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Reproductive isolation in a threespine stickleback hybrid zone

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shape;
sticklebacks;
structure.

Abstract

In many estuarine sites, morphological and genetic differences between anadromous and freshwater threespine sticklebacks are maintained despite breeding in sympatry. Here, we investigate the maintenance of this morphological divergence in a natural hybrid zone in the River Tyne, Scotland. We provide a morphological description of the hybrid zone, and using a Bayesian MCMC approach, identified distinct anadromous and freshwater genetic clusters. Anadromous and freshwater sticklebacks breed in spatial and temporal sympatry in the lower reaches of the River Tyne. The frequency of hybrids within these sites (33%) indicates prezygotic isolation is not complete, and suggests that assortative mating is not strong. However, significant heterozygote deficit and cytonuclear disequilibrium in juveniles collected from sympatric sites confirms that barriers to gene flow exist between the morphs in the wild. In addition, we found no evidence of a directional bias in hybridisation, although hybrids with anadromous mothers were more common because anadromous females outnumbered freshwater females within the hybrid zone. We discuss the potential contribution of temporal, spatial, and sexual prezygotic barriers to the observed reproductive isolation as well as postzygotic selection against hybrid zygotes or fry.

Introduction

Studies of contact zones between divergent populations can provide insight into how genetic and morphological differentiation is maintained, and thus shed light on factors, which may promote speciation. Since divergence between two subpopulations cannot be maintained in the face of gene flow (Dobzhansky, 1951), the evolution of reproductive isolation and factors preventing gene flow form the prime focus of speciation research. Barriers to gene flow are typically classified in terms of whether they act to prevent zygote formation (prezygotic) or reduce zygote fitness (postzygotic), although some factors may contribute to both (e.g. immigrant inviability, Nosil *et al.*, 2005). Examples include prezygotic barriers such as spatial or temporal differences in breeding season and assortative mating (sexual selection) as well as postzygotic barriers such as hybrid inviability (caused by endogenous factors such as genetic incompatabilites) and hybrid inferiority (caused by exogenous factors such as environmental conditions). Divergence in a hybrid zone can be maintained by one or more of the above factors only if these barriers are strong enough to counteract the homogenising effects of gene flow.

Here, we set out to investigate reproductive isolation between recently diverged anadromous and freshwater sticklebacks in a contact zone. The threespine stickleback (*Gasterosteus aculeatus*) has undergone an adaptive radiation since the retreat of the last Pleistocene ice sheet (approximately 20 000 years ago). Molecular evidence suggests that freshwater populations have arisen by the repeated and independent invasion of freshwater habitats by marine sticklebacks (Taylor & McPhail, 2000; McKinnon *et al.*, 2004). Anadromous fish are born in freshwater, spend most of their lives at sea and return to freshwater to breed. In estuaries and the lower reaches of rivers they often breed sympatrically with resident freshwater sticklebacks. Despite breeding in sympatry,

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these fish have undergone divergence in traits associated with differences in ecology and life history (McPhail, 1994; McKinnon & Rundle, 2002). The consistent differences between forms is most likely a result of parallel adaptive divergence (McPhail, 1994; McKinnon & Rundle, 2002; Colosimo *et al.*, 2005). The above-mentioned characteristics make sympatric populations of this species pair useful for studying factors influencing speciation and the parallel evolution of morphological traits.

The degree of prezygotic isolation between anadromous and freshwater resident sticklebacks appears to vary throughout their distribution from complete prezygotic isolation (Ziuganov, 1995), to no prezygotic isolation in laboratory conditions (Hagen, 1967). Intermediate morphs have been described in many populations (e.g. Hagen, 1967; Hay & McPhail, 2000), but despite the general belief that hybridisation is occurring, only one field based study of a hybrid zone has been reported (Hagen, 1967). A lack of available genetic tools prevented Hagen from investigating prezygotic isolation in the wild, but his laboratory experiments and field observations were instrumental in showing that prezygotic isolation might be affected by ecological factors. These are likely to include factors influencing both the spatial (microhabitat) and temporal occurrence of breeding (Hagen, 1967; McPhail, 1994). Sexual selection in the form of assortative mating can also provide a significant prezygotic barrier to gene flow (e.g. Parsons *et al.*, 1993; Cruz *et al.*, 2004), and there is some evidence that sexual selection may also contribute to prezygotic isolation between anadromous and freshwater morphs (McKinnon *et al.*, 2004; Scott, 2004). However, the lack of assortative mating in some populations (Hagen, 1967; F.C. Jones, C. Brown & V.A. Braithwaite, 2005, submitted) suggests that the contribution of sexual selection to reproductive isolation may vary between geographic locations. The importance of postzygotic barriers to gene flow between the morphs is less clear. There is little evidence to suggest that endogenous factors influence hybrid survival since hybrids from laboratory crosses are viable and interfertile (Hagen, 1967; McPhail, 1994). Recent evidence suggests that environmentally mediated factors (i.e. exogenous factors) influence hybrid survival (e.g. over-winter survival probability) and contribute significantly to reproductive isolation in the wild (Jones, 2005; F.C. Jones, C. Brown & V.A. Braithwaite, 2005, submitted).

In a previous study, we showed that hybridisation between anadromous and freshwater morphotypes collected from the River Tyne, Scotland, occurred readily in experimental pond conditions (F.C. Jones, C. Brown & V.A. Braithwaite, 2005, submitted). Further, after accounting for differences in the fecundity and propensity of different morphs to mate, likelihood-based analyses of observed mating events and number of fry produced revealed that some ponds had a deficit of hybrid fry relative to number of hybridisation mating events. These findings are suggestive of the existence of postzygotic barriers to gene flow between anadromous and freshwater morphs.

At present, most of our understanding of factors influencing reproductive isolation between anadromous and freshwater sticklebacks comes from studies conducted in the laboratory, despite the fact that environmental factors may play an important role in species interactions. Moreover, data on the distribution of genotypes in the wild and the bimodality of natural hybrid zones can be invaluable (Jiggins & Mallet, 2000), and are currently lacking for sticklebacks. With the genetic tools available today, field studies of hybrid zones can provide evidence of nonrandom mating between genetically distinct sympatric morphotypes and offer a powerful approach for understanding factors influencing reproductive isolation and maintenance of morphological differences between the members of this species pair.

Here, we describe the contact zone between anadromous and freshwater sticklebacks in the River Tyne, Scotland and investigate the strength and nature of reproductive isolation between the morphs in the wild. First, we investigated morphological differences between anadromous and freshwater sticklebacks using geometric morphometric and traditional morphological analyses. Then, we developed a suite of genetic markers to discriminate between anadromous and freshwater individuals. Our primary objective was to examine the spatial and temporal distribution of sticklebacks in the river, test for the presence of genetic structure, and identify hybrid/introgressed individuals based on genetic ancestry. Due to the migratory life history of anadromous sticklebacks, it is possible that the observed divergence between morphs in any given site results not from barriers to gene flow at that site but rather from influx of unique anadromous migrants from geographically distant sites each year. Therefore, our secondary objective was to investigate reproductive isolation in the subset of sites identified in objective one where anadromous and freshwater sticklebacks breed sympatrically. We looked for evidence of reproductive isolation by testing for a heterozygote deficit in juveniles. Based on our previous pond assortative mating experiment (F.C. Jones, C. Brown & V.A. Braithwaite, 2005, submitted), we predicted that we would observe a weak heterozygote deficit in juveniles from the natural population. In addition, to investigate the nature of reproductive isolation we looked for evidence of directional hybridisation by performing tests for cytonuclear disequilibrium on hybrid individuals.

Materials and methods

Field work and sample collection

Field work was conducted at eight sites along the River Tyne, East Lothian, Scotland (56″1.2′N 2″34.1′W) during

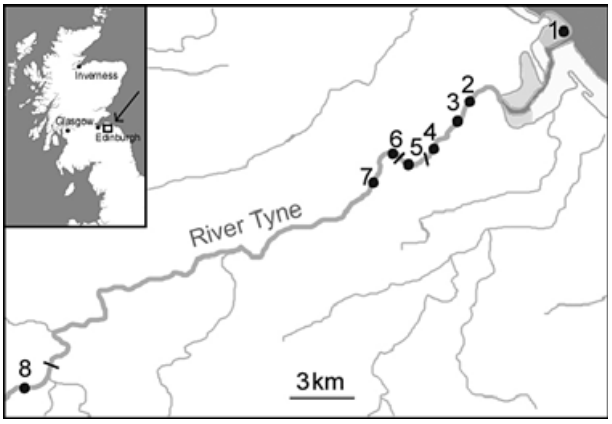

Fig. 1 Map of sample sites (1–8) along the River Tyne, Scotland. Solid black lines across the River indicate the location of weirs.

the years 2001–2003 (Fig. 1). Site 1 was in rock pools at the mouth of the river, sites 2 and 3 were under tidal influence and 4–8 were freshwater sites. Several weirs were constructed across the river during the 19th century (Fig. 1), all of which are likely to affect the movement of within-stream fauna. The lowest weir, located between sites 4 and 5, contains a fish ladder, which may facilitate the upstream movement of migratory fish. In July 2001, July and September 2002, and then on a monthly basis from January to December 2003, sticklebacks were collected using wire mesh minnow traps set overnight. At each site, four traps were placed on each side of the river (eight in total) roughly 5 m apart, equating to samples being collected from approximately 20 m of river. Morphological measurements were taken and the fish were fin-clipped for genetic analysis, photographed, tagged with a visible elastomer tag (colour specific to the site; North West Marine Technologies), and released back into the river (Table 1, UK Home Office Licence 60/2954). Tagging enabled us to identify previously sampled individuals, investigate changes in population size and determine the extent of within river movement. Young of the year collected in late September 2002, and adults from January to March 2003 were preserved in ethanol instead of being tagged and released. We recorded the reproductive status of each female sampled and collected data on the reproductive status of males from digital photos (described below). Individuals sampled from September 2002 to December 2003 were used in genetic structure analysis (see below).

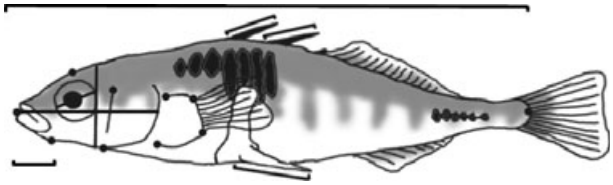

Fig. 2 Morphological measurements taken from sticklebacks. Black points indicate landmarks for which x and y coordinates were digitized. Black lines indicate measurements of standard length, spine lengths, head depth, head length, eye diameter and jaw length. Lateral plate counts were also taken.

Morphology

Lateral plate counts of the left side of the body were performed in the field on sedated fish (MS222 anaesthetic in $NaHCO_3$ buffer). The length of first, second dorsal and left pelvic spines was measured using callipers accurate to 0.05 mm. Standard length, snout length, head depth, head length and eye diameter of the fish was calculated from a digital image with reference to a background 5 mm grid using the software tpsDig (Rohlf, 2001). In addition, we recorded the x and y coordinates of 11 landmarks on the digital photographs (Fig. 2). These landmarks were concentrated around the head region

Table 1 The number of individuals from which morphological data was collected for each site and month in 2003. Note, these samples sizes do not reflect the total number of individuals caught, but a random sample up to $n = 57$ of those individuals caught at each site in each month.

	2003														
	Adults								Juveniles						
Site	January	February	March	April	May	January	July	August	July	August	September	October	November	December	Total
1	0	0	0	0	34	20	16	1	0	0	0	0	0	0	61
2	4	16	7	14	38	50	1	0	39	50	27	5	3	6	260
3	7	30	8	23	42	50	5	0	32	50	50	30	13	15	355
4	57	54	12	50	45	35	7	0	22	50	50	30	30	23	465
5	8	18	15	43	31	28	1	0	44	40	50	30	30	16	354
6	49	15	39	50	44	26	39	0	11	50	50	30	30	30	463
7	35	23	8	30	36	50	39	0	11	50	44	30	30	30	416
8	43	24	8	18	9	7	8	0	1	50	–	30	30	30	258
Total	203	180	97	228	269	266	116	1	160	341	271	185	166	150	2632

rather than the body to avoid the gravidity of females affecting our analyses of shape. Landmark configurations were also used to calculate centroid size (defined as square root of the sum of squared distances of a set of landmarks from their centroid), which provided a useful measure of body size.

Genotyping

DNA extractions were performed on fin-clips using a chelex extraction protocol (Walsh *et al.*, 1991) with 0.2 mg/mL proteinase K. A suite of markers was chosen with the aim of discriminating between anadromous and freshwater morphs and identifying hybrid/introgressed individuals. A total of 1961 fish from all eight sites (Table 2) were genotyped at seven polymorphic microsatellite loci (selected from the linkage map constructed by Peichel *et al.*, 2001), one mitochondrial single nucleotide polymorphism (SNP, located within the cytochrome *b* gene), and three nuclear SNPs positioned in introns of targeted genes (ATP1a2 intron 1, Myosin Heavy Chain intron 5 and beta Androgen Receptor intron 2, see Table 3 and details below). In addition, fish were sexed using labelled primers that amplify the 3′ untranslated region of the Iso-citrate dehydrogenase (Idh) gene where a sex-linked insertion-deletion exists (K. Peichel, unpublished data, Table 3).

Using previously published mtDNA sequence data (Genbank accession number AP002944), we designed species-specific primers (forward and reverse primers labelled with different coloured fluorescent dyes) to amplify a 483 bp region of the cytochrome *b* gene. Fish from sites 1 and 8 were discriminated at a single diagnostic SNP by restriction fragment length polymorphism (RFLP) assay. The enzyme *Hph I* was used to digest the PCR product at 37°C for 3 h, producing two different coloured fragments in freshwater fish and one fragment in anadromous fish (Table 3).

Three nuclear SNPs were identified by designing primers for conserved exon regions spanning introns, using multiple alignments of sequences from different fish taxa. In some cases, primer design was aided by identifying candidate genes from the stickleback expressed sequence tagged (EST) database (Genbank) using the Blast search algorithm. Double stranded sequences of each intron locus (between 400 and 800 bp) were obtained from 14 fish (seven from each of sites 1 and 8) and SNPs were identified. Because diagnostic restriction enzymes were not available, we used allele-specific PCR and primer-induced fragment length variation to identify both homozygous and heterozygous individuals for all three nuclear intron loci as described by Hansson & Kawabe (2005). Our protocol differed as follows: positive and negative controls in the form of one heterozygous and two homozygous individuals were included in each PCR batch, forward primers for each locus were labelled with different coloured fluorescent dyes, and separate PCR reactions were performed for each forwards primer.

For all loci, PCRs were performed in a 10 μL volume containing 1 mM dNTPs, 0.4 μM of each primer, 0.4 units of BioLine *taq* polymerase, 1 × BioLine Buffer, 2.0 μL of DNA and with varying $MgCl_2$ concentrations (Table 3). Amplification cycles consisted of 2 min denaturation at 94°C, followed by 25 cycles of 30 s at annealing temperature (Table 3), 1 min at 72°C and 45 s at 90°C, and finished with 4 min extension at 72°C. Combined PCR products from all 12 loci were run with internal size standard (ABI GS500-Liz; Applied Biosystems) on an ABI 3730 capillary sequencer (Applied Biosystems) using a single capillary per individual. Resulting electropherograms were then analysed using GENEMAPPER software V3.0 (Applied Biosystems). Details of sample sizes of fish genotyped from each site each month for the purposes of this study are shown in Table 2.

Table 2. Sample sizes of fish genotyped from each site each month. Age Class A = Adult, Age class J = Juvenile.

Year	2001	2002			2003											
Age class	A	A	J		A					J						
Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Jul	Aug	Sep	Oct	Nov	Dec	Grand Total
Site																
1	19	–	–	–	–	34	20	16	1	–	–	–	–	–	–	90
2	–	–	–	45	14	38	50	1	–	39	50	27	5	3	6	278
3	–	–	–	45	23	42	50	5	–	32	50	50	30	13	15	355
4	75	18	40	50	50	45	35	7	–	22	50	50	30	30	23	525
5	–	–	–	45	–	–	–	–	–	–	–	50	–	–	–	95
6	–	–	–	45	–	–	–	–	–	–	–	50	–	–	–	95
7	51	21	6	45	30	36	50	39	–	11	50	44	30	30	30	473
8	–	–	–	–	–	–	–	–	–	–	50	–	–	–	–	50
Total	145	39	46	275	117	195	205	68	1	104	250	271	95	76	74	1961

Table 3 Details of loci used to determine genetic ancestry and sex (Peichel *et al.*, 2001; Jones *et al.*, present study; Peichel, unpublished).

Locus	Primer sequence (5′–3′)		Product size (bp)	PCR conditions‡	Reference
Microsatellite loci					
STN9	F	PET GCGAAACGTTCATTTCAATTC	106–146	58 (2.0)	Peichel *et al.* (2001)§
	R	†AAAATTAATCGTTAGCACCCCTA			
STN26	F	NED GTATCGAAGTCTGAAGGCCG	106–126	60 (0.5)	Peichel *et al.* (2001)
	R	GTACAGCATGTGGTCGATGG			
STN94	F	PET GGCACGTCTCTCACTTTGAC	183–216¶	53 (1.5)	Peichel *et al.* (2001)§
	R	TNGATTTTACATTNTANCCTGGAC			
STN96	F	FAM ACACCTTCGGCTCCATATCC	218–280	58 (3.0)	Peichel *et al.* (2001)
	R	CGCAGCTCTCTGCTTTGC			
STN130	F	FAM TTCGGCTTATTTTCTTACCTGC	122–166	56 (0.5)	Peichel *et al.* (2001)§
	R	†ATGTTGTAGGCGAGGACAGGATG			
STN152	F	VIC ATGGAATATCGACAGAGCCG	228–346	57 (3.0)	Peichel *et al.* (2001)
	R	GTGCGGTCTGCTCATCAAGG			
STN208	F	VIC GAGTGGTTTCAAGCTGTGAGC	103–183	53 (1.5)	Peichel *et al.* (2001)
	R	CGCCTGTTCTTTACAAAGCC			
Nuclear intron SNPs					
ATP1a2	F	PET TCTAAAAAATCTTTGTCCAACCC	79	60 (0.5)	Jones *et al*. (Present study)
	F	NED ATCTAAAAAATCTTTGTCCAACCA			
	R	GACCTGGGAGACGAAGAGTAAA			
BAR2	F	FAM AACATTACGGCATATTTTGTACTAAC	184	59 (0.5)	Jones *et al*. (Present study)
	F	VIC CAACATTACGGCATATTTTGTACTAAT			
	R	TGCGAAGTTATCATCCCTAAAGA			
Myo3-1HC	F	VIC TGAAGGTGTATCATCTGCTAATTTT	90	60 (0.5)	Jones *et al*. (Present study)
	F	FAM TTGAAGGTGTATCATCTGCTAATTTG			
	R	TGGATGACTCTTTTGGTGTTGA			
Mitochondrial SNP					
Cyt-b	F	FAM CCCTCCTTGGACTTTGCTTA	157, 326 (FW)	57 (1.5)	Jones *et al*. (Present study)
	R	NED TGAACAAGTGTGGCACCAG	483(AN), 226, 257(AN)		
Sex marker					
Idh 3′UTR	F	GGGACGAGCAAGATTTATTGG	270 (male), 300 (all)	59 (1.5)	Peichel (Unpublished)
	R	PET TATCGTTAGCCAGGAGATGG			

FAM, VIC, NED, PET represent fluorescent labels.
†7 bp 5′ tail.
‡annealing temperature (°C) followed by $MgCl_2$ concentration (mM) in parentheses.
§Primers redesigned using Genbank sequence for resizing purposes. Product sizes are given for freshwater (FW) and anadromous (AN) cytochrome *b* RFLP haplotypes, and for male specific (male) and all individuals (all) sex fragments.
¶1 bp length variation present.

Statistical analysis

Morphology

We examined the differences in morphology between adult fish collected from sites 1 (rock pools) and 8 (upstream) in April–July 2003. To investigate differences in overall body shape we used a geometric morphometric approach. After performing a generalized procrustes alignment of landmark configurations to remove non-shape variation (e.g., due to the position, orientation and size of the fish), we extracted partial warp scores from the remaining variation in landmark configurations. These shape variables were then analysed as dependent variables in a MANOVA with sex and site as independent factors using the program tpsRegr (Rohlf, 2004). Visualisations of shape differences were obtained using thin-plate splines to map the deformation in shape from sites 1 (anadromous) to 8 (freshwater) fish. To compare differences in specific morphological and meristic traits we performed *t*-tests comparing individuals of each sex from sites 1 and 8 separately. Because, we found significant differences in size, as indicated by both standard length and centroid size, statistical tests were performed on size adjusted trait scores (residuals from a regression of the trait against standard length). Finally, using morphometric, morphological (e.g. head length and depth) and meristic measurements (e.g. lateral plate number) collected from adult fish in all sites, we examined the distribution of morphotypes in the river and looked for evidence of morphotypes existing in sympatry. This analysis involved two steps. First, a discriminant function analysis was performed to discriminate between fish from sites 1 and 8, and coefficients for canonical variables were extracted. These coefficients were then used to calculate the canonical score for each individual collected from all eight sites in the river. In this way, the morphology of fish from sites

2 to 7 is scored in terms of similarity to anadromous fish from site 1 or freshwater fish from site 8. We examined the distribution of canonical scores within each site for evidence of geographic overlap of morphotypes.

Adult reproductive condition was scored based on female gravidity, male 'redness' and male eye colouration to determine the temporal overlap in breeding season of the genetic groups (see below). A female's reproductive condition was scored in the field as being gravid or not based on the presence of a distended abdomen. Male reproductive condition was scored from analysis of photos taken in the field. Photos were randomized and thus scored blind with respect to site, date and an individual's genetic ancestry (see below). The area and intensity of red pigmentation was scored on a scale of 0 (no red pigmentation present) to 3 (intense red pigmentation over large area). In addition a male was given a score of 1 if his eyes were blue and 0 if they were not. Redness and blue eye colouration scores were added to give each male a reproductive score ranging from 0 to 4. We examined the reproductive status of genetically anadromous, hybrid and freshwater adults that were sampled each month from sites 1 to 4 and 7. Individuals from sites of sympatry (sites 2–4) were later pooled.

Genetic structure analysis

The presence of (a) distinct genetic clusters in our entire data set of nuclear genotypes (1961 individuals, Table 3), and (b) within-site structuring, was tested using STRUCTURE v2.0 (Falush *et al.*, 2003). This analysis utilized the model-based clustering algorithm of Pritchard *et al.* (2000) to cluster individuals based on their multilocus nuclear genotypes and accounted for the correlations between linked loci that arise in admixed populations (Falush *et al.*, 2003). One of the advantages of this approach is that it is possible to test for distinct genetic clusters without having any *a priori* assumptions about an individual's population of origin. For both analyses we estimated the posterior probabilities of there being K distinct genetic clusters within our data sets, where $K =$ [1–8], and $K =$ [1–3] for analysis (a) and (b), respectively. We tested for up to eight clusters in analysis (a) to incorporate the possibility of there being genetic distinctions between fish in each of the eight sites. This was done assuming an uniform prior for K, was repeated three times to assess convergence of $\mathrm{LnP}(X|K)$, and involved a burn-in period of 100 000 replicates followed by 1 000 000 replicates for each run. The number of clusters was determined by the value of K with largest posterior probability following the guidelines of Pritchard *et al.* (2000). In the case of analysis (b), if two genetically distinct clusters existed in sympatry in a single site (i.e. K to equal 2), then we call such sites 'sympatric sites'. Alternatively, if samples collected from a single site were from a randomly mating population we would expect K to equal 1.

Since, we found $K = 2$ in our analysis of the entire data set (see results), we were able to assign individuals to distinct genetic clusters and identify putative hybrids using the individual ancestry assignment scores (q) obtained from the above analysis. These scores correspond to the probability of an individual having ancestry in one of the two putative source populations. We examined the spatial and age class distribution of ancestry scores in the river by plotting histograms of scores in each of the eight sites for adults (April–July) and juveniles (July–December) separately. Finally, 90% confidence intervals around these estimates were used to classify individuals as either 'freshwater' (confidence intervals around q incorporate 0 but not 1), 'hybrid' (confidence intervals around q incorporate 0.5 but not 0 or 1), 'anadromous' (confidence intervals around q incorporate 1 but not 0), resulting in conservative identification of hybrids. These classifications were used as indicators of an individual's nuclear genetic composition in analyses of female gravidity and cytonuclear disequilibrium.

Tests for Hardy–Weinberg equilibrium and cytonuclear disequilibrium

Having determined that anadromous and freshwater sticklebacks exist in sympatry in sites 2–4, and additionally, that genetically hybrid individuals and individuals of intermediate morphotypes were present in these sites (see results), we used the genotypes of juveniles collected in July and August to determine the mating patterns of the adult breeding population. First, we looked for evidence of reproductive isolation by testing for departures from Hardy–Weinberg equilibrium in sites 1–4, 7 and 8. Specifically, we tested for heterozygote deficiency using the program FSTAT (Goudet, 2002). Significance was assessed by randomisation tests (where alleles were randomized among individuals within samples 50 000 times). We chose not to include young of the year sampled from September to December to exclude as far as possible the potential effects of the anadromous migration out to sea and of selection against young of the year, on genotype frequencies.

Next, we tested for cytonuclear disequilibrium, associations between maternally inherited mitochondrial genotypes and biparentally inherited nuclear genotypes, using the program CNDM (Basten & Asmussen, 1997). In these tests, significant disequilibrium indicates nonrandom association between the nuclear and mitochondrial genotypes. Detection of significant cytonuclear disequilibrium within the anadromous and freshwater nuclear genotypes is an indication of reproductive isolation, and significant cytonuclear disequilibrium within the heterozygous or hybrid nuclear genotype may provide evidence of directional hybridisation or biased hybrid inviability. Cytonuclear disequilibrium was tested using the nuclear genetic ancestry coefficient, q, estimated from the multilocus assignment test employed in STRUCTURE (see

above), and mitochondrial haplotypes of juveniles collected from pooled sites 2–4 in the months of July and August. Significance of overall departures from random genotypic associations was assessed using Monte Carlo Markov Chain randomizations involving 100 batches of 1000 observations whilst the significance of individual disequilibria was assessed using Fisher's exact test.

Results

We sampled a total of 2632 sticklebacks from the River Tyne in 2003 (Table 1). Sticklebacks were present in the rock pools at site 1 in the months of May–August but were absent at all other times of the year. During July 2003 both adults and juveniles were caught in our traps. Adults virtually disappeared from our samples in August 2003 (as a result of mortality or migration back out to sea). Juveniles less than 25 mm standard length were not caught in the traps and, therefore, are not represented in our samples. In the lower reaches of the river, we frequently caught both anadromous and freshwater morphotypes in the same trap.

Morphology

We found a significant difference in the shape of adult fish sampled from sites 1 and 8, and significant differences in shape between sexes (MANOVA: site $F_{54,221.3} = 10.078$, $P < 0.0001$, sex $F_{34,150} = 5.508$, $P < 0.0001$). In addition, we found a significant interaction between sex and site suggesting that shape variation between sites differs for each sex (sex × site $F_{16,76} = 2.525$, $P < 0.0037$). Anadromous fish had a more robust head shape than freshwater fish (Fig. 3). In both sexes, fish from site 1 were significantly larger (centroid size) and longer (standard length) than fish from site 8 (Table 4). We found females from site 1 to have significantly longer dorsal and pelvic spines and deeper heads than females from site 8, after adjusting for standard length. In contrast, males from sites 1 and 8 did not differ significantly in these traits. Both males and females from site 1 possessed significantly more lateral plates than males and females from site 8. Examination of the distribution of canonical scores within each of the eight sites (Fig. 4a) revealed that both anadromous and freshwater morphotypes were sampled from sites 2 to 4, and that fish from sites 5 to 7 were of freshwater morphotype only. Lateral plate number has been used in previous studies as an indicator of ancestral status (e.g. Hagen, 1967; Hay & McPhail, 1975, 2000; McKinnon *et al.*, 2004; Scott, 2004) so we plotted the distribution of lateral plate morphotypes in each of the sites (Fig. 4b). It is notable that in sites 2–4 we observed individuals of intermediate morphology (Fig. 4a, b).

Genetic structure

Three different mitochondrial haplotypes were detected and proved to be completely diagnostic between anadromous fish from site 1 and freshwater fish from site 8. Haplotypes 1 and 2 were found only in fish from site 1, haplotype 2 being very rare (site 1 frequency was 2%, representing 0.001% of the entire sample), haplotype 3 was found in 100% of fish from site 8. None of the nuclear markers were completely diagnostic between fish from sites 1 and 8. At the three nuclear intron loci (MyoHC, ATP1a2 and bAR2), the frequency of 'anadromous' alleles was 80, 90 and 100% in site 1, and 41, 0 and 77% in site 8, respectively. microsatellite loci showed considerable overlap in allele size between sites 1 and 8, although significant differences in allele frequency were detected at all loci (not shown).

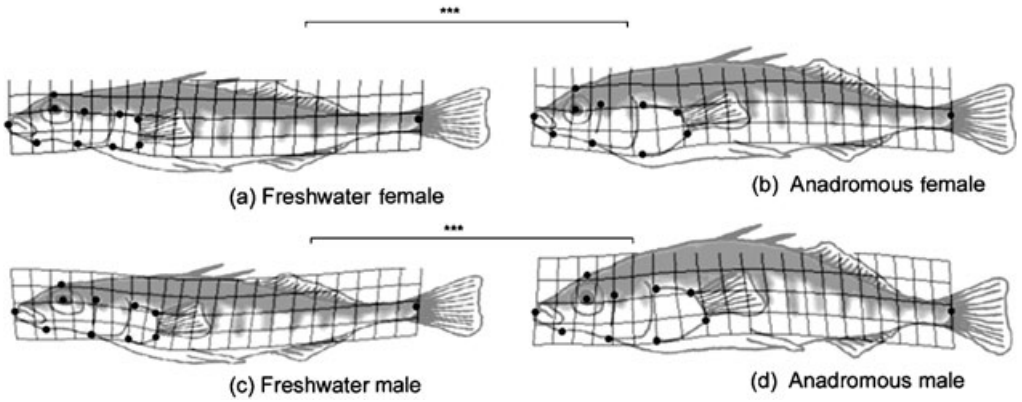

Fig. 3 Visualization of shape differences between anadromous and freshwater fish. For each sex, grids show the deformation in shape of an average consensus configuration into freshwater configuration (a) and (c) and anadromous configuration (b) and (d). Deformations have been magnified 3× to emphasise the shape differences. The background represents superimposition of a fish graphic into the estimated landmark configurations, but only changes in the position of the landmarks should be considered. ***Represents significant differences in a MANOVA of partial warp scores at the $P < 0.0001$ level.

Table 4 Morphological differences between anadromous (site 1) and freshwater (site 8) sticklebacks from the River Tyne. Measurements of spine lengths, and lateral plate counts were collected in the field, using callipers accurate to 0.05 mm. All other measures were calculated from digital photographs. Measurements for all traits are in millimetres, except lateral plates, which is a count, and centroid size which is calculated as the square root of the sum of squared distances of a set of landmarks from their centroid. For each sex, values in table represent mean trait values but statistical tests (*t*-tests) were performed on residuals from a regression of the trait against standard length to examine differences in size adjusted traits between fish from site 1 and site 8.

			Standard length		First dorsal spine		Second dorsal spine		Pelvic spine		No. lateral plates	
Site	Sex	N	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	Females	33	61.211	0.63‡	5.491	0.09	5.947	0.09‡	9.240	0.13‡	27.848	0.19‡
8		22	47.029	1.56***	3.727	0.13*	4.070	0.14**	6.000	0.20**	4.682	0.18***
1	Males	49	53.850	0.62‡	5.235	0.09 NS	5.363	0.10 NS	8.571	0.11 NS	27.408	0.19‡
8		27	40.502	0.70***	3.672	0.12 NS	4.063	0.08 NS	5.539	0.11 NS	5.593	0.28***
			Eye diameter		Head depth		Head length		Snout length		Centroid size	
1	Females	13	4.595	0.06 NS	10.292	0.08‡	16.333	0.17 NS	4.575	0.06 NS	54.720	0.70‡
8		22	3.555	0.08 NS	7.457	0.21**	12.398	0.34 NS	3.547	0.10 NS	40.748	1.36***
1	Males	33	4.528	0.04 NS	9.978	0.13 NS	16.569	0.19 NS	5.041	0.10 NS	48.102	0.53‡
8		27	3.587	0.07 NS	6.836	0.14 NS	11.935	0.27 NS	3.389	0.10 NS	34.945	0.61***

SE represents standard error.
NS $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
‡Tests that remain significant after sequential Bonferroni correction (Sokal & Rohlf, 1995).

STRUCTURE analysis of the entire data set (analysis a) revealed the most likely number of distinct genetic clusters in the River Tyne to be two (Fig. 5). About 97% of fish sampled from site 1 were assigned to cluster 1, whilst 95% of fish sampled from site 8 were assigned to cluster 2. This, along with significant differences in allele frequency between sites 1 and 8, is indicative that anadromous and freshwater fish are of distinct gene pools. Analysis of genetic structure within each of the eight sites (analysis b) revealed the most likely number of genetic clusters to be two in sites 1–4, but only one in sites 5–8 (see Table S1, supplementary information). These results suggest that anadromous and freshwater morphs exist in sympatry in each of the lower four sites. Examination of the distribution of q in each of the sites provides further support for the existence of both genetically anadromous and freshwater fish in sites 2–4 but does not uphold the presence of two classes of genetically distinct individuals in site 1 (see Fig. 4c). These conflicting results for site 1 are best explained by a spurious result from the within-site genetic structure analysis due to the small sample size ($n = 90$). We observed a higher richness (number of alleles sampled after correcting for sample size) in anadromous fish than freshwater fish (mean allelic richness across loci, pooling samples within site $1 = 10.966$, site $7 = 6.389$). This, coupled with a small sample size, would reduce the power of a cluster analysis based on multilocus genotypes. We conclude, from this analysis that sites 2–4 represent sites of spatial overlap of anadromous and freshwater sticklebacks and hereafter call these 'sympatric sites'.

In addition, we observed substantial temporal overlap in breeding seasons (Fig. 6). In sympatric sites, genetically freshwater females were in reproductive condition earlier than genetically anadromous fish, but the breeding season of the two morphotypes coincided for at least three months. A similar pattern was observed in males in sites 2–4 although the end of the male breeding season is difficult to resolve due to the small numbers of males caught in the traps (possibly due to paternal care and nest guarding behaviour). During the peak breeding period (May–June), anadromous fish in sites 2–4 greatly outnumbered freshwater fish (114 anadromous females, 22 freshwater females, 76 anadromous males and 21 anadromous males). We believe the core migration by anadromous fish from the ocean to estuarine habitats occurred between 15 April and 12 May 2003 because we did not observe any sticklebacks in the rock pools until early May. This is consistent with the low proportion of anadromous fish in sites 2–4 in April. The proportion of gravid hybrid females is similar to freshwater females in April but declines earlier and noticeably from April through to June. The breeding season of freshwater females in site 7 is longer than freshwater females in sites 2–4.

None of the 90% confidence interval estimates around q incorporated both 0 and 1, indicating our discriminatory power was strong enough to distinguish between anadromous and freshwater individuals. Further, we were able to identify many hybrid individuals whose confidence interval estimates around q did not encompass the anadromous or freshwater ends of the scale. The majority of hybrid individuals were sampled from sites 2 to 4 where they comprise 33% of the juveniles sampled in July and August. Low frequencies of hybrids were sampled from upstream sites 5 to 8 where they comprise 4–6% of the population sampled.

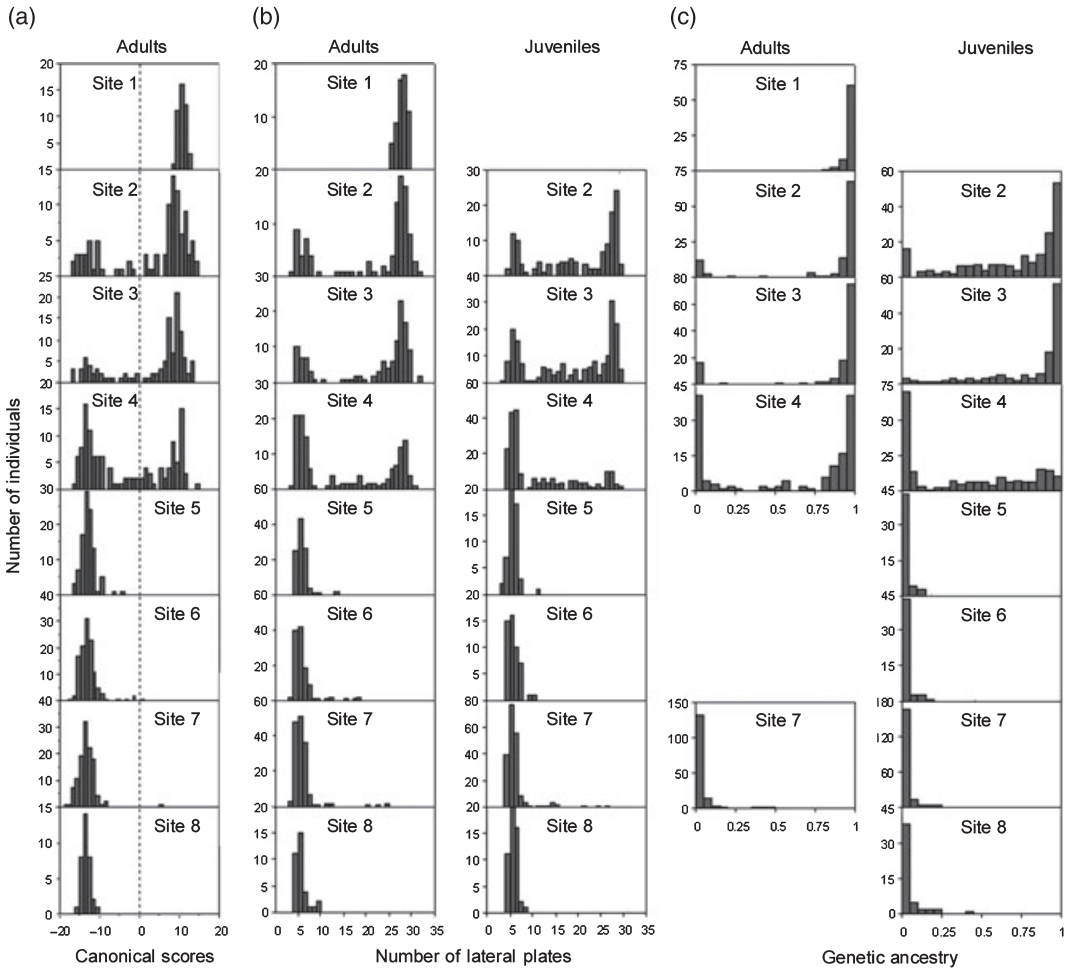

Fig. 4 Distribution of (a) morphological canonical scores in sites 1–8 for Adults (April–June 2003), (b) lateral plates in Adults (April–June 2003) and Juveniles (July–December 2003) and (c) genetic ancestry in sites 1–8, for Adults (April–June 2003) and Juveniles (July–December 2003). Individuals with positive and negative canonical scores are of anadromous and freshwater morphology, respectively. A genetic ancestry score of 0 represents freshwater ancestry and a genetic ancestry score of 1 represents anadromous ancestry. Lateral plate number (b) was used as one of the morphological traits in the canonical analysis (a). Genetic analysis of adult samples from sites 5, 6 and 8 was not performed. Despite efforts, no juveniles were collected from site 1.

Hardy–Weinberg equilibrium and cytonuclear disequilibrium

In sites 2–4, we found that the frequencies of genotypes of juveniles collected in July and August did not represent those expected from a randomly mating population. Over all loci, there was a significant deficit of heterozygotes compared to frequencies expected under Hardy Weinberg equilibrium ($P < 0.001$ in sites 2–4 and significant after sequential Bonferroni correction; Sokal & Rohlf, 1995). In contrast, the frequency of genotypes over all loci in samples of juveniles from upstream sites 7 and 8 did not deviate significantly from those expected under Hardy–Weinberg Equilibrium (Site 7 $0.05 > P > 0.01$; Site 8 $P > 0.05$; neither P values significant after Bonferroni correction). We observed consistent heterozygote deficits at the loci STN152 and STN26 in the sympatric sites, which appeared to be causing the observed overall deficit of heterozygotes since there was no significant deficit in the remaining eight loci. We conducted further analyses to determine if null alleles in the anadromous population may be causing this pattern. F_{IS} values at the locus STN26 in adults sampled from sites 1 and 7 varied, but mean values did not differ greatly in magnitude (site 7 $F_{IS} = 0.09$, site 1 $F_{IS} = 0.11$) and null alleles are therefore unlikely to be causing the observed heterozygote deficit in downstream sites. A different pattern was observed at the locus STN152, where F_{IS} values in adults were found to be consistently low in samples from site 7 (mean site 7 $F_{IS} = 0.00$) but elevated in samples adults sampled from site 1 (mean site 7 $F_{IS} = 0.33$). This pattern would be consistent with the presence

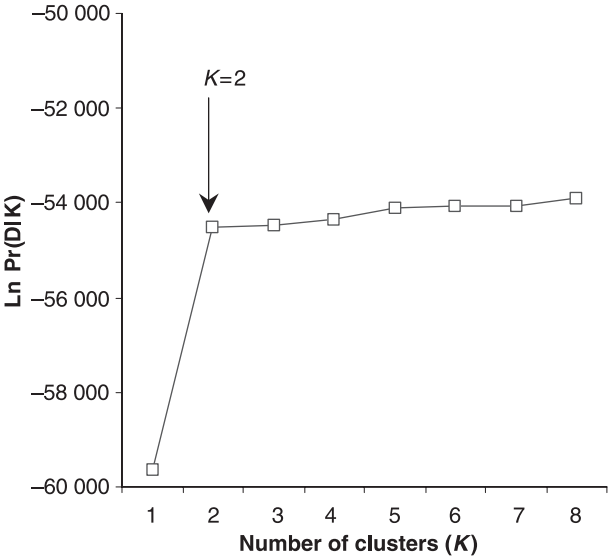

Fig. 5 The Ln probability of there being K genetic clusters in the entire data set of 1961 individuals. Following the guidelines of Pritchard *et al.* (2000), the most likely value of K is interpreted to be the lowest value of K at the start of the plateau (in this case, $K = 2$).

of null alleles at the locus STN152 in the anadromous genepool. It should be noted that repeating the above STRUCTURE analyses excluding the loci STN26 and STN152 did not qualitatively change the results or conclusions.

Juveniles sampled in July and August from sites 2 to 4 with anadromous mitochondrial haplotypes were more common than those with freshwater mitochondrial haplotypes (Table 5). We investigated cytonuclear disequilibrium and detected significant overall departures from random genotypic associations ($P < 0.001$). The freshwater mitochondrial haplotype showed strong association with the freshwater nuclear genotype, and, similarly the anadromous mitochondrial haplotype showed strong association with the anadromous nuclear genotype (Table 5). In contrast, we did not detect significant disequilibria with hybrid genotypes, indicating that the anadromous mitochondrial haplotype is sampled in hybrids as frequently as the freshwater mitochondrial haplotype.

Discussion

Evidence for divergent ecotypes

Genetic and morphological analyses provide two independent lines of evidence of divergence between anadromous and freshwater sticklebacks in the River Tyne.

Anadromous sticklebacks had more lateral plates, were larger, more robustly shaped and females possessed longer spines and deeper heads than freshwater sticklebacks. These findings are consistent with other morphological descriptions (e.g. Hagen, 1967; Walker & Bell, 2000; Jones, 2005), although previous authors have not investigated sexual dimorphism in shape. Significant differences in spine length and head depth between anadromous and freshwater females, but not males, suggests that sex-biased selection may be operating on these traits. Significant genetic differences between anadromous and freshwater sticklebacks also mirrors other studies (Hagen, 1967; McKinnon *et al.*, 2004) and this paper is novel in providing a detailed genetic description of a bimodal anadromous freshwater stickleback hybrid zone. Our findings contrast with that of Higuchi *et al.* (1996) in their study of coexisting anadromous and freshwater fish in Lake Harutori. They found no evidence of a heterozygote deficit in breeding adults and concluded that these forms make up a single breeding population despite noticeable divergence in size.

Evidence for reproductive isolation

The existence of genetic and morphological differences between anadromous and freshwater sticklebacks is not necessarily indicative of reproductive isolation since divergence may have occurred in allopatry and new anadromous individuals may be migrating into the river each year. Once in sympatry, gene flow may still occur. Therefore, to confirm that reproductive isolation exists between the forms, it is important to show either that the adult population does not mate randomly and/or that selection against hybrids occurs. Individuals of both intermediate genetic ancestry and morphology were found in sympatric sites supporting the hypothesis that hybridisation is occurring, is relatively common, and that prezygotic isolation is not complete. Genetic hybrids represent 33% of juveniles sampled from sympatric sites 2–4 in July and August. The observed hybrid frequency is lower than Hagen's (1967) estimate of hybrid frequency in sympatric sites in the Little Campbell River (46%), but higher than 1994 estimates of hybrid frequency between limnetic and benthic morphs in Enos Lake (11%; Taylor *et al.*, 2006, in press). Differences in criteria used to define hybrids in these studies means comparisons must be made cautiously, however, it is possible that the difference between the two anadromous-freshwater populations studied is due to stronger reproductive isolation between sticklebacks in the River Tyne. Our tests for cytonuclear disequilibrium in hybrids revealed no directional bias in hybridisation events. However, in terms of absolute number, we observed many more hybrid juveniles with anadromous mitochondrial haplotypes than freshwater mitochondrial haplotypes (Table 5). This is most likely due to the greater number of anadromous adults in these sites (Table 5) but might also be a result of higher fecundity of anadromous females (Hagen, 1967; F.C. Jones, C. Brown & V.A. Braithwaite, 2005, submitted). Despite the observed

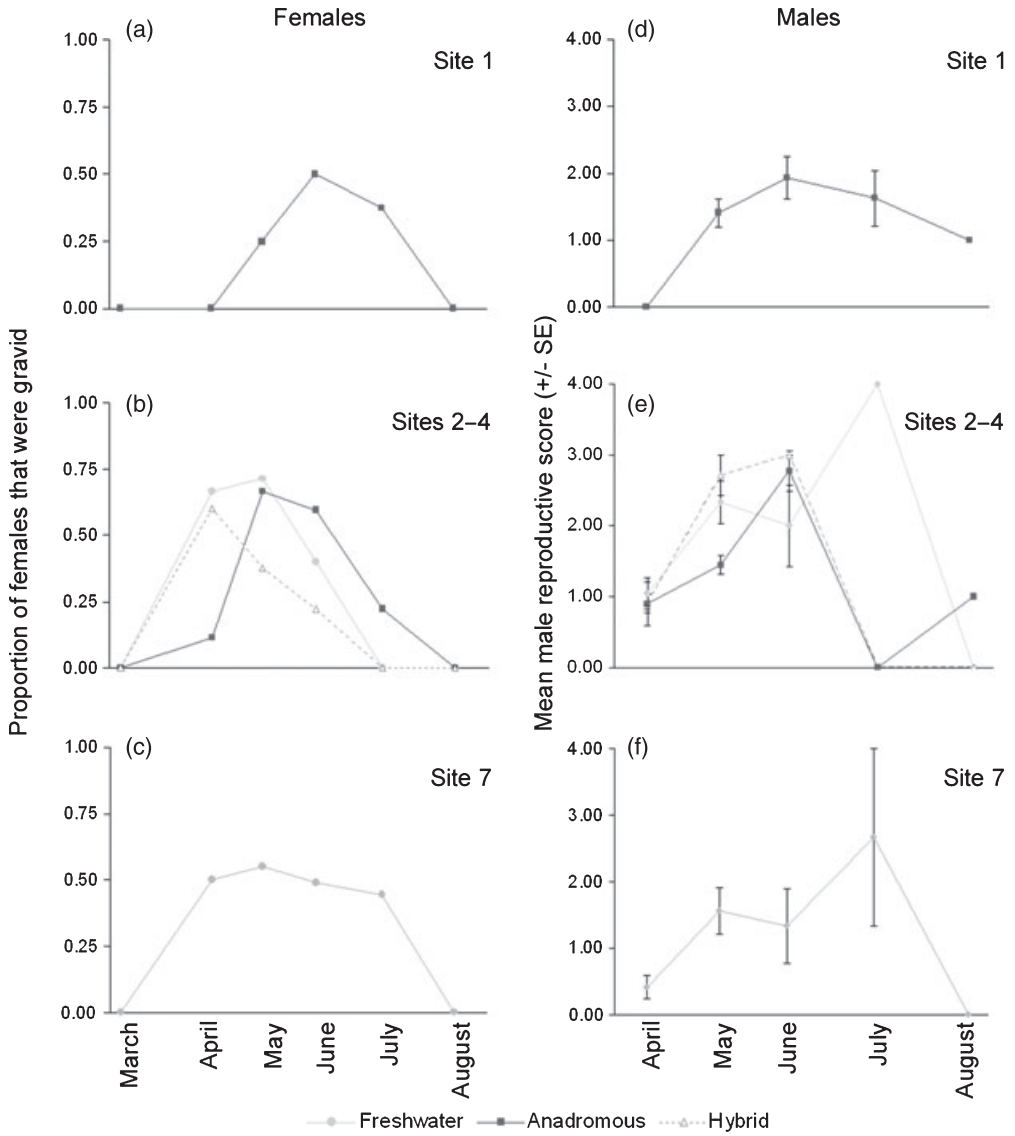

Fig. 6 The temporal distribution of adults in breeding condition in sites 1, 2–4 and 7 [females on left (a–c) and males on right (d–f)]. Sample sizes for plots: (a) 34, (b) freshwater = 85, hybrid = 34, anadromous = 163, (c) 103, (d) 40, (e) freshwater = 45, hybrid = 20, anadromous = 108 and (f) 52. Error bars do not exist for points based on a single male.

frequency of hybridisation, analysis of heterozygote deficit and cytonuclear disequilibrium suggests that mating is not random. Although the heterozygote deficit was not strong across all loci, when combined with the observation of cytonuclear disequilibrium these data provide good evidence for the existence of partial reproductive isolation between anadromous and freshwater sticklebacks. Below we discuss evidence for the contribution of prezygotic barriers (spatial barriers, temporal differences in breeding season and assortative mating) and/or postzygotic barriers (exogenous selection against zygotes/fry) to the observed reproductive isolation.

Prezygotic barriers to gene flow

Prezygotic barriers (primarily microhabitat differences and assortative mating) are thought to play an important role in restricting geneflow between other stickleback species pairs (McPhail, 1994). During the breeding season, anadromous and freshwater sticklebacks overlapped spatially for at least 3 km in the lower reaches of the River Tyne. We found an effect of stream gradient on the upstream migration of anadromous fish in the River Tyne similar to Hagen's (1967) study of the Little Campbell River. A sharp transition in genetic ancestry and morphology was observed between sites 4 and 5

Table 5 Cytonuclear genotypic disequilibria (D^* normalized disequilibria) for each of the three nuclear genetic ancestry groups based on juveniles sampled in July and August from sites 2 to 4 combined. In this analysis, a negative D^* represents an excess of anadromous mitochondrial haplotypes associated with a particular genotype, and a positive value an excess of freshwater mitochondrial haplotypes associated with a particular genotype.

	Number of individuals with mitochondrial haplotype			
	Anadromous	Freshwater	D^*	P value
Number of individuals with nuclear genetic ancestry (q)				
Anadromous	114	5	−0.8380	<0.0001
Hybrid	54	24	0.0902	0.4954
Freshwater	9	33	0.7107	<0.0001

corresponding to the lowest weir on the river. The high density of anadromous fish below the weir and their absence above it suggests that the weir prevents upstream migration despite the presence of a fish ladder. The fragmenting effect of weirs on gene flow has been reported in other river systems (Meldgaard *et al*., 2003). It is likely that this weir is contributing to prezygotic isolation, acting as a barrier to hybridisation between anadromous and freshwater sticklebacks by limiting overlap in spatial distribution. Within sites of overlap (sites 2–4), we observed no differences in habitat preferences on a large scale, since we regularly caught both morphotypes in any given trap. However, this does not preclude the possibility of microhabitat preferences occurring on a very fine scale. In the mosaic hybrid zone between firebellied toads, habitat preference for puddles vs. ponds was found to play an important role in limiting gene flow between *Bombina variegata* and *Bombina bombina* (MacCallum *et al*., 1998). It is unknown whether the microhabitat preference of anadromous-freshwater stickleback morphotypes observed by Hagen (1967) corresponds to nesting location, and at present, no studies of nest location in a hybrid zone have been carried out. Nesting location may affect encounter rate by females and could contribute significantly to prezygotic isolation between the morphs.

A substantial temporal overlap in the breeding season of anadromous and freshwater sticklebacks occurs in the months of April through to July. Despite the greater temporal overlap of breeding seasons in the River Tyne compared to the Little Campbell River (Hagen, 1967), the fact that fewer hybrids were sampled in sympatric sites of the River Tyne (33%) compared to that in sympatric sites of the Little Campbell River (46%) suggests that reproductive isolation is stronger in the River Tyne. It is important to note that even the small temporal difference in the breeding seasons observed in sympatric sites 2–4 may contribute to prezygotic isolation between anadromous and freshwater sticklebacks by limiting the potential for hybridisation in the early and later months of the breeding season.

A previous study of sympatric sticklebacks from the River Tyne conducted in outdoor ponds (F.C. Jones, C. Brown & V.A. Braithwaite, 2005, submitted), found no evidence of assortative mating. However, in the present study we are unable to rule out the existence of assortative mating occurring in the wild, since it may be mediated by ecological conditions that F.C. Jones, C. Brown & V.A. Braithwaite (2005, submitted) did not investigate (e.g. flow, water depth, substrate, and vegetation). Ecologically dependent sexual selection has been found in several other studies (e.g. host plant specialisation in pea aphids, Caillaud & Via, 2000; song-type in song sparrows, Patten *et al*., 2004). A recent study by Taylor *et al.* (2006), in press) suggests that the breakdown of reproductive isolation between limnetic and benthic sticklebacks in Enos Lake, Canada (thought to be primarily prezygotic isolation, Ridgway & McPhail, 1984) may be associated with changes in ecology. Other factors, which may influence assortative mating in the wild include the ratio of anadromous: freshwater females during the peak breeding season (1 : 1 in F.C. Jones, C. Brown & V.A. Braithwaite, 2005, submitted, but 5 : 1 present study), and nesting density.

Postzygotic barriers to gene flow

Jiggins & Mallet (2000) report that bimodal hybrid zones appear to be associated with strong prezygotic barriers to gene flow. The hybrid zone between anadromous and freshwater sticklebacks in the River Tyne is strongly bimodal yet the relatively high frequency of hybrid juveniles suggests that prezygotic barriers are not particularly strong. We believe postzygotic barriers to gene flow are likely to play an equally important role in maintaining the morphological and genetic divergence between anadromous and freshwater morphs in the River Tyne. The sample of juveniles in the present study did not include fry <25 mm standard length, and it is likely that selection against hybrid zygotes or fry may have already contributed towards the observed heterozygote deficit and cytonuclear disequilibrium. Hybrid juveniles in the River Tyne >25 mm had reduced probability of over winter survival, and adult female hybrids had reduced probability of being gravid (Jones, 2005). In addition, Jones *et al.* (2005, submitted) found some evidence supporting the idea that postzygotic barriers to geneflow act on hybrid fry <10 mm.

In sympatric populations of *Drosophila* sister species, prezygotic isolation appears to evolve faster than postzygotic isolation, and when postzygotic isolation evolves, it tends to affect males before females (Coyne & Orr, 1997). The applicability of these conclusions to taxa outside the *Drosophila* group is unknown (for some exceptions see Virdee & Hewitt, 1992; Moyle *et al*., 2004; Saldamando *et al*., 2005). Furthermore, studies of already divergent sister species cannot resolve the factors or processes leading to reproductive isolation since they may be obscured by, and difficult to separate from, those which

have diverged or played a role in divergence after speciation. In this respect, recently diverged 'incipient species' such as the anadromous and freshwater sticklebacks discussed above may provide better insight into processes and factors involved in initiating speciation. Genetic evidence indicates that temporal differences in breeding season may contribute in part to prezygotic isolation between anadromous and freshwater sticklebacks *in situ*. However, this barrier to gene flow is not strong since hybridisation occurs often. Hybrids made up 33% of juveniles sampled in sympatric sites and hybridisation was bidirectional. The roles of microhabitat preference, ecologically dependent assortative mating, and selection against hybrid zygotes/fry remain to be investigated further in sympatric wild populations.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1. Ln likelihood values of there being $K = [1–3]$ genetic clusters within samples collected from each of sites 1 to 8, and the proportion of individuals assigned to each of the 1–3 clusters.

This material is available as part of the online article from http://www.blackwell-synergy.com

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