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Parallel and nonparallel ecological, morphological and genetic divergence in lake–stream stickleback from a single catchment

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Abstract

Parallel phenotypic evolution in similar environments has been well studied in evolutionary biology; however, comparatively little is known about the influence of determinism and historical contingency on the nature, extent and generality of this divergence. Taking advantage of a novel system containing multiple lake–stream stickleback populations, we examined the extent of ecological, morphological and genetic divergence between three-spined stickleback present in parapatric environments. Consistent with other lake–stream studies, we found a shift towards a deeper body and shorter gill rakers in stream fish. Morphological shifts were concurrent with changes in diet, indicated by both stable isotope and stomach contents analysis. Performing a multivariate test for shared and unique components of evolutionary response to the distance gradient from the lake, we found a strong signature of parallel adaptation. Nonparallel divergence was also present, attributable mainly to differences between river locations. We additionally found evidence of genetic substructuring across five lake–stream transitions, indicating that some level of reproductive isolation occurs between populations in these habitats. Strong correlations between pairwise measures of morphological, ecological and genetic distance between lake and stream populations supports the hypothesis that divergent natural selection between habitats drives adaptive divergence and reproductive isolation. Lake–stream stickleback divergence in Lough Neagh provides evidence for the deterministic role of selection and supports the hypothesis that parallel selection in similar environments may initiate parallel speciation.

Introduction

In the last two decades, evolutionary biology has shifted towards a greater scrutiny of the roles that natural selection, drift and gene flow play in speciation (Schluter, 2009). Natural selection is fundamental to ecological speciation where divergent selection between differing environments drives local adaptation (Schluter, 2000; Rundle & Nosil, 2005). In turn, adaptive divergence can lead to reproductive isolation due to evolutionary trade-offs (Schluter, 1993, 1995) and selection against both hybrids and migrants between populations inhabiting contrasting environments (Nosil *et al.*, 2005; Rundle & Nosil, 2005). Despite being central to the concept of ecological speciation, demonstrating that selection has played a role in divergence is notoriously difficult (Kingsolver *et al.*, 2001; Räsänen & Hendry, 2008; Hendry, 2009). However, the repeated evolution of similar phenotypes in evolutionary-independent populations inhabiting similar environments provides convincing evidence of the deterministic nature of selection in speciation (Schluter & Nagel, 1995; Losos *et al.*, 1998; Rundle *et al.*, 2000).

Repeated, parallel evolution of morphologically and ecologically adaptive traits is a defining characteristic of the three-spined stickleback (*Gasterosteus aculeatus* L.) species complex (Bell & Foster, 1994; McKinnon &

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Rundle, 2002; Hendry *et al.*, 2009). Although numerous examples of parallel divergence between stickleback species pairs exist, many are confined to restricted geographical areas (see Hendry *et al.*, 2009; for a review). In contrast, morphological, ecological and genetic divergence between parapatric lake–stream sticklebacks is widespread, having been reported in North America (Lavin & McPhail, 1993; Thompson *et al.*, 1997; Hendry *et al.*, 2002; Hendry & Taylor, 2004) and Europe (Reusch *et al.*, 2001; Berner *et al.*, 2010). The gradient between abutting lake and stream environments is well marked by shifts from pelagic (lake) towards benthic (river) prey resources and changes in environmental factors such as flow regime (Hendry *et al.*, 2002; Berner *et al.*, 2008). As a result, adaptation to these environments results in divergent ecoytpes; lake fish typically exhibit a shallow body depth (BD) and possess longer and more numerous gill rakers; conversely, stream fish are characteristically relatively deeper-bodied and have fewer, shorter gill rakers (Hendry & Taylor, 2004; Berner *et al.*, 2009; Kaueffer *et al.*, 2012).

The spatial structuring of parapatric populations across environmental gradients can potentially increase the rate of divergence in speciation as adaptive divergence leads to a reduction of gene flow (Schilthuizen, 2000; Doebeli & Dieckmann, 2003). The lake–stream model is well suited to closely examining the relationship between adaptation and gene flow; for example, strong phenotypic and ecological divergence is typically correlated with greater neutral genetic differentiation between populations (Hendry & Taylor, 2004; Berner *et al.*, 2009; Kaueffer *et al.*, 2012). Furthermore, experimental evidence has demonstrated that divergent adaptive traits are heritable (Sharpe *et al.*, 2008; Berner *et al.*, 2011) and likely influence fitness (Schluter, 1993; Robinson, 2000; Hendry *et al.*, 2011). Alternatively, quantitative estimates indicate that migration between environments might constrain morphological divergence by as much as approximately 90% (Moore *et al.*, 2007). Thus, although it is difficult to disentangle the extent of cause and effect in terms of adaptation and gene flow, the two processes almost certainly interact (Räsänen & Hendry, 2008).

To date, the majority of comparative studies on parallel lake–stream sticklebacks has focused on populations in British Columbia, Haida Gwaii, Canada and Alaska (Moodie, 1972; Lavin & McPhail, 1993; Hendry & Taylor, 2004; Berner *et al.*, 2008; Aguirre, 2009; Kaueffer *et al.*, 2012), although divergence has also been reported in Europe (Reusch *et al.*, 2001; Berner *et al.*, 2010). It should be noted that some authors prefer the use of the term 'convergent evolution' to 'parallel' (Arendt & Reznick, 2007; Losos, 2011). We feel that the inclusion of parallel is supported in this case as stickleback adaptation has likely arisen from selection on standing genetic variation leading to similar alleles underlying similar phenotypes (Colosimo *et al.*, 2005; Jones *et al.*, 2012). Furthermore, the lack of such variation may potentially constrain parallel evolution in lake–stream systems.

Comparing Canadian and geologically young European lake–stream populations, Berner *et al.* (2010) found the latter had weaker morphological and genetic divergence. The authors suggested that time and genomic constraints – specifically the lack of allelic variants for lake–stream adaptation – might restrict parallel lake–stream divergence in European populations. Although a mid-Pleistocene split (approximately 250 ka BP) between Atlantic and Pacific stickleback clades (Orti *et al.*, 1994; Mäkinen & Merilä, 2008) indicates that historical contingency operating at the genomic level might explain this weaker divergence, Berner *et al.* (2010) examined only very young populations (<150 years) from Central Europe, which were established by human introduction (Lucek *et al.*, 2010).

Studies from evolutionarily older populations in Europe are therefore necessary to clarify the extent of time and genomic constraints on lake–stream divergence in this region. Lough Neagh is the largest lake in the UK and Ireland (383 km^2) and drains a catchment covering approximately 45% of Northern Ireland (Carter, 1993). The catchment is characterized by nine main river systems directly connected to a single lake (see Fig. 1a), and sticklebacks are present throughout. Ireland was rapidly recolonized by sticklebacks following the last glacial maximum (approximately 17 ka BP, M. Ravinet, unpublished data). Populations in Lough Neagh were likely established rapidly following ice retreat in this region (approximately 14–12 ka BP), and thus, studies of stickleback populations in the Lough Neagh catchment can help assess whether lake–stream divergence is constrained in Europe. Furthermore, the unusual configuration of the catchment provides an excellent opportunity to test whether patterns of molecular, ecological and morphological divergence can occur in parallel within a large-scale system, as well as to contribute to our understanding of the generality of lake–stream divergence within the distribution of the species (Schluter & Nagel, 1995; McKinnon & Rundle, 2002).

Given the novel features of Lough Neagh, our study attempted to investigate (i) whether divergent lake–stream stickleback populations could be identified within the Lough Neagh system using morphological, ecological and genetic analysis; (ii) whether multiple patterns of lake–stream divergence occur in a single catchment; (iii) whether the direction and magnitude of divergence is consistent between populations, that is, parallelism and nonparallelism; (iv) whether patterns of adaptive divergence correlated with divergence in selection and decreased gene flow and (v) how divergent are Lough Neagh lake–stream populations in comparison with other European and Canadian systems?

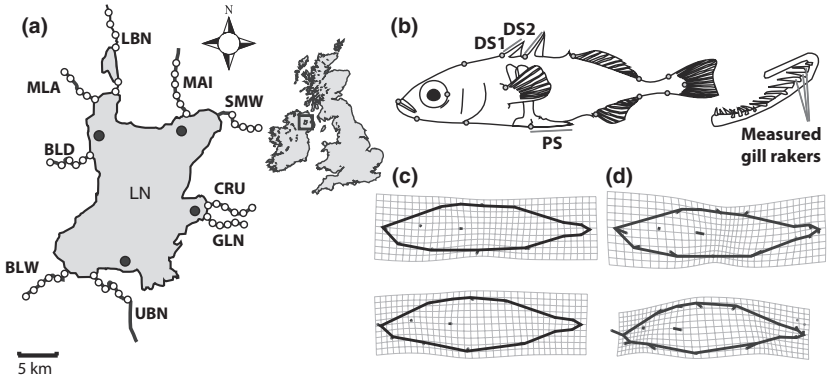

Fig. 1 (a) Map of the Lough Neagh catchment and sample sites for three-spined sticklebacks, black and white circles denote lake and river samples, respectively. Location codes are as follows BLD, Ballinderry; BLW, Blackwater; CRU, Crumlin; GLN, Glenavy; LBN, Lower Bann; LN, Lough Neagh; MAI, Maine; MLA, Moyola; SMW, Sixmilewater; UBN, Upper Bann. N.B. – two sites from further along the Lower Bann are not shown for ease of presentation; (b) Schematic diagram of three-spined stickleback indicating geometric morphometric landmark positions (grey circles) and linear body and gill raker measurements; DS1, 1st dorsal spine; DS2, 2nd dorsal spine; PS, pelvic spine. Deformation grids showing major shape difference along (c) DF_{SHAPE} (scaled to 4.0) and (d) PC_{SHAPE} (scaled to 0.1). In both cases, upper grid shows typical lake and lower river fish shape, and lower grid shows upper river fish.

Materials and methods

Sample collection

Three-spined sticklebacks were collected from across the Lough Neagh catchment (Fig. 1a) between April and May 2010 using unbaited minnow traps. Fish were sampled from four lake sites and, following Berner *et al.* (2009), at five to six sites clinally along each afferent ($n = 8$) and efferent ($n = 1$) river with increasing distance from the lake (total sites, $n = 58$). Sampling sites at equal distances along each river was not possible due to restrictions, and therefore, distance between sites varies between river locations (see Table S1). At each sample site, six traps were deployed for 6–12 h, and upon retrieval, fish were pooled and subsampled randomly, avoiding only gravid females; fish were then euthanized using an overdose of clove oil and immediately placed in 95% ethanol. At each site, an effort was made to capture 30 individuals, although this was not always possible (mean individuals per site, $n = 28$). In addition to stickleback collection, a standardized 3 min kick sample (mesh size = 1 mm, Gordon *et al.*, 1992) was carried out at each site to collect putative prey for isotopic mixing models and a baseline for trophic position estimation (see Stable isotope and dietary analysis).

Shape and trait measurements

Geometric morphometrics was performed on ethanol-preserved individuals to capture body shape variation across the Lough Neagh catchment. Sticklebacks (total = 1502, approximately 30 individuals per site) were pinned out and photographed on the left flank using a CANON EOS 1000D Digital SLR camera with a macro lens and the CANON EOS utility remote operating software. Using tpsDig2 (Rohlf, 2010), 16 landmarks were placed on each image (Fig. 1b), and a Procrustes fit was performed using MorphoJ (Klingenberg, 2011). To correct for allometric shape variation, regression parameters were estimated within groups (with river location as a grouping variable) using multivariate regression of Procrustes coordinates against centroid size. The common within-group relationship was estimated to account for between-group differences in allometry (Reist, 1986; McCoy *et al.*, 2006; Berner, 2011).

Principal components analysis (PCA) of Procrustes values indicated the first two PC axes explained a considerable proportion of shape variance (45.8%), and inspection of the transformation grids suggested that specimen bending caused the greatest variation between individuals. Following Valentin *et al.* (2008), we performed a discriminant function analysis (DFA) on the PC scores using habitat as a grouping variable. The loading coefficient of the first principal shape component (−0.02) on DF1 was low compared with PC2 (0.46, herein PC_{SHAPE}), confirming that bending masked true shape variation. We therefore performed a second DFA on the Procrustes residuals using habitat as a grouping variable to better characterize shape variation.

Trophic and anti-predator traits were also measured ($n = 1489$, approximately 30 individuals per site). The number of lateral armour plates was recorded on the left and right sides of each individual under a dissecting microscope. Gill rakers are bony protrusions from the gill arches that facilitate prey handling and are an important trophic structure (Schluter & McPhail, 1993).

Therefore, gill rakers on the first left branchial arch were counted (Gill raker number, GRN), and then the three largest rakers occurring after the joint between the upper and lower gill limbs were measured (Fig. 1b). Mean gill raker length (GRL) was then calculated from these measurements. Additional measurements of anti-predator traits; 1st dorsal spine (DS1), 2nd dorsal spine (DS2) and the pelvic spine (PS), as well as linear measurements of standard length and BD were taken from digital photographs using ImageJ (Abramoff *et al.*, 2004; see Fig. 1b). All linear trait measurements were size-standardized prior to analysis (see Statistical analysis).

Stable isotope and dietary analysis

Foraging morphology in sticklebacks is strongly correlated with diet and fitness (Schluter & McPhail, 1993; Robinson, 2000; Berner *et al.*, 2008; Matthews *et al.*, 2010). Divergent diets are commonly considered as a proxy for divergent selection acting on foraging traits (Berner *et al.*, 2008). Inferring fish diet is typically carried out using stomach contents analysis (SCA). Although straightforward and inexpensive, SCA can only provide a dietary snapshot and may be biased towards unassimilated or indigestible prey items (Schindler *et al.*, 2004; Grey, 2006). In contrast, stable isotope analysis (SIA) using carbon and nitrogen provides a time-averaged signal of dietary assimilation; for example, in fish muscle, C and N values can reflect diet over approximately 6 months (Hesslein *et al.*, 1993; Grey, 2000; Perga & Gerdeaux, 2005). SIA therefore has the potential to act as an indicator of both habitat use and diet (Harrod *et al.*, 2005, 2010), important factors in promoting divergence between parapatric lake–stream stickleback populations (Berner *et al.*, 2008; Bolnick *et al.*, 2009). Both methods have previously been used successfully to demonstrate divergent selection between lake–stream stickleback populations (Berner *et al.*, 2008; Kaueffer *et al.*, 2012).

For three-spined sticklebacks in the Lough Neagh catchment, five individuals from each site were used for SIA (total $n = 298$). A section of anterior dorsal muscle was dissected from each fish and dried for 48 h at 60 °C, before being ground to a fine powder and weighed out into tin capsules. In addition to fish analysis, approximately five individual representative benthic macroinvertebrates from each site (total $n = 233$) were similarly processed. Samples were then analysed for δ^{13}C, δ^{15}N,% C and% N on a Carlo Erba Elemental Analyser and a Thermo Finnigan Delta Plus XL mass spectrometer at the Duke Environmental Isotope Analysis laboratory (DEVIL) at Duke University, Durham, NC, USA.

Failure to consider potential biases causing variation in isotopic values when performing SIA can lead to inaccurate conclusions (Gannes *et al.*, 1997). For example, lipids are ^{13}C-depleted, and varying lipid content between individual fish can complicate estimates of carbon source contribution (Kiljunen *et al.*, 2006). We therefore lipid-corrected stickleback δ^{13}C values following Kiljunen *et al.* (2006). We used mean trophic enrichment factor (TEF) values of 1.6‰ (± 0.6) for δ^{13}C and 4.1 ± 1.4‰ δ^{15}N based on a previous study of tropic fractionation in three-spined sticklebacks (J. Grey, Queen Mary University of London, unpublished data), providing the most realistic estimates of trophic position and source contribution in a sensitivity analysis (M. Ravinet, unpublished data).

Baseline-corrected consumer δ^{15}N values can provide estimates of trophic position (Vander Zanden & Rasmussen, 1999; Post, 2002). As such, trophic position (T_{pos}) estimates were calculated using the two-end mixing model equations following Post (2002). We also estimated the contribution of lake and stream prey to diet using Stable Isotope Analysis in R (SIAR), a linear mixing model which uses Bayesian inference to account for variation and uncertainty in both source contributions and TEFs (Parnell *et al.*, 2010). Here we estimated the proportional contribution of lake vs. river prey sources to individual fish sampled from across the catchment. We used three end-member sources in the model: lake zooplankton, lake benthic macroinvertebrates and river benthic macroinvertebrates – all of which are putative prey for sticklebacks (Hynes, 1950). Given that benthic macroinvertebrates differed in isotopic values between rivers (see Results), river-specific values were used in analyses of river fish. For lake fish, a global analysis was conducted using the mean values of all river invertebrates.

The utility of SIA is greatly increased through the parallel use of SCA (Harrod *et al.*, 2010; Kahilainen *et al.*, 2011) conducted on the same individuals used for SIA. Stomachs were dissected in 70% ethanol under a binocular microscope, and proportion of stomach contents was recorded using the points method (Hynes, 1950). Stomach contents were classified as either benthic (Gammarids, Trichoptera, Simulidae, Chironomidae, Asellus, Coleoptera, Notonectidae, Baetidae, Nematoda, Unionidae, Gastropoda, Tipulidae, Hemiptera, Ostracoda, Odonata), limnetic (Cladocera, *Chaoborus* larvae) or other (digested matter, fish eggs). We then calculated the percentage proportion of each prey category as a percentage of total stomach fullness (Schluter & McPhail, 1992; Berner *et al.*, 2008; Kaueffer *et al.*, 2012).

Parallelism, determinism and nonparallelism

By examining evolutionary responses of populations to a common environmental gradient, it is possible to quantify both the parallel and nonparallel aspects of adaptation (Langerhans & DeWitt, 2004; Berner *et al.*, 2010; Kaueffer *et al.*, 2012). To do so, we used the multivariate analysis of variance (MANCOVA) approach

outlined by Langerhans & DeWitt (2004) on body shape (size-corrected Procrustes coordinates), gill raker morphology (GRL, GRN), anti-predator morphology (DS1, DS2, PS and mean lateral plate number) and stable isotope values (δ^{13}C, δ^{15}N). Briefly, the approach involves using a MANCOVA model with distance and river location as factors in addition to a distance × location interaction. The orthogonal axes produced by this approach can then estimate the effect sizes of parallel and nonparallel divergence. We conducted two sets of MANCOVA analyses, one with all rivers and one with variant rivers only. For each model, we calculated the Wilk's η^2, a percentage value measuring the proportion of explained variance (Langerhans & DeWitt, 2004). The first factor in each model represents the shared evolutionary response to distance along each river from the lake, thus representing parallel adaptation. The second factor therefore represents differences in adaptation as a result of location and can thus be interpreted as the deterministic influence of different river environments. Finally, the interaction term represents the idiosyncratic response of populations to the distance gradient, that is, nonparallel adaptation.

Statistical analysis

For all analyses, individual rivers were considered as locations (river location herein), whereas sample sites along rivers were considered as individual sites. Habitat classes – lake, lower river and upper river – were also used to aid interpretation; the first site on each river was considered to be lake habitat, the second two were classed as lower river and finally the last three sites were categorized as upper river habitat (see Table S1).

To account for the effects of body size on measured morphological traits, an ANCOVA-based size correction method was applied to each linear trait (Reist, 1986; McCoy *et al.*, 2006; Berner, 2011). To account for the high correlation between linear anti-predator traits (i.e. DS1, DS2 and PS), a PCA was performed to summarize the variation, and the major principle component (PC_{AP}) was used in subsequent analyses. When analysed separately, anti-predator traits produced identical results (data not shown).

For morphological traits (shape principal components, PC_{AP}, lateral plate number, GRL, gill raker number), generalized linear mixed models (GLMMs) with site as a random effect were used to examine variation in traits between habitat classes. To ensure the most appropriate model was applied, nested models were compared using Akaike Information Criteria and ANOVA (Zuur *et al.*, 2007). Stable isotope values were examined using GLMMs and MANOVA. Generalized linear models (GLMs) were applied to three end-member proportion data modelled in SIAR, with a quasi-binomial distribution. All statistical analyses were carried out using R 2.14.1 (R Development Core Team, 2012).

Population structuring and genetic differentiation

Genetic population structure has been previously observed between lake–stream sticklebacks (Hendry & Taylor, 2004; Berner *et al.*, 2009; Kaueffer *et al.*, 2012) and is commonly correlated with measures of adaptive divergence. To detect whether population structure existed in the Lough Neagh catchment, we used nine microsatellite markers. DNA was first extracted from caudal fin clips following a salt extraction method (Aljanabi & Martinez, 1997). A total of 538 samples (approximately 10 individuals per site) were then screened for the microsatellite loci in two multiplex reactions. PCR amplification was carried out using Top-Bio PPP mastermix (Top Bio, Prague, Czech Republic); total reaction volume was 3.5 μL with 1.5 μL mastermix, 1 μL template DNA (1–5 ng) and 0.035 μL (10 pM) of each primer with the remainder volume made up with ddH_20. Identical thermocycler conditions were used for both multiplexes, 110 °C heated lid, denaturation at 95 °C for 15 min and then 20 cycles of 95 °C for 30 s, 57 °C for 1.5 min and 72 °C for 1.5 min, with a final extension of 60 °C for 30 min. Genetic screening was then performed on a 96 capillary 3730xl DNA Analyzer (Applied Biosystems Inc). Raw fragment profiles for each individual were then manually genotyped using GENEMAPPER v4.1 (Applied Biosystems Inc., Foster City, CA, USA).

Deviance from Hardy–Weingberg Equilibrium (HWE) was tested at two hierarchical levels using ARLEQUIN 3.5.1.2 (Excoffier & Schneider, 2005) using sequential Bonferroni corrections and the Benjamini and Yekutieli (2001) False Discovery Rate (FDR) Method (Rice, 1989; Narum, 2006). Individuals were first pooled and tested as a single panmictic population before being split into river location and retested. Pairwise F_{ST} values were estimated between upper river, lower river and lake populations using ARLEQUIN 3.5.1.2. As F_{ST} may not always accurately reflect genetic differentiation for microsatellite data (Hedrick, 2005a; Jost, 2008), we also calculated pairwise values of Jost's D (Jost, 2008) in SMOGD (Crawford, 2010).

To detect population structure along lake–stream transitions, we used STRUCTURE 2.3.3 (Pritchard *et al.*, 2000). Data sets consisted of individuals sampled in a given river location and all individuals sampled from the lake. Ten runs of k 1–7 were conducted using a burn-in of 100 000 and a subsequent 100 000 Monte Carlo Markov Chain (MCMC) iterations. Independent runs were grouped using CLUMPP (Jakobsson & Rosenberg, 2007), and the most probable k was assessed using the log-likelihood values and the Evanno *et al.* (2005) method.

Isolation by adaptation

The extent of adaptive and genetic divergence can increase with the strength of divergent selection

between environments (Hendry & Taylor, 2004; Renaut *et al.*, 2010). Correlations between phenotypic and neutral genetic differentiation can therefore indicate a pattern of isolation by adaptation (IBA), thus suggesting selection has a role in divergence and that adaptive differences between populations contribute to reproductive isolation (Nosil *et al.*, 2008). To test the IBA hypothesis in Lough Neagh, we calculated six matrices of pairwise distance measures (see Table 1). In addition to divergence measures, both geographical distance (i.e. measured along river courses) and difference in elevation between sites were included as potential explanatory variables for phenotypic, ecological and genetic differentiation. Euclidean distance for site means for ecological and phenotypic distances were first calculated and then tested using partial and pairwise Mantel tests (Storz, 2002; Rosenblum *et al.*, 2007) implemented in the VEGAN package for R (Oksanen *et al.*, 2011). Significance values for both pairwise and partial tests were estimated using 1000 permutations of the test matrices. All ecological, morphological and microsatellite data are available on the Dryad Digital Repository (doi:10.5061/dryad.bn43b).

Comparative lake–stream divergence

The relatively lower levels of phenotypic and genetic divergence observed in European lake–stream compared to those from British Columbia may reflect both time and genomic constraints (Berner *et al.*, 2010). Examining lake–stream divergence in a comparative context with other populations can provide insight as to whether such constraints act in the Lough Neagh system. We first chose two lake–stream comparative studies examining multiple lake–stream systems in Central Europe (Berner *et al.*, 2010) and British Columbia (Kaueffer *et al.*, 2012). F_{ST} values were collated from the summary tables present in both studies, and morphological data sets were downloaded from the Dryad Digital Repository (doi: 10.5061/dryad.1960 and doi: 10.5061/dryad.6vq04). We then selected two divergent adaptive morphological traits (gill raker number and GRL) measured in these systems and our own. To calculate divergence in body shape, we used a pooled shape data set consisting of populations used by Berner *et al.* (2010) and individuals from Lough Neagh. We then performed a common PCA on shape differences, extracting the PC explaining the greatest variation. We additionally used these populations to calculate the size-corrected BD (inter-landmark distance between landmark placed on the insertion of the DS1 and that at the insertion of the PS to the pelvic girdle; anterior BD in Berner *et al.*, 2008). Using a pooled data set, we calculated an indicator of phenotypic divergence, P_{ST} (Spitze, 1993; Storz, 2002) following the method used by Kaueffer *et al.* (2012). P_{ST} is analogous to F_{ST} and provides a unitless measure of divergence that can be compared between different measurement units and populations (Kaueffer *et al.*, 2012); in this case, it provided an unbiased measure of divergence between lake–stream pairs suitable for comparisons across evolutionary-independent systems. We additionally examined whether patterns of phenotypic divergence were correlated with genetic divergence between lake–stream pairs.

Table 1 Description of pairwise matrices used in Mantel tests.

Matrix	Description
Physical distance	River distance measured in km
Elevation distance	Difference in elevation measured in m above sea level
Ecological distance	Euclidean distance between site centroids for $\delta^{13}C$ and $\delta^{15}N$ values
Shape distance	Euclidean distance between mean site DF_{SHAPE} values
Gill raker distance	Euclidean distance between mean site gill raker lengths
Genetic distance	Pairwise linearized F_{ST} values between sites

Results

Body shape

A DFA on Procrustes coordinates produced an axis (DF_{SHAPE} herein) that accounted for 76.5% of the variance between habitat groups (Fig. 1c), and MANOVA confirmed this was highly significant (Wilk's $\lambda = 0.82$, $F_{2,1499} = 4.75$, $P < 0.0001$). GLMMs revealed this was also the case with PC_{SHAPE} ($R^2 = 0.15$, $F_{2,42} = 7.55$, $P = 0.0016$).

Both PC_{SHAPE} and DF_{SHAPE} indicated a subtle deepening of the body, shortening of the caudal peduncle and shortening of the snout in fish from the upper river (Fig. 1c,d; snout change remained even when testing sexes separately – see Fig. S1). With site treated as a random effect for the entire catchment-level data set, DF_{SHAPE} varied significantly with distance from the lake ($F_{1,1367} = 15.79$, $P = 0.0001$) and location ($F_{8,1367} = 13.59$, $P < 0.0001$); furthermore, a significant interaction term indicated that the relationship between distance and shape varied between rivers ($F_{8,1367} = 13.56$, $P < 0.0001$; overall model $R^2 = 0.40$). Within-river models revealed shape changed significantly with distance in six rivers ($P < 0.0001$, see Table S2), but no relationship was present in either the afferent Rivers Blackwater ($P = 0.290$) and Upper Bann ($P = 0.126$) or the efferent Lower Bann River ($P = 0.439$). All rivers demonstrating significant shape change had a positive slope coefficient, indicating a shift towards a deeper body shape with increasing geographical distance from the lake.

Foraging and anti-predator morphology

Across the catchment, mean GRL varied significantly with both distance from the lake ($F_{1,1341} = 5.30$,

$P = 0.022$) and between river locations ($F_{8,1341} = 4.38$, $P < 0.0001$); furthermore, the relationship with distance varied between rivers ($F_{8,1341} = 10.77$, $P < 0.0001$, overall model $R^2 = 0.16$). Within six of the nine rivers, mean GRL decreased significantly with distance from the lake ($P < 0.001$ in all cases). Notably, this pattern also occurred in the efferent river, the Lower Bann (see Table S3). As with body shape, GRL showed no change over distance in the Blackwater ($P = 0.852$) and Upper Bann ($P = 0.295$). No such shift was seen in the Maine ($P = 0.286$, see Table S3). Although GLMM analysis of gill raker number showed a similar pattern of significant relationships, the overall model explained a low proportion of the variance (distance: $F_{1,1341} = 7.45$ $P = 0.006$; location: $F_{8,1341} = 62.40$, $P < 0.0001$; distance × location: $F_{8,1341} = 21.55$, $P < 0.0001$, overall model $R^2 = 0.06$).

Variation in lateral plate numbers was considerable (1–16); model selection revealed that mean lateral plate number differed significantly only between river locations (GLM: $F_{8,1382} = 3.84$, $R^2 = 0.02$, $P = 0.0002$). As no obvious trend in plate morphology could be detected between habitat types, it was not considered further. In contrast, other features of anti-predator morphology showed consistent patterns of variation across Lough Neagh. PCA on anti-predator traits (DS1, DS2, PS) produced a single axis (PC_{AP}) explaining 85.8% of the variance. Loadings and correlation coefficients from the PCA indicated that all three traits contributed strongly to PC_{AP} and that variation along this axis represents a decrease in standardized trait length (Table 2). Across the catchment, PC_{AP} varied significantly with distance ($F_{1,1366} = 12.12$, $P < 0.0001$) and between river locations ($F_{8,1366} = 17.43$, $P < 0.0001$). Furthermore, as with other traits, there was a significant interaction between distance and location ($F_{8,1366} = 14.18$, $P < 0.0001$, Overall $R^2 = 0.24$). Within-river local GLMs confirmed a decrease in standardized anti-predator trait length with increasing distance from the lake ($P < 0.0001$) except in the Blackwater, Lower Bann and Upper Bann ($P > 0.05$ in all cases, see Table S3).

Stable isotope and dietary analysis

Macroinvertebrate mean $\delta^{13}C$ and $\delta^{15}N$ values differed significantly between both river locations (MANOVA; Wilk's $\lambda = 0.152$, $F_{10,403} = 62.63$, $P < 0.0001$) and habitats (Wilk's $\lambda = 0.922$, $F_{10,403} = 8.32$, $P < 0.0001$). Furthermore, a significant interaction term indicated that the difference between habitats varied between rivers (Wilk's $\lambda = 0.78$, $F_{13,403} = 4.13$, $P < 0.0001$). Examining the output of simple linear models (see Table S4) revealed that mean $\delta^{13}C$ (± SD) differed significantly between the lake (−30.9 ± 2.6‰) and both river habitats (lower: −28.7 ± 2.0‰; upper: −29.0 ± 2.7‰, Tukey HSD, $P < 0.0001$). There was no difference, however, between river habitats ($P = 0.774$). A similar pattern of large differences between lake and river values occurred for $\delta^{15}N$ ($P < 0.0001$), although in this case river environments also tended to differ from one another ($P = 0.049$, mean $\delta^{15}N$ ± SD; lake: 14.9 ± 2.4‰, lower river: 9.5 ± 2.1‰, upper river: 8.7 ± 2.1‰). Within the lake itself, macroinvertebrates and zooplankton were isotopically distinct (MANOVA, Wilk's $\lambda = 0.92$, $F_{1,203} = 9.27$, $P = 0.0001$).

Isotopic values of sticklebacks were highly variable across the catchment, ranging from −32.5‰ to −24.1‰ for $\delta^{13}C$ and between 11.6‰ and 22.8‰ for $\delta^{15}N$. Both isotopic values differed significantly between habitats (MANOVA, Wilk's $\lambda = 0.292$, $F_{2,272} = 14.93$, $P < 0.0001$); notably, stickleback $\delta^{15}N$ values (mean ± SD) for the lake and lower river (21.0 ± 0.3‰ and 21.0 ± 0.7‰, respectively) overlapped but were ^{15}N enriched relative to those from the upper river (16. 8 ± 3. 7‰, Fig 2a). Mean $\delta^{13}C$ and $\delta^{15}N$ values also differed between river locations (Wilk's $\lambda = 0.290$, $F_{9,272} = 25.85$, $P < 0.0001$), and a highly significant interaction term (Wilk's $\lambda = 0.283$, $F_{13,272} = 18.34$, $P < 0.0001$) suggested isotopic shifts between habitats differed between rivers. Global and local models supported the MANOVA output and further demonstrated that stickleback $\delta^{13}C$ and $\delta^{15}N$ values did not change with distance in the Blackwater, Lower Bann or Upper Bann ($P > 0.05$, see Table S5).

Stickleback individual trophic position varied considerably across Lough Neagh: mean trophic position (± SD) differed between habitat types (GLMM: $F_{2,46} = 30.30$, $R^2 = 0.93$, $P < 0.0001$); there was no clear difference between lake (3.64 ± 0.07) and lower river fish (3.62 ± 0.13; $P = 0.586$); however, the upper river (2.91 ± 0.62) differed significantly from both ($P < 0.0001$). Trophic position varied significantly with distance (GLMM: $F_{1,193} = 6.42$, $P = 0.012$); between river locations, trophic position also tended to vary ($F_{8,43} = 1.98$, $P = 0.072$). This was supported by a significant interaction between distance and location ($F_{8,193} = 3.24$, $P = 0.002$, overall $R^2 = 0.92$). Local GLMs (see Table S6) again revealed that the distance had no effect on trophic position for the Blackwater, Lower Bann and Upper Bann. SIAR estimated contributions of sources (lake zooplankton, lake benthic macroinvertebrates and river benthic macroinvertebrates) to stickleback diet all showed a significant relationship with distance from the lake ($P < 0.05$ in all cases, see Table S6), and all GLMMs had a significant interaction term ($P < 0.0001$), suggesting this relationship varied

Table 2 Loadings and correlation coefficients of measured anti-predator traits on PC_{AP}.

Trait	Loadings	Correlation coefficient
1st dorsal spine length	−0.58	−0.93
2nd dorsal spine length	−0.58	−0.94
Pelvic spine length	−0.56	−0.90

between rivers. Local models revealed that contributions of both lake zooplankton and lake benthic macroinvertebrates decreased with distance from the lake, whereas the opposite occurred for percentage contribution of benthic macroinvertebrates ($P < 0.0001$, see Table S6 and Fig. 2b–d). However, the Blackwater, Lower Bann and Upper Bann showed no such relationships. An isotope biplot of global source means and all stickleback $\delta^{13}C$ and $\delta^{15}N$ values also strongly supported the hypothesis that upper river sticklebacks feed primarily on river benthic macroinvertebrates (see Fig. 2a). Stomach content analysis revealed a high proportion of benthic prey in sticklebacks from all habitats; however, lake fish had a higher mean proportion of limnetic prey ($R^2 = 0.12$, $F_{2,227} = 19.86$, $P < 0.0001$, see Fig. 2e).

Parallelism, determinism and nonparallelism

For shape, gill raker morphology, anti-predator morphology and diet, MANCOVA analysis revealed that distance, river location and an interaction between distance and river location were highly significant model terms in variant rivers ($P < 0.001$ in all cases, see Table 3). Similar results were obtained using all rivers in the model (see Table S7). We detected a strong signal of shared divergence for all traits amongst river populations with increasing distance from the lake [greatest partial variance explained (PVE) in all cases, see Table 3 and Fig. 3]. Canonical variates (CV) show a clinal trend of divergence from the lake trait means with increasing distance from the lake (see Fig. 3). However, three river locations remain largely invariant in all cases (Lower Bann, Upper Bann and Blackwater, Fig. 3). CV loadings reveal that shape CV represents a deepening of the body (see Fig. 1c); gill raker CV represents a shortening of mean raker length; anti-predator CV represents a decrease in spine lengths and diet CV represents a shift towards riverine isotope values.

Diet in particular showed a strong parallel response across this gradient (PVE = 62%). For each trait, variation in response occurred between river locations (see Fig. 3). In some cases, this was considerable, for example, location accounted for 21.5% of variance in gill raker morphology within the MANCOVA model. In contrast, anti-predator morphology showed a low level of variation between locations (2.4%). Finally, highly significant interaction terms in each of the MANCOVA models (see Table 3) confirmed that the response to distance differed between river locations and that this could account for some of the phenotypic variation present. For the majority of traits, a nonparallel response explained the lowest proportion of variance, with the exception of diet (52%, see Table 3).

Microsatellite analysis and population structuring

Across the entire catchment, all nine microsatellite loci were out of HWE ($P < 0.05$) following Bonferroni corrections and three following FDR (see Table S8), indicating population substructuring. Grouping individuals by location varied this pattern; for example, the lake population showed no significant deviation at any loci. Deviation was present, however, in rivers such as the

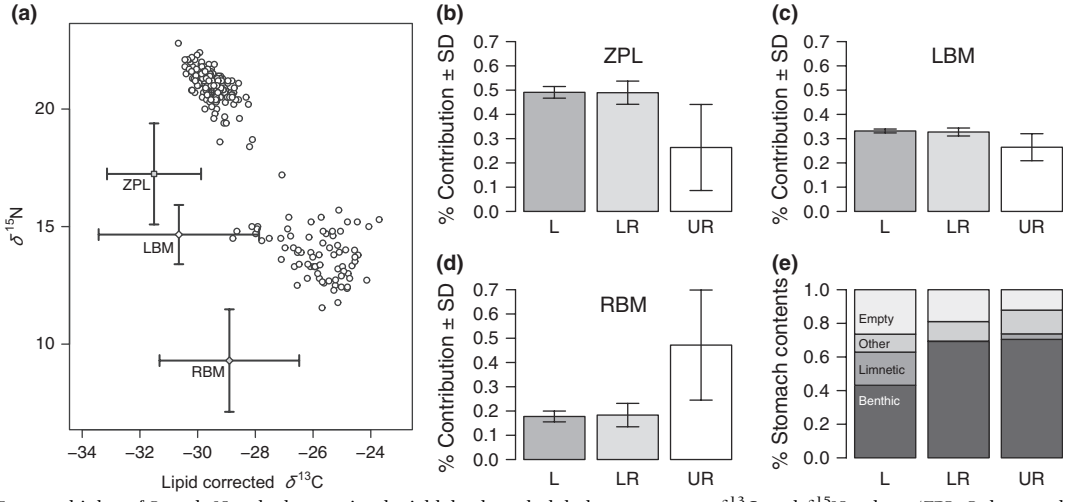

Fig. 2 (a) Isotope biplot of Lough Neagh three-spined stickleback and global source mean $\delta^{13}C$ and $\delta^{15}N$ values (ZPL, Lake zooplankton; LBM, lake benthic macroinvertebrates; RBM, river benthic macroinvertebrates; error bars indicate standard deviation of $\delta^{13}C$ and $\delta^{15}N$ filled circles indicate mean values for prey categories, and open circles indicate stickleback individuals); Mean dietary proportions ± SD of (b) lake zooplankton, (c) lake benthic macroinvertebrates and (d) river benthic macroinvertebrates estimated using Stable Isotope Analysis in R; (e) mean percentage proportion of stomach contents for each habitat class (L, lake; LR, lower river; UR, upper river; error bars indicate standard deviation in all cases).

Table 3 Results of MANCOVA analysis on body shape, gill raker morphology, anti-predator morphology and stable isotope values in variant rivers.

	Wilk's λ	F	d.f.	P	PVE
Shape					
Distance	0.85	7.54	1,32	< 0.0001	15.1
Location	0.36	5.74	8,256	< 0.0001	11.9
Distance × location	0.66	2.25	8,256	< 0.0001	5.1
Gill raker morphology					
Distance	0.62	249.35	1,2	< 0.0001	38.0
Location	0.62	44.39	5,10	< 0.0001	21.5
Distance × location	0.83	15.37	5,10	< 0.0001	8.6
Anti-predator morphology					
Distance	0.77	61.22	1,4	< 0.0001	22.8
Location	0.94	2.51	5,20	0.0002	1.5
Distance × location	0.91	4.01	5,20	< 0.0001	2.4
Diet					
Distance	0.14	144.32	2,4	< 0.0001	62.4
Location	0.27	27.14	6,12	< 0.0001	48.3
Distance × location	0.23	18.79	10,20	< 0.0001	51.9

PVE, partial variance explained, calculated as η^2.
N.B. this value can sum to > 1 (See Materials and methods).

Glenavy, Maine, Moyola and Sixmilewater (see Table S8), again suggesting population substructuring, most probably due to the Wahlund Effect (Hedrick, 2005b).

STRUCTURE analysis revealed no pattern of population structure within the lake itself (see Fig. 4). However, population structure was present across lake–stream transitions in five of the nine river inlets (i.e. greatest support for $k = 2$). The clearest evidence of difference between upper river and lower river populations occurred in the Glenavy and Sixmilewater although less obvious structuring was observed in the Ballinderry, Maine and Moyola. F_{ST} values between the upper and lower parts of each of these rivers were > 0.04 (see Table 4) Glenavy showed the clearest structuring and had the highest levels of divergence ($F_{ST} = 0.08$ and Jost's $D = 0.25$) of all upper and lower river comparisons. The highest differentiation values however occurred between geographically isolated upper river populations, for example, the upper Glenavy and upper Maine ($F_{ST} = 0.14$, Jost's $D = 0.31$). Where no population clustering could be detected across the lake–river transition, pairwise F_{ST} values were low (Blackwater and Upper Bann = 0.00, respectively, Crumlin = 0.02, Lower Bann = 0.01, Table 4).

Isolation by adaptation

Pairwise Mantel tests revealed that no measures of phenotypic, ecological or genetic difference were correlated with geographical distance ($P > 0.05$ in all cases, see Table 5). In contrast, all measures of divergence except for shape ($P = 0.03$) were correlated with elevation after Bonferroni corrections ($P < 0.013$). Tests between divergence measures also remained significant following corrections, indicating that correlations between ecological and adaptive divergence measures were high (see Table 5). In particular, a high correlation was present between shape and gill raker distance ($r = 0.927$, $P = 0.001$). When controlling for neutral genetic differentiation, partial Mantel tests demonstrated that both

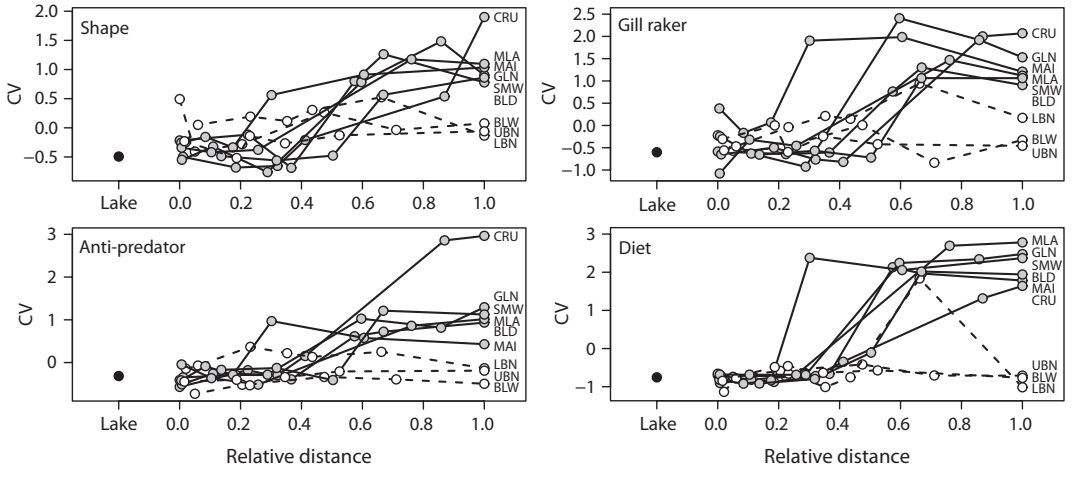

Fig. 3 Clinal trends in mean canonical variates of body shape, anti-predator traits, gill raker morphology and diet with increasing distance from the river. Black circles indicate mean value for the lake population, white circles indicate variant river populations (i.e. where a trait varies significantly with distance in local and global generalized linear modelss) and dark grey circles denote invariant rivers. Note, distance is plotted as relative distance from the lake to aid visualization. River location codes are as follows; BLD, Ballinderry; BLW, Blackwater; CRU, Crumlin; GLN, Glenavy; LN, Lough Neagh (lake); LBN, Lower Bann; MAI, Maine; MLA, Moyola; SMW, Sixmilewater; UBN, Upper Bann.

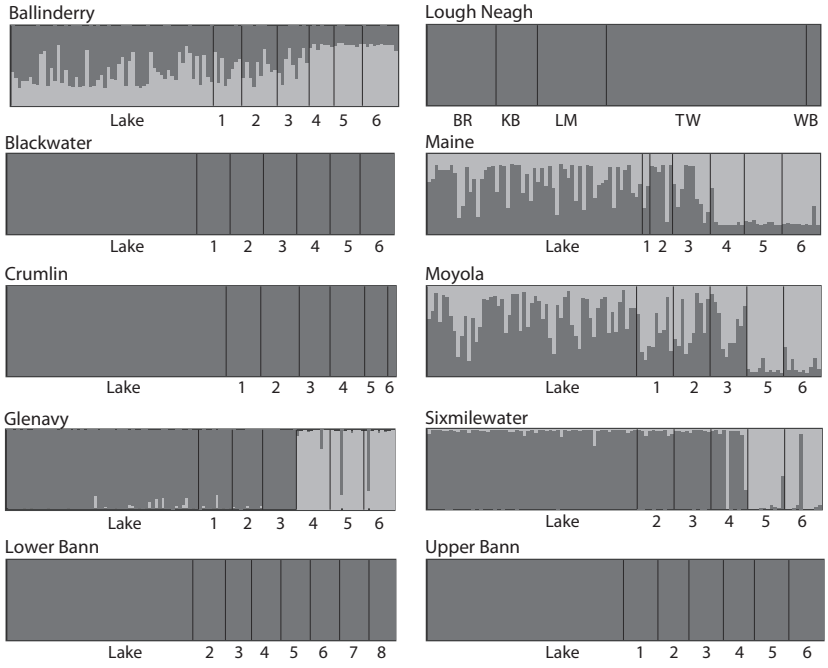

Fig. 4 STRUCTURE plots per river location (max possible $k = 2$, dark grey = lake and lower river cluster, light grey = upper river cluster).

body shape ($r = 0.80$, $P = 0.001$) and gill raker ($r = 0.81$, $P = 0.001$) divergence were strongly correlated with ecological distance. Additionally, ecological, gill raker and shape distance were all strongly positively correlated with F_{ST} values (see Fig. 5).

Comparative lake–stream divergence

Comparative P_{ST} and F_{ST} values revealed that Lough Neagh populations were generally more phenotypically and genetically divergent than those seen in Central Europe, although highest levels of divergence are seen in populations from British Columbia (Table 6). Additionally, although a single Central European population, Constance South exhibited an F_{ST} value comparable to Canadian populations (0.11), P_{ST} values were on a similar scale to the Irish populations (see Table 6, Fig. 6). The most divergent Lough Neagh pairs (Ballinderry, Glenavy, Maine, Moyola and Sixmilewater) exhibited F_{ST} values of 0.04–0.07, comparable to levels seen in the Pye, Robert's and Village Bay systems in Canada (see Fig. 6). However, P_{ST} values for body shape were much higher in the Canadian systems (mean ± SD 046 ± 0.10) than those in the variant Irish systems (0.09 ± 0.08). Divergence in gill raker traits varied considerably between the different regions, for example, similar P_{ST} values for gill raker number were seen in British Columbia and Ireland; however, the highest value occurred in Constance South (0.68, see Fig. 6). Positive correlations between F_{ST} and P_{ST} were found for body shape ($r = 0.89$, t = 7.18, d.f. = 14, $P < 0.0001$), BD ($r = 0.82$, t = 5.28, d.f. = 14, $P = 0.0001$) and gill raker number ($r = 0.51$, t = 2.36, d.f. = 16, $P = 0.031$). No such association was apparent with GRL ($P = 0.158$).

Discussion

Our study reveals the presence of multiple, divergent lake–stream populations of three-spined sticklebacks in the Lough Neagh system. Within the same catchment, we also detected nonvariant river populations, indistinguishable from their lake conspecifics. Our findings suggest that the extent of lake–stream divergence in the Lough Neagh system is comparable to that occurring in some populations from British Columbia. This suggests that other processes besides genomic constraints – that is, absence of allelic variants for lake–stream adaptation in ancestral populations – may hinder lake–stream divergence in European populations. Patterns of trait divergence and differences in diet were generally consistent between the multiple river populations and lake fish in Lough Neagh, and we detected an appreciable pattern of shared divergence with distance from the lake. However, we also detected variation in phenotypes due to differences in river location and how populations responded to lake–river transitions, suggesting a marked nonparallel component. The significant neutral genetic differentiation between some river populations and the lake supports the hypothesis that divergent selection between environments maintains reproductive isolation between parapatric ecotypes.

Table 4 Pairwise F_{ST} (below diagonal) and Jost's D (above diagonal) between lake, lower and upper river sites across the Lough Neagh catchment.

	Lake	BLD_L	BLD_U	BLW_L	BLW_U	CRU_L	CRU_U	GLN_L	GLN_U	LBN_L	LBN_U	MAI_L	MAI_U	MLA_L	MLA_U	SMW_L	SMW_U	UBN_L	UBN_U
Lake		0.00	0.12	0.00	0.00	0.00	0.04	0.00	0.22	0.01	0.00	0.00	0.19	0.00	0.24	0.00	0.17	0.00	0.00
BLD_L	0.00		0.10	0.00	0.00	0.00	0.03	0.00	0.23	0.00	0.00	0.00	0.21	0.00	0.18	0.00	0.13	0.00	0.00
BLD_U	**0.04**	**0.03**		0.16	0.11	0.10	0.24	0.10	0.40	0.15	0.13	0.15	0.26	0.13	0.29	0.09	0.31	0.08	0.15
BLW_L	0.00	0.00	**0.04**		0.00	0.00	0.03	0.01	0.22	0.00	0.00	0.00	0.18	0.00	0.15	0.00	0.11	0.00	0.00
BLW_U	0.00	0.00	**0.03**	0.00		0.02	0.04	0.01	0.24	0.01	0.00	0.01	0.13	0.00	0.18	0.00	0.12	0.00	0.00
CRU_L	0.00	0.00	**0.04**	0.00	**0.01**		0.03	0.00	0.18	0.00	0.01	0.00	0.15	0.02	0.19	0.02	0.12	0.00	0.02
CRU_U	**0.02**	**0.02**	**0.07**	**0.02**	**0.02**	**0.02**		0.07	0.14	0.07	0.02	0.01	0.11	0.01	0.29	0.03	0.11	0.03	0.06
GLN_L	0.00	0.00	**0.04**	0.01	**0.01**	0.00	0.02		0.25	0.02	0.01	0.00	0.19	0.00	0.14	0.02	0.13	0.00	0.01
GLN_U	**0.06**	**0.07**	**0.10**	**0.07**	**0.07**	**0.07**	**0.07**	**0.08**		0.29	0.23	0.23	0.31	0.24	0.45	0.25	0.38	0.21	0.22
LBN_L	0.01	0.00	**0.04**	0.00	0.00	0.01	0.04	0.01	**0.07**		0.01	0.00	0.22	0.02	0.16	0.01	0.14	0.01	0.01
LBN_U	0.00	0.00	**0.04**	0.00	0.00	0.01	0.02	0.01	**0.06**	0.01		0.01	0.18	0.00	0.21	0.00	0.18	0.00	0.00
MAI_L	0.00	0.00	**0.04**	0.00	0.00	0.00	0.02	0.01	**0.07**	0.00	0.01		0.18	0.00	0.25	0.00	0.13	0.00	0.02
MAI_U	**0.05**	**0.06**	**0.09**	**0.05**	**0.05**	**0.06**	**0.05**	**0.06**	**0.10**	**0.06**	**0.05**	**0.06**		0.13	0.38	0.22	0.20	0.13	0.18
MLA_L	0.00	0.00	**0.04**	0.00	0.00	0.01	0.01	0.00	**0.07**	**0.01**	0.00	0.00	**0.05**		0.16	0.00	0.11	0.00	0.00
MLA_U	**0.07**	**0.06**	**0.09**	**0.05**	**0.05**	**0.06**	**0.09**	**0.05**	**0.14**	**0.05**	**0.06**	**0.07**	**0.11**	**0.05**		0.21	0.23	0.18	0.18
SMW_L	0.00	0.00	**0.03**	0.00	0.00	**0.01**	**0.01**	**0.01**	**0.08**	**0.01**	0.00	0.01	**0.06**	0.00	**0.06**		0.13	0.00	0.00
SMW_U	**0.05**	**0.04**	**0.09**	**0.04**	**0.04**	**0.05**	**0.04**	**0.04**	**0.11**	**0.05**	**0.05**	**0.04**	**0.06**	**0.04**	**0.08**	**0.04**		0.14	0.11
UBN_L	0.00	0.00	**0.03**	0.00	0.00	0.00	0.01	0.00	**0.07**	0.01	0.00	0.00	**0.04**	0.00	**0.05**	0.00	**0.04**		0.00
UBN_U	0.00	−0.01	**0.04**	0.00	0.00	**0.01**	**0.03**	0.01	**0.06**	0.01	0.00	**0.01**	**0.06**	0.00	**0.05**	0.00	**0.04**	0.00	

Emboldened text indicates F_{ST} values significant to $P < 0.05$.

BLD, Ballinderry; BLW, Blackwater; CRU, Crumlin; GLN, Glenavy; LBN, Lower Bann; MAI, Maine; MLA, Moyola; SMW, Sixmilewater; UBN, Upper Bann.

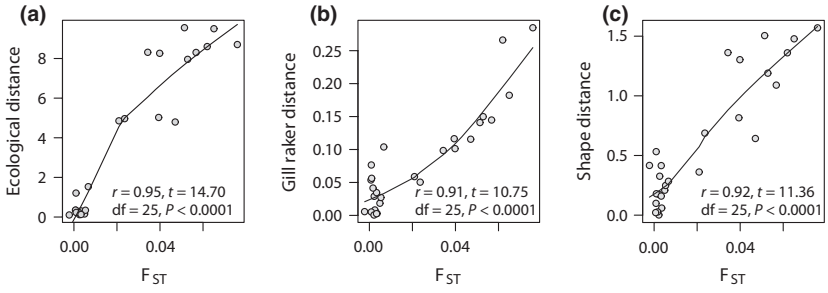

Fig. 5 Correlations between F_{ST} and pairwise distance measures of (a) ecological distance, (b) gill raker distance and (c) shape distance. Individual points are pairwise comparisons between sites for both F_{ST} and distance measurement; lines represent loess smoothing (smoothing parameter = 0.66) to indicate direction of correlation.

Table 5 Pairwise Mantel tests between ecological, morphological and genetic divergence measures.

	r	*P*-value
Geographical distance		
Elevation	0.077	0.228
Ecological	0.020	0.402
Shape	−0.033	0.573
Gill raker	0.034	0.353
F_{ST}	0.065	0.310
Elevation		
Ecological	0.448	0.003
Shape	0.324	0.032
Gill raker	0.418	0.010
F_{ST}	0.601	0.004
Ecological		
Shape	0.875	0.001
Gill raker	0.893	0.001
F_{ST}	0.659	0.001
Shape		
Gill raker	0.927	0.001
F_{ST}	0.573	0.002
Gill raker		
F_{ST}	0.642	0.001

Strong correlations between adaptive divergence metrics, ecological divergence and genetic differentiation give further weight to this hypothesis, indicating that IBA might be occurring in Lough Neagh.

Parallel morphological, ecological and genetic divergence in Lough Neagh

Shifts towards greater BD and reduced GRL with increasing distance from the lake habitat were detected in six of the nine main-stem rivers within the Lough Neagh catchment. These patterns are consistent with phenotypic divergence reported between lake–stream fish in Canadian populations (Hendry & Taylor, 2004; Berner, 2009; Kaueffer *et al.*, 2012). Trait differences between Lough Neagh lake–river populations are therefore similar to their Canadian counterparts, likely a result of divergent natural selection between these environments.

Body depth has a functional role in foraging and adaptation to divergent environments. Deeper-bodied sticklebacks, for example, display increased manoeuvrability (Walker, 1997; Hendry *et al.*, 2011), probably a fitness advantage when foraging in structurally complex benthic environments (Langerhans, 2008). In the Lough Neagh catchment, body shape deepened with increasing distance from the lake and was also strongly associated with feeding on riverine benthic prey resources. River sticklebacks in Lough Neagh also demonstrated a shift in trophic traits consistent with those occurring in other lake–stream systems. In the populations where GRL and number decreased with distance from the lake, SIA and SCA indicated that these fish also fed increasingly on river benthic macroinvertebrates. Shifts in body and trophic morphology, concordant with shifts in diet, suggest similar selective pressures observed in other lake–stream systems drive adaptive divergence in Lough Neagh.

Unlike other adaptive traits, comparative studies have generally been unable to detect consistent parallel divergence in anti-predator straits across the lake–stream axis (Hendry & Taylor, 2004; Kaueffer *et al.*, 2012). Dorsal and PS lengths showed a consistent pattern of shortening with increasing distance from the lake within the Lough Neagh catchment, suggesting a basis for adaptive divergence between these traits in this system. Experimental and observational evidence suggests that increased predation on sticklebacks by benthic invertebrates results in selection for shorter pelvic dorsal spines, whereas predation from piscivorous fish and birds leads to longer spines (Reimchen, 1994; Marchinko, 2009). Increased numbers of benthic invertebrates are expected in the river environments across Lough Neagh, and therefore divergent predation regimes may drive parallel divergence in these traits within this system. The lack of divergence in lateral plate armour is surprising however, as this is the only

Table 6 P_{ST} and F_{ST} values calculated from morphological traits and neutral genetic markers for pairwise comparisons of lake–stream populations in Lough Neagh, Central Europe and British Columbia.

Region	System	Body shape	Body depth	Gill raker no.	Gill raker length	F_{ST}
Lough Neagh	Ballinderry	0.020	0.228	0.095	0.126	0.040
	Blackwater	0.008	0.113	0.023	0.028	0.003
	Crumlin	0.000	0.016	0.000	0.048	0.021
	Glenavy	0.144	0.277	0.603	0.445	0.062
	Lower Bann	0.004	0.003	0.075	0.089	0.001
	Maine	0.088	0.197	0.589	0.251	0.053
	Moyola	0.232	0.274	0.000	0.314	0.065
	Sixmilewater	0.077	0.266	0.000	0.149	0.047
	Upper Bann	0.011	0.010	0.000	0.060	0.001
Central Europe	Constance South	0.439	0.353	0.097	0.682	0.110
Berner *et al.* (2010)	Constance West	0.101	0.054	0.000	0.449	0.030
	Geneva	0.000	0.263	0.000	0.036	0.000
British Columbia	Beaver	0.563	0.797	0.016	0.320	0.192
Berner *et al.* (2010), Kaueffer *et al.* (2012)	Boot	0.575	0.621	0.635	0.466	0.178
	Joe's	0.378	0.212			
	Misty	–	–	0.298	0.014	0.121
	Pye	0.359	0.673	0.652	0.036	0.069
	Robert's	0.433	0.500	0.027	0.149	0.045
	Village Bay	–	–	0.467	0.030	0.046

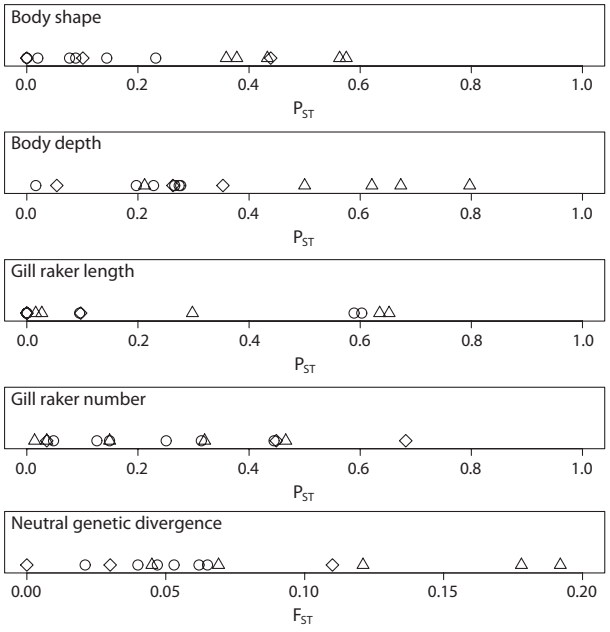

Fig. 6 Comparison of P_{ST} and F_{ST} values between independent systems with lake–stream divergence in British Columbia (open triangles), Central Europe (open diamonds) and Ireland (open circles).

anti-predator trait that consistently varies in other lake –stream systems (Berner *et al.*, 2010; Kaueffer *et al.*, 2012). However, it should be noted that freshwater stickleback populations in Ireland do not vary considerably for this trait (M. Ravinet, unpublished data).

In Lough Neagh, stable isotope values, modelled dietary contributions and trophic position indicated a shift towards a more benthic diet in river populations with increasing distance from the lake. Similar patterns using stable isotopes have also been demonstrated in lake–stream sticklebacks elsewhere (Kaueffer *et al.*, 2012). One of the most striking differences between stickleback SIA values in the Lough Neagh catchment are the large ranges in values (δ^{13}C: 7.0‰; δ^{15}N: 11.3‰). Lough Neagh is a hypereutrophic lake that has experienced considerable cultural eutrophication over the last 50 years (Foy *et al.*, 1995), and this is likely responsible for exaggerated differences in isotopic baselines across the catchment (Anderson & Cabana, 2005, 2006). Furthermore, the lake has a relatively small littoral zone with large areas of open water (Carter, 1993). In our study, these elevated values acted as a useful biomarker for lake habitat use. High trophic levels in lake fish likely reflect the fact lake sticklebacks feed on proportionally more zooplankton including carnivorous copepods and cladocerans, resulting in cryptic trophic levels (Harrod & Grey, 2006; Santer *et al.*, 2006). This may also explain higher trophic levels observed in other lake –stream stickleback systems (Kaueffer *et al.*, 2012).

In contrast with the stable isotope data, SCA was less conclusive, although a significant decrease in the proportion of limnetic prey was seen between lake fish and those from other habitat classes. Indeed, SCA revealed that benthic invertebrates represented the main prey resource for sticklebacks across the catchment. It should be noted however that the small samples sizes used for SCA (n = 5 per site) may reduce the power of this analysis; additionally, individuals

remained in traps for 6–12 h prior to retrieval, likely leading to digestion of some stomach contents. This may explain the high proportion of empty stomachs in our data set and would possibly increase the bias towards benthic prey (i.e. soft-bodied limnetic prey items such as zooplankton are more rapidly digested). Additionally, digestion in our study impacted our ability to resolve the taxonomy of Cladocera, and we were unable to distinguish whether benthic taxa, for example, chydorids were present. The disparity between SIA and SCA in this study highlights the need to apply both methods with care when attempting to infer divergent selection. SCA provides only a short dietary 'snapshot' and is not necessarily indicative of long term dietary trends (Schindler *et al.*, 2004). This may prove a particular problem in lake–stream stickleback systems as lake sticklebacks feed on both limnetic and benthic prey, and pelagic sticklebacks in particular make use of littoral resources during the breeding season (Gow *et al.*, 2006; Berner *et al.*, 2008). Furthermore, sampling in our study occurred during the stickleback breeding season – hence, it is possible that this could account for the discrepancy between measures of diet.

Associations between adaptive trait shifts and genetic divergence are well observed in Canadian lake–stream stickleback populations (Hendry & Taylor, 2004; Berner *et al.*, 2009; Kaueffer *et al.*, 2012). Within the Lough Neagh catchment, we found no evidence of population structure in the lake itself; however, genetic structuring was present in the upper regions of the Ballinderry, Glenavy, Maine, Moyola and Sixmilewater. High genetic differentiation values were present between the upper river, lake and lower river populations in these river systems, suggesting limited gene flow across lake–stream transitions. Measures of genetic divergence were higher still between isolated upper lake systems (i.e. Upper Moyola and Upper Glenavy, $F_{ST} = 0.14$, Jost's $D = 0.45$). Although we cannot accurately determine evolutionary relationships between these river populations, high divergence suggests the possibility that they have arisen independently, at least in some cases. Nonetheless, we cannot discount a dispersalist hypothesis (Thompson *et al.*, 1997); several rivers in the Lough Neagh catchment are connected to one another via the wider drainage system.

Parallelism, determinism and nonparallelism

Taking a quantitative approach to parallel lake–stream divergence in Lough Neagh, we detected a strong pattern of shared phenotypic response to increasing distance from the lake. Shared, parallel divergence across the lake–stream transition explained the greatest proportion of phenotypic variance within Lough Neagh, suggesting that selective determinism in this system is driving parallel morphological divergence.

For lake–stream stickleback divergence, we hypothesize that selective determinism likely operates across nested hierarchical levels. For example, at the highest level, this drives a similar evolutionary pattern in body shape and trophic morphology. Repeated divergence in these traits between catchments and continents argues in favour of this (Hendry & Taylor, 2004; Berner *et al.*, 2010; Kaueffer *et al.*, 2012; this study). The extent of determinism on certain traits may also vary between catchments, that is, parallel shifts towards shorter anti-predator traits in Lough Neagh but not in other catchments (Kaueffer *et al.*, 2012).

However, our analysis also detected the occurrence of nonparallel divergence within the Lough Neagh catchment. The majority of such nonparallel divergence was attributed to differences in river location, suggesting that environmental variation between rivers within Lough Neagh may drive stream–stream phenotypic divergence. Although we did not quantify environmental differences between the streams in the system, the rivers do differ in character, and it is possible that within-catchment determinism may be responsible for phenotypic variation at this level. This is further supported by the extremely low effect of location in explaining variation in anti-predator traits (PVE = 1.5%) in contrast to other traits such as body shape (PVE = 21.5%). The proportion of benthic invertebrates present is probably greater with increasing distance from the lake in all river systems, thus resulting in a stronger shared response and a lower nonparallel component.

For nearly all traits measured here, the unique response (i.e. a distance × location interaction) of populations to increasing distance from the lake was < 10% of PVE. Unique responses to environmental gradients are expected to be low when populations are closely related and environmental differences between locations are relatively minor (Langerhans & DeWitt, 2004). Low unique response in the Lough Neagh system indicates that whereas environmental differences between river locations may drive some phenotypic variance, the parallel phenotypic convergence is still marked. However, we did detect a strong unique response in diet inferred from SIA. This likely reflects strong baseline isotopic differences between rivers.

It should be noted however that our test for parallelism in the Lough Neagh catchment is a crude attempt to do so, and we cannot rule out the possibility of alternative explanations for phenotypic divergence between river locations. Further work using a common garden approach to quantify environmental effects on phenotype and a more thorough attempt to quantify environmental differences between river locations is needed.

Isolation by adaptation and gene flow in Lough Neagh

Correlations between neutral genetic difference and measures of phenotypic divergence independent of

geographical distance are indicative of a pattern of IBA (Rosenblum *et al.*, 2007; Nosil *et al.*, 2008). In the Lough Neagh catchment, geographical distance could not account for phenotypic or genetic divergence between stickleback populations. Instead, our results were consistent with an isolation-by-adaptation hypothesis, suggesting that divergent natural selection between lake and river environments has led to reproductive isolation in this system. Similar patterns of increasing genetic divergence with phenotypic differentiation have previously been observed in Canadian lake–stream stickleback populations (Deagle *et al.*, 2011; Kaueffer *et al.*, 2012; Roesti *et al.*, 2012).

Despite this, causality cannot be inferred from correlation alone, and we are not able to conclusively confirm whether adaptive divergence is causing isolation or vice versa (Räsänen & Hendry, 2008; Hendry, 2009). Certainly, we cannot rule out the possibility that physical barriers to gene flow present within Lough Neagh contribute to adaptive divergence. For example, the significant correlation between elevation and all divergence measures indicates that differences in river flow regime might act as a barrier, preventing migration of lake fish to the upper river reaches and therefore facilitating divergence. This would certainly explain why lower river populations are so phenotypically and genetically similar to those in the lake. Gene flow constraint is particularly notable in the River Glenavy, where the strongest genetic structuring (see Fig. 4) and greatest phenotypic divergence are present. A large waterfall (approximately 10 m in height) is present between the upper and lower populations, impassable to sticklebacks and therefore a major barrier to gene flow.

We are not aware of similar barriers on the other rivers; however, for two of the rivers where phenotype, ecological and genetic divergence is almost nonexistent (Blackwater and Upper Bann), there is no drastic change in river gradient and likely no shift in flow regime – thus these rivers remain extremely slow-flowing and are more 'lake-like' than others in the catchment. It seems likely that in these rivers there is little divergent selection and no barriers to gene flow to facilitate adaptive divergence, as has been observed in systems from British Columbia (Berner *et al.*, 2008, 2009). Lower river stickleback populations in Lough Neagh also exhibit this pattern, and we suspect that a combination of high gene flow and fewer ecological differences (i.e. less divergent selection) between the lower river and lake environments contributes to the greater phenotypic and genetic similarity between individuals.

In contrast, the third invariant river the Lower Bann is the only outflow of Lough Neagh and therefore might be expected to be most similar to the lake system, due to high gene flow. Phenotypic and genetic similarity between lake stickleback populations and outlets have been observed repeatedly in British Columbia (Berner *et al.*, 2009; Roesti *et al.*, 2012). We sampled sites at large distances along this river (total sampled length = 38.4 km, see Table S1) and could detect no clear shifts in phenotype or genetic structuring, despite strong shifts in flow regime and obvious habitat differences. Higher levels of gene flow and migration are therefore a more likely explanation for the lack of divergence along the Lower Bann. Therefore, the patterns observed in Lough Neagh are consistent with those seen in British Columbia where high phenotypic and genetic divergence exists between some populations but lower divergence occurs in populations that experience higher gene flow (Hendry & Taylor, 2004; Moore & Hendry, 2005).

There are some additional caveats to consider when using an isolation-by-adaptation approach with neutral markers. In particular, neutral markers may only meet the predictions of ecological speciation (i.e. greater genetic differentiation between populations in different habitats) when gene flow is intermediate and selection high (Thibert-Plante & Hendry, 2010). Neutral markers may therefore fail to detect ecological speciation when it is occurring or indeed cause false positives – that is, confusing genetic divergence between ecologically different populations caused by drift instead of divergent selection. Given the strong correlations present between trait divergence and genetic divergence, we think that the latter is likely in the Lough Neagh system. In particular, divergence between ecologically similar populations is almost nonexistent in some cases. Nonetheless, we cannot discount the possibility that divergence is present at some level in these populations but that we are not able to detect it with our markers. Markers linked to genomic regions under selection may be better suited to this (Nosil *et al.*, 2008; Thibert-Plante & Hendry, 2010), and there is a need to examine the Lough Neagh populations in this context.

Comparative lake–stream divergence

Previous research on lake–stream stickleback populations has indicated that the strongest divergence between these parapatric forms occurs in British Columbia, whereas much lower divergence is found in populations from Central Europe. Comparative analysis indicates that lake–stream divergence in the Lough Neagh populations is typically intermediate between these two extremes, although this was not the case for measures such as body shape. However, in several Lough Neagh rivers, both phenotypic and genetic divergence occurs at a similar extent to that seen in some populations from British Columbia.

Our findings provide an additional perspective to those of Berner *et al.* (2010) who examined populations from Central Europe but found considerably lower divergence in comparison with Canadian populations. These authors suggested that genomic constraints may be responsible for this lower divergence – that is, that

allelic variants necessary for lake–stream adaptation have been lost in European populations. We cannot directly test this hypothesis without high-density genomic data; however, appreciable levels of divergence in the Lough Neagh system suggest that if present, genomic constraints are not as restrictive as first thought.

Lower divergence in lake–stream systems from Central Europe may therefore be as a result of younger population age (approximately 150 years old, Lucek *et al.*, 2010). This is further supported by high levels of genetic differentiation between lake–stream populations from older populations elsewhere in Europe (F_{ST} = 0.18, Reusch *et al.*, 2001). As an older system most probably colonized by stickleback following ice retreat after the LGM in Ireland, Lough Neagh lake–stream divergence has been able to evolve without time constraints. It should be noted that one Central European catchment, Constance South, exhibited higher phenotypic and neutral genetic differentiation than most Irish populations, suggesting that strong divergent selection might overcome time constraints. Nonetheless, divergence in European populations is still not as high as that seen in some systems from British Columbia (i.e. the Misty system). Divergence is therefore potentially limited by a combination of both temporal and genomic constraints; further comparative work using genomic data is required to assess the extent of the roles these two factors play.

Conclusion

Our work has confirmed that shifts in adaptive traits, foraging behaviour and allele frequencies occur along at least five of the lake–stream transitions present in the Lough Neagh system. Thus, Lough Neagh provides the opportunity to study multiple instances of lake–stream divergence within a single catchment and to assess the extent of parallel divergence occurring at this level. A shared parallel adaptive response to increasing distance from the lake is present between river populations in Lough Neagh. However, differences in river environment likely contribute to phenotypic variation between river locations, thus confirming nonparallelism in our system. Examining the processes responsible for lake–stream divergence in Lough Neagh suggests IBA may be occurring. However, interplay between gene flow and the strength of divergent selection is likely to be responsible for the extent of adaptive divergence in this system. Comparative analysis of lake–stream stickleback populations from across the world suggests that Lough Neagh populations are as divergent as some from British Columbia. This suggests an interaction between both time and genomic constraints accounts for regional differences in lake–stream divergence. By identifying this novel system for parapatric stickleback divergence, we hope that future lake–stream research will take the Lough Neagh stickleback populations into account.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Table S1 Table of all site/location data.
Table S2 Body shape ANOVA table.
Table S3 Foraging and anti-predator trait ANOVA tables.
Table S4 Macroinvertebrate isotope ANOVA.
Table S5 Stickleback global GLMMs and local GLMs.
Table S6 Global GLMMs and local GLMs on estimated proportions of end member sources to diet.
Table S7 MANCOVA test for parallelism using all samples (invariant and variant rivers).
Table S8 Results for deviation from Hardy–Weingberg Equilibrium for whole catchment and by river location (using Bonferroni corrections and False Discovery Rate).
Figure S1 Deformation grids showing shape differences in female and male individuals along PC1; note the change in snout length.

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