009, 2010). Limnetic-foraging stickleback tend towards higher gill raker number and length compared to conspecifics foraging on benthos. Furthermore, variation in gill raker number and length within and among populations consistently exhibits a strong genetic component (Hagen 1973; Gross & Anderson 1984; Lavin & McPhail 1987; Schluter 1996; Hatfield 1997; Hermida *et al.* 2002; Aguirre *et al.* 2004). The traits were quantified after at least two months of preservation. We counted the total number of gill rakers on the first branchial arch at 50× magnification under a stereomicroscope. Gill raker length was expressed as the average length of the rakers two to four counted from the joint with the dorsal arch bone (Berner *et al.* 2008). The same measurements, made by the same person, were also available for the Canadian specimens.

We next quantified variation in body shape by using geometric morphometrics. Body shape also plays an important role in the exploitation of limnetic versus benthic-foraging habitats. Limnetic stickleback have shallower bodies and longer caudal peduncles (Reimchen *et al.* 1985; Schluter & McPhail 1992; Lavin & McPhail 1993; Robinson 2000; Hendry & Taylor 2004; Aguirre 2009; Berner *et al.* 2009), which presumably reduces friction drag and hence facilitates foraging on suspended prey in the open water (Webb 1984; Blake 2004). Benthic stickleback, in contrast, tend towards deep bodies and short caudal peduncles. This appears to maximize manoeuvrability and thereby facilitate foraging on complex bottom substrates. Shape divergence has been shown to exhibit a strong genetic component in stickleback (Schluter *et al.* 2004; Albert *et al.* 2008; Sharpe *et al.* 2008). For shape analysis, we used digital photographs of a specimen's left body side in natural position. On every image, 16 landmarks were digitized using tpsDig v2 (Rohlf 2001). The landmark configuration was the same as in Berner *et al.* (2009), with the exception that the slider (semilandmark) was omitted. We then used TpsRelw (Rohlf 2001) to superimpose all individual landmark configurations in a single analysis (European and Canadian combined), to calculate centroid size, and to obtain the weight matrix (summarizing all uniform and localized attributes of shape variation) along with its principal components (relative warps).

While our main interest was in foraging morphology, stickleback populations in both European watersheds proved polymorphic for the number of bony plates along their body. We therefore included this phenotype in our morphological analysis. Ancestral marine stickleback display a full complement of lateral plates, while freshwater populations typically display a dramatic reduction in plate number (Bell & Foster 1994). While the selective forces acting on lateral plates are probably multifarious and remain only partly understood, differential predation pressures and escape opportunities between habitats probably play a key role in lateral plate evolution (Reimchen 1994). Specifically, invertebrate predation (Marchinko 2009) and the availability of shelter (Reimchen 1992) seem to promote plate reduction in freshwater habitats. Furthermore, lateral plate morphs have a relatively simple genetic architecture; most of the variation is accounted for by regulatory changes in the *Ectodysplasin* (*Eda*) pathway (Cresko *et al.* 2004; Colosimo *et al.* 2005). We assigned each specimen to one of three lateral plate morphs (Cuvier & Valenciennes 1829; Hagen & Gilbertson 1972): low-plated: displaying only 0–3 plates posterior of the pelvic girdle; fully plated: displaying a continuous series of plates along the entire flank, the ones on the tail forming a keel; partially plated: exhibiting a gap of 2–11 plates between the pelvic girdle and the caudal keel. Only the European specimens were subjected to lateral plate analysis, as the Canadian samples were invariably low-plated.

Statistical analysis

Our first prediction was a general reduction in the number and length of gill rakers in stream fish relative to lake fish. As gill raker length (but not number) scales with overall body size, this trait had first to be size-adjusted. For this, we ran all 16 samples together in a general linear model (GLM) with gill raker length as response, sample site as factor and centroid size as covariate. Individual residuals from the common within-group slope (Reist 1986) were then added to the predicted gill raker length for each population at grand mean centroid size, thus maintaining the original measurement unit. Variation in gill raker number and length was then analysed in univariate random permutation tests (9999 iterations), which made no assumption about the statistical distribution of the data (parametric tests produced quantitatively very similar results). We carried out three separate permutation tests, each using as test statistic *F*-values from a GLM (Manly 2007). The first GLM, for the GEN lake-stream pair, included only the factors habitat (lake versus stream), sex and their interaction. The second model analysed the COW and COS sample pairings together with lake-stream pair, habitat, sex and all interactions as factors. A third GLM with the same structure was used to analyse the Canadian samples.

For body shape, we predicted generally deeper bodies and shorter caudal peduncles in stream compared to lake stickleback. This was examined by com-

bining two sets of analyses. First, we tested for significant shifts in body shape by analysing the weight matrix in three separate multivariate GLMs (for GEN, for COW and COS combined, and for all Canadian lake-stream pairs combined). For GEN, habitat and sex were entered as factors, and centroid size as covariate (including all interactions). The other two models included lake-stream pair as an additional factor. The second shape analysis compared all stickleback samples simultaneously along a few key dimensions. We here analysed the entire weight matrix in a single global model with continent (Europe versus Canada), habitat and sex as factors, centroid size as covariate, and all interactions. We then extracted the canonical variate for the continent, habitat and sex factor in the model and visualized them using TpsRegr (Rohlf 2001). Effect sizes for these factors (i.e., the proportion of explained partial variance) were estimated by Wilks' partial eta-squared (η^2) (Langerhans & DeWitt 2004). In addition to canonical variates analysis, we explored major axes of shape variation within the full data set based on the principal components of the weight matrix (relative warps).

Finally, we predicted a habitat-related trend in lateral plate morphs for the European stickleback, with a higher frequency of low-plated fish in streams when compared to lakes. The reason is that we expected invertebrate predation to be more severe and shelter from vertebrate predators more available in shallow streams than in the lakes. This prediction was tested for each European lake-stream pair separately by performing 9999 random permutations of plate phenotypes over the habitats and using the chi-square value as test statistic. All analyses and graphics were performed in R v2.9.2 (R Development Core Team 2009). Codes are available on request. All morphological data are available on the Dryad Digital Repository (doi:10.5061/dryad.1960).

Genetics

Patterns of genetic differentiation within and among the Canadian watersheds have been presented in detail elsewhere (Thompson *et al.* 1997; Hendry & Taylor 2004; Berner *et al.* 2009). Our genetic analyses thus focused primarily on the European fish. In a first step, we quantified the sequence variation in a fragment of the mitochondrial control region (D-loop) to characterize geographic population structure among the European samples at a coarse scale, and specifically to test for the independent history of stickleback in the two watersheds. To place the European populations in a broader phylogeographic context, this analysis also included specimens from two Pacific-derived freshwater populations (Salinas River, California; Misty Lake, Vancouver Island).

DNA was extracted from fin tissue following the method described in Bruford *et al.* (1998). We then sequenced a 328 basepair (bp) fragment of the mitochondrial D-loop for a subsample of 2–13 individuals per locality. The D-loop fragment was amplified using standard primers [L-Pro-F (Meyer *et al.* 1994), TDK-D (Lee *et al.* 1995)] and the cycling conditions given in Baric *et al.* (2003). PCR products were sequenced on an ABI3130xl sequencer (Applied Biosystems). We then used jModelTest v0.1.1 (Posada 2008) to identify the most appropriate model of sequence evolution (HKY+I; Hasegawa *et al.* 1985; Posada 2008). The most probable genealogical relationship was identified using the maximum-likelihood method implemented in PAUP* v4.0 (Swofford 2003) and translated into a haplotype genealogy for visualization [see Salzburger *et al.* (2003) for details].

To explore the population structure in the European samples at a higher resolution and to test for molecular signatures of habitat-related reproductive isolation in particular, we next quantified allelic variation at six rapidly evolving microsatellite markers for 20 individuals per sample. To allow for a comparison with patterns seen in the Canadian watersheds, we chose exactly the same set of markers as in Berner *et al.* (2009). These markers were Stn67 (chromosome 6), Stn159 (13), Stn171 (15), Stn195 (18), Stn207 (20) and Stn238 (4) (for primer combinations see Peichel *et al.* 2001). PCR multiplex amplification with labelled primers was carried out using the QIAGEN multiplex kit following the manufacturer's protocol. PCR products were run on an ABI3130xl sequencer (Applied Biosystems) and fragment lengths scored by hand in Peak Scanner v1.0 (Applied Biosystems). Fragment length consistency and the absence of contamination were confirmed by running the same two reference individuals and two blinds on all plates. The quality of the microsatellite data was then examined with MICRO-CHECKER (Van Oosterhout *et al.* 2004). No deviation from Hardy–Weinberg expectation was found in any marker by sample combination. For Stn238, however, a null allele was indicated in the two Lake Constance samples. We therefore carried out all analyses with and without this marker. This did not materially influence any conclusion so that we report all analyses with Stn238 included. File conversion for the different programs used in the microsatellite analysis was carried out using CREATE (Coombs *et al.* 2008).

We first used the microsatellite data to quantify genetic differentiation within all European sample pairings by Weir & Cockerham's (1984) F_{ST} estimator implemented in Genetix v4.05 (Belkhir *et al.* 2004). *P*-values were determined using 999 permutations. As the F_{ST}

metric is dependent on actual levels of within-population variation, we also transformed the data set using RECODEDATA v0.1 (Meirmans 2006) and calculated standardized F_{ST}, defined as the proportion of differentiation relative to the maximum possible for a given level of within-population heterozygosity (Hedrick 2005).

We next explored the hypothesis that neighbouring lake and stream samples within the European watersheds form distinct populations by using the Bayesian genetic clustering software Structure v2.3.1 (Pritchard *et al.* 2000; Hubisz *et al.* 2009). All six European samples were run in a common analysis using the admixture and independent allele options and 20 000 iterations as burnin and run length [different settings, including the more sensitive clustering algorithm incorporating locality information (Hubisz *et al.* 2009) produced similar results]. Five replicate simulations were performed for K = 1–6 (the assumed number of populations). Population structure was interpreted following the recommendations in Pritchard & Wen (2004) and using the ΔK approach (Evanno *et al.* 2005). For comparison, a similar Structure analysis was then performed for each of the five Canadian sample pairs separately by using data for the same six microsatellite markers available from previous work (Berner *et al.* 2009). Sample size was here also 20 individuals per locality.

Finally, we used the microsatellite data to test for recent bottlenecks (reductions in population size) in the European samples. That is, we examined whether the colonization of streams from lakes (or possibly vice versa) may have involved only a relatively small number of founder individuals. The genetic signature of recent bottlenecks is a heterozygosity excess relative to the level expected from the number of alleles under mutation–drift equilibrium (Nei *et al.* 1975; Cornuet & Luikart 1996). We tested for a bottleneck in each of the six samples by using the program Bottleneck (Piry *et al.* 1999). We used the recommended two-phase mutation model with 93% stepwise and 7% multistep mutations, a variance of 12, 5000 iterations in the coalescent simulations and Wilcoxon's signed-rank test.

The above D-loop and microsatellite analyses quantified population differentiation in genomic regions evolving primarily by neutral processes (mutation and drift). Because our European study populations differed in lateral plate morph frequency and this phenotype is known to be determined strongly by the *Eda* pathway (see above), we took the opportunity to also study genomic differentiation in the neighbourhood of a locus under selection. Specifically, low-plated freshwater stickleback that diverge from full-plated marine ancestors at geographically independent locations usually (but not always) do so through the fixation of a shared old *Eda* variant (Colosimo *et al.* 2005). As a result, stickleback *Eda* gene sequences from evolutionarily independent populations typically cluster by plate morph rather than by geographic relatedness. We here tested whether this also holds for our European populations by sequencing *Eda* for a subsample of 2–12 specimens per locality. These samples were chosen to include primarily fully and low-plated individuals, but partially plated individuals where also considered where possible. The *Eda* sequence analysis again included individuals from Salinas River (N = 6) and Misty lake (N = 5). We sequenced 1005 bp of *Eda*, representing the entire coding region of both known splice variants except for a 108 bp fragment adjacent to the first exon–intron boundary. This was excluded because we placed a reverse primer in the first exon. Primer combinations and PCR cycling conditions are given in supporting online Table S1. DNA sequencing was carried out on an ABI3130xl sequencer (Applied Biosystems). Sequence analysis and visualization were performed as described for the D-loop fragment, except that sequence evolution was selected to follow the TPM2 mutation model (Kimura 1981; Posada 2008). Both D-loop and *Eda* sequences are deposited on GenBank under the accession numbers HQ184698-HQ184746 and HQ184747-HQ184850. The microsatellite data are available on Dryad (doi:10.5061/dryad.1960).

Results

Morphology

We detected no differentiation in gill raker number within or among European stickleback samples (Fig. 2)

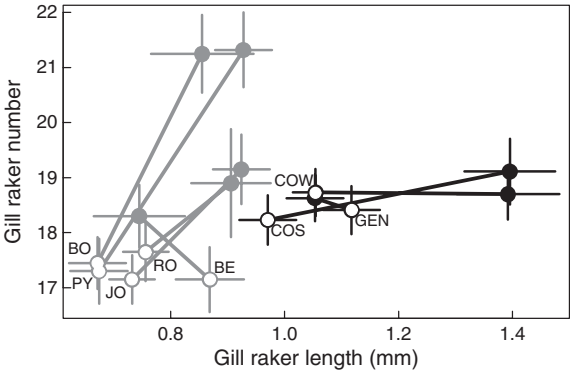

Fig. 2 Differences in gill raker morphology among lake (filled symbols) and stream (open symbols) stickleback from Europe (black) and Canada (grey). Error bars are parametric 95% confidence intervals. Gill raker length was size-adjusted to grand mean centroid size. Labels next to the stream symbols indicate the lake-stream pair [codes for the European pairs see Table 1; the Canadian pairs are Beaver (BE), Boot (BO), Joe's (JO), Pye (PY), and Robert's (RO)].

(all model terms $P \geq 0.095$; full statistical tables for all analyses are given in supporting online Table S2). In contrast, habitat-related divergence in Canadian fish was very strong overall ($P = 0.0001$), with substantial variation also seen among the replicate lake-stream pairs (pair $P = 0.0001$, pair-habitat interaction $P = 0.0001$). The European samples were clearly intermediate in gill raker number relative to the extremes observed in Canada. There was no indication of sexual dimorphism for this trait in any model (all sex main effects $P \geq 0.12$).

Our prediction of shorter gill rakers in stream relative to lake stickleback was strikingly confirmed for both COW and COS (habitat $P = 0.0001$, pair-habitat interaction $P = 0.065$). This pattern paralleled a general but slightly weaker trend in Canadian fish (habitat $P = 0.0001$, pair $P = 0.045$, interaction $P = 0.0001$). Habitat-related divergence in gill raker length was negligible (albeit significant, $P = 0.035$) for GEN. Interestingly, the range of European sample means showed no overlap with the Canadian lake and stream means; the latter populations consistently displayed shorter gill rakers on average. This trait also showed very consistent sexual dimorphism (all $P \leq 0.002$; not illustrated): males displayed longer (14% on average) gill rakers in all 16 samples.

All three separate GLMs analysing body shape variation found significant shifts associated with habitat (GEN $P = 0.006$; COW/COS $P < 0.0001$; Canadian pairs $P < 0.0001$; details see supporting online Table S2). Canonical variates analysis for the habitat factor ($P < 0.0001$) in the global GLM made clear that stream fish tended towards shorter snouts and caudal peduncles, and greater overall body depth compared to lake fish (Fig. 3). However, lake-stream divergence in European stickleback was consistently very low in magnitude along this canonical variate compared to the divergence seen in Canada. The global GLM also detected strong body shape differences between the continents ($P < 0.0001$). Variation along the corresponding canonical variate resembled the habitat-related shifts in that European stickleback displayed relatively shorter snouts and caudal peduncles, and greater body depth (Fig. 3). The proportion of partial variance explained by the continent factor (partial $\eta^2 = 0.93$) was roughly 50% greater than for the habitat factor (0.67). Finally, the global GLM indicated substantial sexual dimorphism in body shape ($P < 0.0001$). Males displayed larger heads and longer snouts than females (supporting online Fig. S2), consistent with recent findings from other stickleback systems (Albert *et al.* 2008; Aguirre & Akinpelu 2010). This trend was evident in all 16 samples but more pronounced in European fish. Sex was comparable in effect size to the continent and habitat factors (partial $\eta^2 = 0.79$). Patterns of shape change between the habitats, continents and sexes revealed by the major relative warps (RWs) were qualitatively similar to those obtained from canonical variates analysis and are presented in Appendix 1. For instance, RW1 essentially captured the shifts in body depth and caudal peduncle length within the Canadian lake-stream pairs and among the continents, while the sex-related shifts in head morphology appeared on RW2.

The fully plated phenotype dominated in both samples from Lake Constance (Fig. 4). In contrast, the COW stream sample displayed a strikingly lower ($P = 0.0001$) proportion of fully plated fish. Stream fish from COS, however, did not differ in plate morph frequencies from their lake counterparts ($P = 0.377$). In Lake Geneva stickleback, the fully and partially plated morphs were not frequent but still occurred at an appreciable frequency, while the stream population was

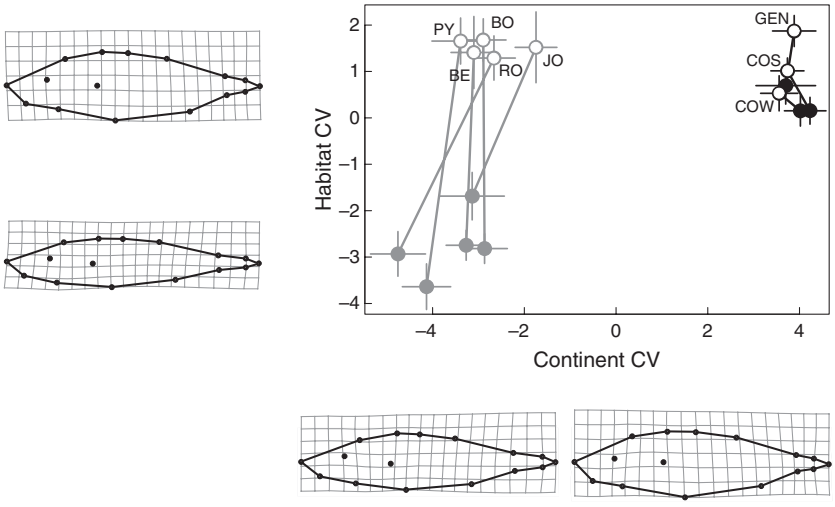

Fig. 3 Divergence in stickleback body shape between the continents (Europe black, Canada grey), and between lake and stream habitats (filled and open symbols), expressed as the canonical variate (CV) for the continent and habitat factor in the global general linear model (GLM) (details see text). The deformation grids visualize the lowest and highest observed CV scores (continent: −7.4/7.4; habitat: −6.2/4.1). Error bars are 95% confidence intervals. Lake-stream pairs are labelled as in Fig. 2. Note that for ease of presentation the two CVs are plotted on orthogonal axes although they are not orthogonal in trait space.

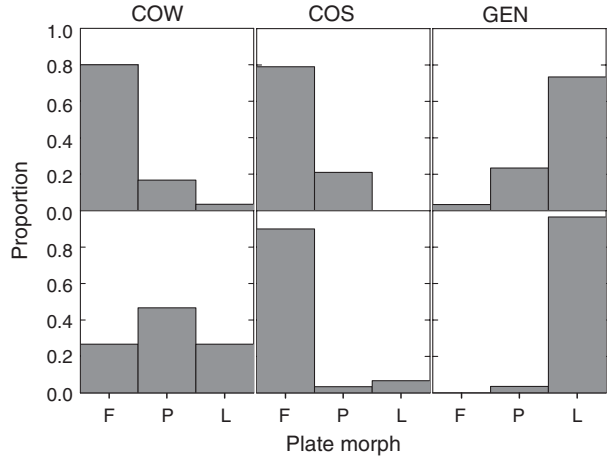

Fig. 4 Relative proportions of fully (F), partially (P) and low-plated (L) stickleback in the lake (top) and stream (bottom) samples from the three European habitat pairs. The shift towards lower armour in the stream is significant in Constance West (COW) and Geneva (GEN).

essentially fixed for the low-plated phenotype (only a single partial morph observed; $P = 0.029$).

Genetics

Stickleback from the Lake Constance and Lake Geneva watersheds shared no D-loop haplotypes, although one of the two haplotypes found in the Lake Geneva watershed differed from the most common Lake Constance haplotype by a single substitution only (Fig. 5a). In contrast to this differentiation between the European watersheds, common haplotypes were shared among samples from different localities *within* the watersheds. The only exception was two private sequence variants in the NID sample. The Pacific-derived freshwater samples (Salinas River and Misty Lake) displayed substantial D-loop variation but were all very different from European haplotypes. Overall, the D-loop sequence data were consistent with the view that stickleback from the two European watersheds derive from separate introductions (see also Lucek *et al.* 2010), but still originate from a common Atlantic source relatively distinct from Pacific stickleback.

Concordant with the D-loop data, microsatellite allele frequencies indicated very strong differentiation between stickleback from the Lake Constance and Lake Geneva watersheds (F_{ST} for all sample pair combinations >0.38, standardized >0.84; Table 2). Differentiation within the Lake Constance watershed, however, was low overall. In particular, F_{ST} between the two lake samples (ROM, FUS) was zero. This was also true for the GEN lake and stream sample pair. These patterns were mirrored by the joint analysis of the six European

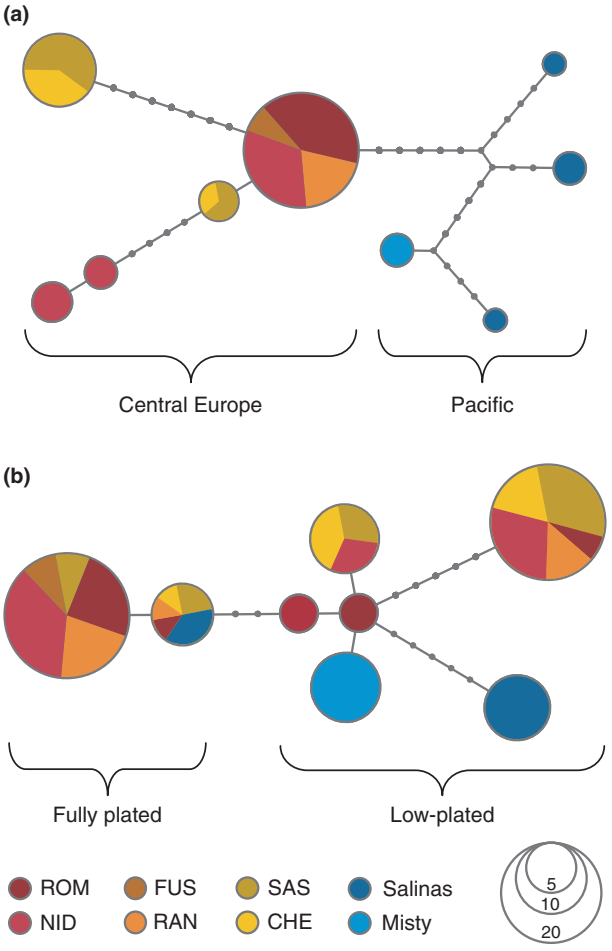

Fig. 5 Haplotype genealogy for (a) the mitochondrial D-loop fragment (based on 26 SNPs found in 49 total sequences) and (b) the *Ectodysplasin* (*Eda*) gene (14 SNPs in 104 total sequences). Lines connecting nodes represent single nucleotide differences (substitutions or deletions). Circle sizes indicate the number of times a haplotype was recovered. Shown are data for the six European samples and two Pacific-derived freshwater populations (Salinas River and Misty lake). For locality codes to the European fish see Table 1.

populations using STRUCTURE: the program unambiguously identified two as the most likely number of distinct populations, thereby perfectly separating stickleback from the Lake Constance and Lake Geneva watersheds (details not presented). Lake and stream samples within watersheds thus did not emerge as genetically distinct populations, even when Structure was applied to each of the three habitat pairings separately. Our microsatellite analysis therefore provided no evidence for ecologically based reproductive isolation in neutral genomic regions. In striking contrast, re-analysis of the same set of markers in Canadian stickleback using Structure indicated that lake and stream fish formed distinct populations ($K = 2$) in each of the five

Table 2 Genetic differentiation among the six European stickleback samples ($N = 20$ individuals; site codes see Table 1), based on polymorphism at six microsatellite loci. The upper semimatrix presents Weir & Cockerham's (1984) F_{ST} estimator calculated on the raw data. *P*-values based on 999 permutations are given in parentheses (bold if <0.01). The lower semimatrix presents F_{ST} standardized by the maximum possible differentiation for the observed levels of within-population variation (Hedrick 2005). Note that strong differentiation is observed mainly between but not within the two drainages

	ROM	NID	FUS	RAN	SAS	CHE
ROM		0.03 (0.056)	0.00 (0.563)	0.12 (**0.001**)	0.40 (**0.001**)	0.42 (**0.001**)
NID	0.07		0.03 (0.028)	0.12 (**0.002**)	0.39 (**0.001**)	0.41 (**0.001**)
FUS	0.00	0.08		0.11 (**0.001**)	0.38 (**0.001**)	0.40 (**0.001**)
RAN	0.29	0.29	0.28		0.40 (**0.001**)	0.42 (**0.001**)
SAS	0.90	0.86	0.88	0.88		0.00 (0.409)
CHE	0.90	0.85	0.87	0.89	0.00	

watersheds. This agreed with the previous results based on larger sample sizes obtained by combining multiple clinal samples (Berner *et al.* 2009). Finally, the bottleneck analysis found no evidence for a recent reduction in population size in any of the six European samples (all one-tailed *P* values for heterozygosity excess ≥0.34).

The *Ectodysplasin* (*Eda*) gene revealed a pattern of genetic differentiation quite different from those obtained by the D-loop fragment and the microsatellite analyses. *Eda* sequences clustered primarily by plate phenotype and to a lesser extent by geography (Fig. 5b). In particular, all samples except for Misty Lake (fixed for the low-plated phenotype) shared at least one of two closely related haplotypes that were consistently associated with the fully plated phenotype when in homozygous state. Similarly, European individuals typically shared a common haplotype associated with the low-plated phenotype when homozygous, although additional low-plated variants occurred. Both Pacific-derived populations harboured a private *Eda* variant associated with the low-plated phenotype. Without exception, partially plated specimens proved heterozygous in the sense that they represented a combination of a low-plated and fully plated *Eda* haplotype.

Discussion

We explored whether the colonization of lake and stream habitats by stickleback recently introduced to Central European watersheds has been accompanied by phenotypic and genetic divergence, and used data from Canadian lake-stream systems as a benchmark for evaluating this divergence. Our main findings are that morphological divergence in European lake-stream pairs was generally very low compared to Canadian watersheds, although gill raker length did exhibit strong predicted habitat-related shifts. Moreover, clear overall differences in morphology were evident between the two continents. European lake-stream pairs also did not display the strong habitat-related divergence in neutral marker frequencies seen in Canadian stickleback. We now discuss how these findings shed light on both determinism and constraints in adaptive divergence, and their relevance for understanding processes acting in the earliest stages of speciation.

Deterministic divergence in morphology

The most striking pattern of morphological divergence in European stickleback was the reduction in gill raker length in stream when compared to lake fish within the Lake Constance watershed. The direction of this shift was predicted from the typical divergence seen in the Canadian systems. Correlative and experimental work in stickleback has identified gill raker length as a key morphological determinant of foraging success in limnetic versus benthic-foraging environments (Bentzen & McPhail 1984; Lavin & McPhail 1986; Schluter 1993, 1995; Robinson 2000; Svanbäck & Bolnick 2007; Araujo *et al.* 2008; Berner *et al.* 2008, 2010). Previous evidence further suggests that the observed differences in gill raker length have a substantial genetic basis, as opposed to reflecting primarily phenotypic plasticity. First, this has been found by quantitative genetic investigations in other stickleback systems (Lavin & McPhail 1987; Schluter 1996; Hatfield 1997). Second, several studies have attempted to induce phenotypic plasticity in gill raker length by raising stickleback on limnetic versus benthic prey in the laboratory. While some adaptive plasticity was observed in one of these studies (Day *et al.* 1994), its magnitude was modest when compared to the genetically based difference between the experimental populations, and subsequent experiments failed to induce significant plasticity (Day & McPhail 1996; Wund *et al.* 2008; Berner & Hendry, unpublished data). Nevertheless, we do not dismiss the possibility that some of the divergence reflects plasticity, which should be quantified in future work.

Overall, we thus find strong evidence that gill raker length has evolved in response to resource-mediated

divergent selection within the Lake Constance watershed. This view is strengthened by the very similar shifts observed in *both* COW and COS. Given that the mouths of the two tributaries are far apart (20 km) and that stickleback from the two streams do not cluster together based on microsatellite frequencies or D-loop haplotypes, it is unlikely that the consistent shifts in both habitat pairs originate from direct dispersal among streams. Moreover, the bottleneck analysis certainly argues against a small number of colonizers. The most parsimonious explanation for the repeated evolution of short gill rakers in these streams is therefore independent and rapid sorting of heritable variation segregating within an essentially panmictic (as indicated by the microsatellite markers) Lake Constance population.

A similar conclusion may be drawn for the lateral plates in both European watersheds. In two of the three habitat pairs (COW and GEN), lake fish displayed a significantly lower frequency of low-plated phenotypes compared to their stream counterparts. Plate phenotypes are genetically based (e.g. Colosimo *et al.* 2005), and the direction of the observed frequency shifts was predicted from presumed differences between lake and stream habitats in the importance of vertebrate versus invertebrate predation and the availability of shelter (Reimchen 1992, 1994; Marchinko 2009). Overall, our study thus makes a case for at least some deterministic morphological evolution on a contemporary timescale.

Stasis, maladaptation, and possible causes

While our analysis documents some adaptive lake-stream divergence in European stickleback, most traits instead showed evolutionary stasis between these two habitats. In particular, European fish failed to diversify in gill raker number, and none of the European stream samples displayed the short gill rakers typical of Canadian stream fish. Moreover, lake-stream divergence in body shape was in the expected direction but marginal in magnitude compared to Canadian watersheds. Several not mutually exclusive explanations may account for these striking inconsistencies across the continents.

First, ecological conditions in lakes and streams might differ systematically between Europe and Canada, resulting in distinct phenotypic optima and hence different divergence patterns. This scenario is unlikely to explain the stasis observed in foraging traits. Preliminary stomach content data from NID and CHE stickleback (D. Berner and W. Salzburger, unpublished data) make clear that European stream fish exploit very similar (benthic) resources as Canadian stream fish (primarily macro-invertebrates such as chironomid and ephemeroptera larvae; Berner *et al.* 2008). The corresponding data are lacking for the European lake fish. However, both Lake Constance and Lake Geneva are very large (≥535 km^2) and deep and offer little littoral habitat, conditions selecting for a limnetic lifestyle and corresponding trophic morphology in stickleback (Moodie & Reimchen 1976; Bolnick & Lau 2008). Furthermore, a limnetic lifestyle at least in Lake Constance stickleback is indicated directly by occasional captures in gillnets far offshore (A. Lunardon, personal communication). We therefore expected a similar dichotomy in foraging environments and associated divergent selection on trophic morphology as in Canadian watersheds. The generally weak lake-stream divergence seen in European stickleback, and their distinct overall position in phenotype space compared to Canadian fish, thus lead us to hypothesize that European fish might be *relatively* maladapted at least in some trophic traits (e.g. gill raker design in streams, and perhaps body shape in lakes). Extensive direct information on ecological conditions (e.g., resource availability, competitors, predation regimes) experienced by stickleback is required, however, to evaluate this hypothesis more conclusively.

A second possible explanation for the weak lake-stream divergence in Europe might be excessive gene flow between the environments. Indeed, previous work suggests that local adaptation to stream habitats can be severely constrained by gene flow from the lake (Moore *et al.* 2007; Berner *et al.* 2009). However, we considered only inlet streams in Europe as opposed to outlet streams in Canada, and we sampled the former at substantially greater distance from the lake. Thus, it would have been more likely for gene flow to constrain adaptive divergence in the Canadian pairs than in the European ones.

A third possible explanation for evolutionary stasis in European lake-stream stickleback is their young age. Selective differences between lakes and streams might be comparable across continents, but European fish may *not yet* have adapted strongly to them. Indeed, the split between the partly reproductively isolated parapatric lake-stream populations on Vancouver Island has been estimated to typically date back thousands of generations (Berner *et al.* 2009; see also Caldera & Bolnick 2008), as opposed to some 150 generations or less for our European fish. This explanation appears challenged by the very rapid morphological evolution (i.e., within years or decades) sometimes seen in stickleback (e.g., Bell *et al.* 2004; Kitano *et al.* 2008; Gelmond *et al.* 2009). All examples of rapid evolution, however, concern armour traits exhibiting a simple genetic architecture. The typical pace of divergence in gill raker traits or body shape is unknown.

Finally, both the incongruence in divergence patterns between our European and Canadian watersheds and the overall phenotypic differences at the continent level

might be attributable to fundamental differences in genetic architecture. Allelic variants that promote adaptation to colonized freshwater habitats and that segregate around Vancouver Island—perhaps because of recurrent gene flow from freshwater to the ocean (Schluter & Conte 2009)—might be lacking in the focal European populations. Such genetic constraints might plausibly result from founder effects directly associated with the introduction of the species to the focal European watersheds, and perhaps also from historical contingency at a larger geographic scale.

Taken together, our comparative study provides evidence for a mixture of deterministic and constrained evolution in our European lake-stream stickleback pairs. Although the precise cause(s) for the latter remain(s) to be resolved, insufficient time for divergence and differences in genetic architecture between the study regions emerge as strong candidates.

Progress towards complete speciation

Adaptive lake-stream divergence has been shown to mediate at least partial reproductive isolation in Canadian stickleback, resulting in the maintenance of relatively abrupt shifts in heritable morphology and neutral marker frequencies in remarkably close parapatry (Berner *et al.* 2009). The underlying reproductive barriers remain to be elucidated but probably include adaptive habitat choice and perhaps reproductive timing and performance trade-offs leading to selection against immigrants and hybrids (reviewed in Hendry *et al.* 2009). Based on these observations, we also expected to find habitat-related differentiation in microsatellite marker frequencies within the European lake-stream pairs. This expectation was not supported, as differentiation in all lake-stream pairs was very low. Note that the slightly stronger differentiation seen in the COS habitat pair (Table 2) probably simply reflects isolation by distance or founder effects because here the geographic distance between the lake and stream localities (28 km; see Table 1) was substantially greater than in the other European (or Canadian) pairs (9 km or less). In short, our microsatellite marker data do not indicate substantial genetic differentiation associated with habitat transitions in European stickleback.

Combined with the adaptive divergence seen at least in some heritable morphological traits (gill raker length, plate morph), this finding clearly indicates a heterogeneous (or mosaic) pattern of genomic differentiation (Wu 2001; Gavrilets & Vose 2005; Via 2009) in the European fish. Divergent selection seems to keep allele frequencies distinct between the habitats for some ecologically relevant loci (or hitchhiking regions) but not neutral markers. In the COW pair, for instance, the *Eda* haplotypes associated with the fully plated phenotype occurred at a frequency of 0.9 in the lake but only 0.5 in the stream (taking into account that partially plated individuals were heterozygous for *Eda* haplotypes). And yet, neutral marker differentiation between these localities was negligible.

We propose two not mutually exclusive explanations for discordance in genetically based phenotypic versus neutral genetic differentiation. First, strong and generalized reproductive barriers across the habitat transition may not have emerged in Europe because adaptive divergence is also relatively weak, and/or restricted to few traits (Rice & Hostert 1993; Hendry 2009; Nosil *et al.* 2009). This may allow for enough gene flow to homogenize the genome except in regions under particularly strong divergent selection. (We note that absolute physical barriers to dispersal between habitats are unlikely in all three European lake-stream pairs.) The observed patterns of morphological and genetic differentiation would then reflect a balance between gene flow and divergent selection (Barton & Hewitt 1985; Jiggins & Mallet 2000; Wu 2001; Gavrilets & Vose 2005; Via 2009).

The second possibility is that rapid divergent local adaptation (possibly involving traits not considered in our study, such as habitat choice or reproductive behaviour) has indeed built up some generalized barrier to gene flow between the European lakes and streams, but that time has been too short for drift and mutation to substantially shift neutral marker frequencies between the habitats. Indeed, a similarly high genetic differentiation (based on seven microsatellites) as in our Canadian watersheds has been reported from *older* (thousands of years) parapatric lake and stream stickleback in Europe (Northern Germany; Reusch *et al.* 2001). We explored the possibility of time constraints to neutral genetic differentiation in the European fish in an *ad hoc* analysis using individual-based simulation. Specifically, we modelled divergence in population pairs under drift, mutation and different migration schemes over 150 generations, the approximate upper age limit of the European study populations. The simulations were carried out using both EASYPOP v1.7 (Balloux 2001), and an own algorithm for R (R Development Core Team 2009) that explicitly considered the microsatellite diversity observed in the Lake Constance population. Both approaches revealed that 150 generations of divergence are unlikely to be sufficient for driving substantial genetic differentiation between lake and stream populations even in the presence of very strong reproductive barriers (the simulation methods and results are presented in detail as supporting online Appendix S1). Our simulations and empirical analyses together therefore support more extensive recent simulation studies

arguing that neutral markers are poorly suited for informing on the existence and strength of reproductive barriers if they emerge very rapidly (Thibert-Plante & Hendry 2009, 2010; Labonne & Hendry 2010). Exploring these early stages of speciation therefore requires the direct quantification of components of reproductive isolation, such as selection against migrants or reduced dispersal between habitats, using experimental approaches.

Conclusions

Our study comparing young lake-stream stickleback from Europe to populations residing in Canadian watersheds has provided evidence for some adaptive divergence in morphology that is nevertheless much lower than would be expected to be fully adaptive. European lake-stream pairs also did not exhibit the strong differentiation in neutral genomic regions predicted from Canadian populations. Taken together, our analysis suggests weak progress towards complete speciation in the European fish. We propose that limited time and genetic variation might presently preclude the strong adaptive divergence required for generalized reproductive barriers to emerge. Alternatively, such barriers may already exist but not yet be detectable by neutral markers. While the present data do not allow us to infer the relative importance of time and genetic constraints on divergence, our work does implicate these factors as determinants of progress towards complete speciation. This insight was possible only through a comparative analysis of adaptive divergence replicated in nature. Future work involving QTL mapping, time series and the direct quantification of reproductive barriers in lake-stream stickleback on both continents promises to shed further light on mechanisms acting early in the origin of species.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1 Individual-based simulations exploring neutral genetic divergence expected for the young European Stickle back pairs.

Fig. S1 Map of Switzerland indicating the localities in the Lake Constance and Lake Geneva watersheds where the six European stickleback samples were taken.

Fig. S2 Sexual dimorphism in stickleback body shape.

Table S1 Primers and cycling conditions used for the PCR-amplification of the stickleback *Eda* gene

Table S2 Statistical tables for the tests performed for gill raker number, gill raker length and geometric morphometric body shape

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Appendix 1

Variation in stickleback body shape along the three dominant relative warps (RWs), together capturing 60% of the total shape variation (RW1: 27%, RW2: 22%, RW3: 11%; all subsequent RWs captured less than 8.6% variation and are not presented). The deformation grids visualize the lowest and highest observed RW scores in the data set (RW1: −0.0530/0.0598, RW2: −0.0372/0.0489, RW3: −0.0396/0.0322). Sample means and associated 95% confidence intervals are shown separately for males and females. Black and grey bars indicate lake and stream fish. Codes for the European pairs (labelled in black) are given in Table 1; the Canadian pairs (grey labels) are Beaver (BE), Boot (BO), Joe's (JO), Pye (PY), and Robert's (RO). Note that RW1 and RW3 reflect primarily habitat-related shape shifts (weak in the European pairs), whereas RW2 is strongly driven by sexual dimorphism. Also, RW1 and RW2 display strong differences between the continents.