MOLECULAR ECOLOGY

Molecular Ecology (2014) **23**, 3226–3240 doi: 10.1111/mec.12820

Gene expression plasticity evolves in response to colonization of freshwater lakes in threespine stickleback

MATTHEW R. J. MORRIS,* ROMAIN RICHARD,* ERICA H. LEDER,† ROWAN D. H. BARRETT,‡ NADIA AUBIN-HORTH§ and SEAN M. ROGERS*

Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, AB, Canada T2N 1N4, †Division of Genetics and Physiology, Vesilinnantie, Department of Biological Sciences, University of Turku, 20014 Turku, Finland, ‡Redpath Museum and Department of Biology, McGill University, 859 Sherbrooke Street West, Montreal, QC, Canada H2J 3G5, §Département de Biologie, Institut de Biologie Intégrative et des Systèmes, Université Laval, 1030 avenue de la Médecine, Québec, QC, Canada G1V 0A6

Abstract

Phenotypic plasticity is predicted to facilitate individual survival and/or evolve in response to novel environments. Plasticity that facilitates survival should both permit colonization and act as a buffer against further evolution, with contemporary and derived forms predicted to be similarly plastic for a suite of traits. On the other hand, given the importance of plasticity in maintaining internal homeostasis, derived populations that encounter greater environmental heterogeneity should evolve greater plasticity. We tested the evolutionary significance of phenotypic plasticity in coastal British Columbian postglacial populations of threespine stickleback (*Gasterosteus aculeatus*) that evolved under greater seasonal extremes in temperature after invading freshwater lakes from the sea. Two ancestral (contemporary marine) and two derived (contemporary freshwater) populations of stickleback were raised near their thermal tolerance extremes, 7 and 22 °C. Gene expression plasticity was estimated for more than 14 000 genes. Over five thousand genes were similarly plastic in marine and freshwater stickleback, but freshwater populations exhibited significantly more genes with plastic expression than marine populations. Furthermore, several of the loci shown to exhibit gene expression plasticity have been previously implicated in the adaptive evolution of freshwater populations, including a gene involved in mitochondrial regulation (PPARAa). Collectively, these data provide molecular evidence that highlights the importance of plasticity in colonization and adaptation to new environments.

Keywords: adaptation, Baldwin effect, ecological genomics, parallel evolution, phenotypic plasticity, temperature

Received 21 March 2014; revision received 27 May 2014; accepted 28 May 2014

Introduction

A wealth of evidence has accumulated to show that the environment not only selects phenotypic variants with high fitness (Grant *et al*. 1976; Rundle *et al*. 2000), but is also integral to the development of the phenotype (Baldwin 1896; Woltereck 1909; Bradshaw 1965; Lewontin 1974; Agrawal 2001; West-Eberhard 2003; Moczek *et al*. 2011). The same genotype may produce alternative environmentally induced phenotypes, a phenomenon referred to as phenotypic plasticity. Yet, the exact role of phenotypic plasticity in adaptive evolution remains a largely unresolved problem in evolutionary biology.

Baldwin (1896, 1902) proposed that genotypes that exhibit adaptive plasticity in response to novel environmental conditions may be more likely to survive than nonplastic genotypes, conferring plasticity to

Correspondence: Matthew R.J. Morris, Fax: (403) 289-9311; E-mail: mrjmorri@ucalgary.ca

subsequent generations (Simpson 1953). Thus, plasticity can 'buffer' populations against evolutionary change during colonization, by producing phenotypes of relatively high fitness (Chevin & Lande 2010; Pavey *et al.* 2010; Thibert-Plante & Hendry 2011; Kovach-Orr & Fussmann 2013). Baldwin also theorized that selection on standing genetic variation or de novo mutations could result in the evolution of plasticity, with possible evolutionary scenarios including the loss of plasticity in novel stable environments (genetic assimilation – Waddington 1953; Crispo 2007), the loss of plasticity as an adaptation to resist phenotypic change (genetic compensation – Grether 2005) or the formation of adaptive plasticity (Bradshaw 1965; West-Eberhard 2003; Pigliucci *et al.* 2006; for the role of nonadaptive plasticity, see Ghalambor *et al.* 2007; Fitzpatrick 2012; Morris & Rogers 2013). These two hypotheses are not mutually exclusive: pre-existing plasticity could permit colonization and thereby inhibit evolution for some phenotypes, but fitness could be improved after colonization by the evolution of plasticity in other phenotypes. Comparisons between ancestral and derived populations have provided evidence for the importance of plasticity during colonization (Yeh & Price 2004; Torres-Dowdall *et al.* 2012; Vedder *et al.* 2013; but see Reed *et al.* 2013) and the evolution of plasticity (Day *et al.* 1994; Aubret *et al.* 2004; Parsons & Robinson 2006; Wund *et al.* 2008; Pfennig & McGee 2010; Schwander & Leimar 2011; Latta *et al.* 2012; Svanbäck & Schluter 2012; Yampolsky *et al.* 2012; see also Morris & Rogers 2014 and references therein), but the role of gene expression in colonization success and adaptation to new environments remains unexplored (but see McCairns & Bernatchez 2010).

One of the primary drivers of adaptation to new environments in all organisms is temperature (Powers *et al.* 1991; Somero 2010; Hoffmann & Sgrò 2011; Amarasekare & Savage 2012). Temperature changes can disrupt internal homeostasis in fish (Guderley *et al.* 1994; Hayward *et al.* 2007; Tomanek 2008; Kammer *et al.* 2011; Schulte 2014). Phenotypic plasticity in terms of behaviour and physiology (Kammer *et al.* 2011) may help maintain homeostasis, but widespread temperature-induced changes in gene expression also play an integral role (West-Eberhard 2003; Gracey *et al.* 2004; Podrabsky & Somero 2004).

Species that underwent the postglacial colonization of freshwater lakes from the marine environment are uniquely suited to the study of the role of phenotypic plasticity in a novel environment (Rogers *et al.* 2013). The threespine stickleback (*Gasterosteus aculeatus*) is an abundant species in both marine and freshwater habitats across the Northern Hemisphere. Since the end of the last glacial advance, marine stickleback have invaded newly created freshwater habitats along the west coast of North America (Bell 1976; Bell & Foster 1994). In British Columbia, Canada, this began as late as 13 000 years ago (Clague *et al.* 1982; Hutchinson *et al.* 2004). Novel environmental conditions within these freshwater bodies, including salinity, temperature, water density, and predator and parasite diversity, likely contributed to the subsequent phenotypic and genetic differentiation of freshwater stickleback populations from their marine ancestors (e.g. Lindsey 1962; Bell 2001; Wund *et al.* 2008; Schluter & Conte 2009; McCairns & Bernatchez 2010; Jones *et al.* 2012; Rogers *et al.* 2012, 2013; Bell & Aguirre 2013). The marine stickleback populations have remained relatively unchanged, at least morphologically and possibly genetically, since this period of divergence (Bell 1977; Walker & Bell 2000; Hohenlohe *et al.* 2010; but see caveats to this assumption in the discussion). The invasion of lakes from the marine environment provides researchers with the unusual benefit of being able to test predictions about the course of stickleback evolutionary history, through comparisons of contemporary 'ancestral' and 'derived' stickleback populations or multiple independently derived freshwater populations (e.g. Bell 1995; Walker & Bell 2000; Kristjánsson *et al.* 2002; Bell *et al.* 2004; Colosimo *et al.* 2005; Barrett *et al.* 2008; Chan *et al.* 2010; Furin *et al.* 2012).

When marine stickleback invaded freshwater lakes in coastal British Columbia, they encountered new and broader temperature regimes that led to the rapid evolution of thermal tolerance. The average mean monthly surface temperature in British Columbia varies between 6.5 and 16.5 °C in the marine environment and 4 and 21 °C in freshwater lakes (Barrett *et al.* 2011). Freshwater stickleback evolved lower temperature tolerances that reflect their colder habitats, but marine and freshwater stickleback both tolerate temperatures higher than currently required (Barrett *et al.* 2011). Furthermore, marine stickleback released in experimental ponds evolved within three generations cold tolerance similar to freshwater stickleback (Barrett *et al.* 2011). Understanding the role of gene expression plasticity in such rapid adaptation remains an important goal of molecular ecology research (Andrew *et al.* 2013; Narum *et al.* 2013). Environmentally sensitive variation in transcript abundance at the genome-wide scale can now be measured as a plastic molecular phenotype (Aubin-Horth & Renn 2009; Pavey *et al.* 2010; Smith *et al.* 2013), facilitating the testing of these hypotheses for thousands of phenotypes while identifying candidate loci for plasticity. Measuring phenotypes at this level of organization is crucial because temperature has wide-ranging effects on physiological processes.

In this study, we used a species-specific microarray to compare temperature-induced gene expression plasticity in laboratory-reared families of two marine and

two freshwater populations of threespine stickleback raised under temperatures nearing the critical minimum and maximum temperatures experienced in the marine habitat (7 and 22 °C). These particular marine and freshwater populations were the same used in Barrett *et al.* (2011). Similarities in temperature-induced plasticity between stickleback of different origins would lend support to the hypothesis that the marine colonists of freshwater lakes displayed plasticity in their new environment that facilitated their survival and buffered against further evolutionary change. An increase in freshwater stickleback plasticity would support the non-mutually exclusive hypothesis that stickleback evolved physiological mechanisms to cope with the greater seasonal temperature extremes of the freshwater environment. Using microarrays can identify candidate loci for both aspects of the Baldwin effect.

Materials and methods

Experimental organisms

Wild threespine stickleback (*Gasterosteus aculeatus*) were sampled from two freshwater populations (Cranby Lake, CLF, on Texada Island, 49°42′00″, 124°30′00″, and Hoggan Lake, HLF, on Gabriola Island, 49°36′00″, 124°01′20″) and two marine locations representing putative populations (Oyster Lagoon, OLM, 49°36′48.6″N, 124°1′46.88″, and Little Campbell River, LCM, 49°1′4″, 122°45′52″) along the southern coast of British Columbia, Canada from 2006 to 2008 (Fig. 1). We chose these four populations following recent evidence of more extreme seasonal temperatures in these freshwater habitats, greater temperature tolerance breadth in these freshwater populations and demonstrations of rapid adaptation to freshwater temperatures in freshwater pond-reared OLM stickleback (see Barrett *et al.* 2011). Adults were crossed at the University of British Columbia (Vancouver, BC, Canada) in summer 2009 to produce families (freshwater = 9 families total, marine = 13 families total) of pure lines of F1 individuals. Families were pooled together. These families were also used in Barrett *et al.* (2011), the predecessor to this study. Juveniles were fed live brine shrimp nauplii with the start of active swimming. On 13 September 2009, upon attaining a length of approximately 2 cm, juveniles were switched to a diet of chironomid larvae ('bloodworms'). Temperature was maintained at 17 °C.

On 25 September 2009, juveniles from all four populations were shipped to the University of Calgary

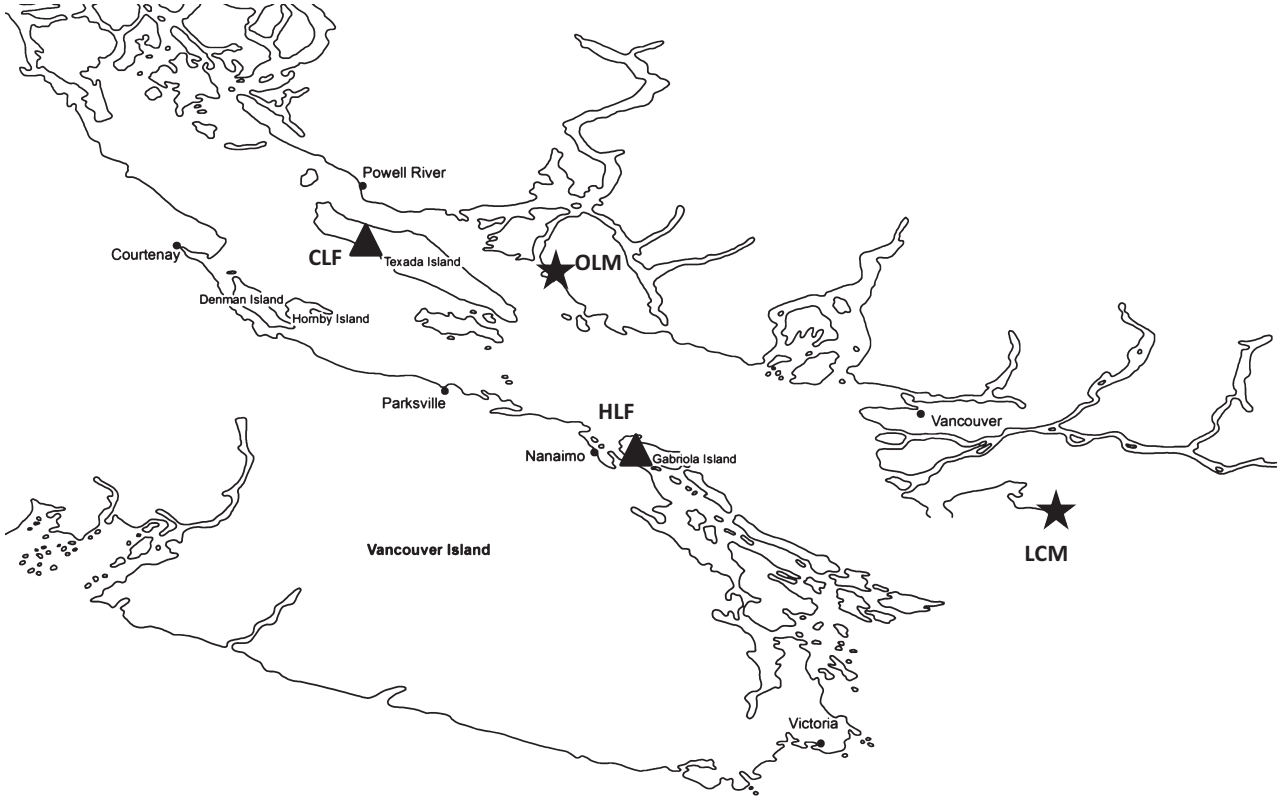

Fig. 1 Geographical location of threespine stickleback populations used in this experiment. Stars indicate marine sampling sites, triangles indicate freshwater sampling sites. CLF, Cranby Lake (freshwater); HLF, Hoggan Lake (freshwater); OLM, Oyster Lagoon (marine); LCM, Little Campbell River (marine).

(Calgary, AB, Canada). The number of individuals from each family was unknown. From September 2009 to February 2010, all stickleback were kept in 114-L (30-gallon) tanks in a flow-through system under a 12:12 h light: dark cycle in the Life and Environmental Sciences Animal Resources Centre (LESARC) at the University of Calgary in accordance with the Canadian Council on Animal Care. Juveniles were fed frozen bloodworms to satiation twice daily. Temperatures were maintained at 18 °C.

On 2 March 2010, juvenile fish were injected subepidermally with a fluorescent elastomer tag (Northwest Marine Technology, Inc.) to denote their population and were then added to 114-L (30 gallon) tanks (20 fish per tank). They were allowed to acclimate post-tagging for 20 days. To commence the experiment, fish from each population were matched for length. Fish were selected to produce similar sample means and variances for each population (HLF 43.6 mm ± 4.7 SD; CLF 48.8 mm ± 5.7 SD; OLM 41.2 mm ± 3.8 SD; LCM 42.3 mm ± 4.4 SD).

Experimental design

On 23 March 2010, tagged fish from each population were randomly assigned to a 7 or 22 °C temperature treatment, these temperatures being within the tolerance range of all populations (Barrett *et al.* 2011). Marine fish were kept separate from freshwater fish, at densities of 20 fish (10/population) per tank with three replicate tanks per origin per temperature. Insufficient numbers of LCM meant only two of the three marine replicates contained this population, for either temperature, with nonexperimental stickleback used to maintain densities. Fish were fed frozen bloodworms ad libitum twice daily. Each tank was equipped with its own filter, air pump, water supply and temperature regulator. Salinity was maintained at 5–6 ppt to promote health and maintain proper development in both marine and freshwater stickleback (Heuts 1947; Ahn & Gibson 1999).

Temperature experiment

The cold (7 °C) treatment was maintained by JBJ Arctica 1/10 hp titanium aquarium chillers (one per tank), and the warm (22 °C) treatment was maintained by Fluval E50 electronic heaters (one per tank). Temperatures were recorded daily with an Accu-Temp Instant Read digital thermometer. The temperature treatments were tightly regulated by chillers and heaters, although the daily chiller variance was lower (chillers: average 7.3 °C ± 0.2 SD; heaters: average 22.1 °C ± 0.8 SD).

All fish experienced the same thermal history in terms of growing degree days, where GDD = sum of [daily temperature readings − base temperature] with base set at 0 °C (Neuheimer & Taggart 2007), rather than an equal number of Julian calendar days. This approach avoided sampling fish at different developmental stages, as variation in length-at-age and development in fish correlates strongly with thermal history (Neuheimer & Taggart 2007). The 22 °C treatment ended on 11 June 2010 (average of 1699 GDD). The 7 °C treatment ended on 17 November 2010 (average of 1704 GDD). The lengths of the warm-water fish did not significantly differ from those of the cold-water fish at the end of the experiment (2-sample *t*-test: HLF $P = 0.71$, CLF $P = 0.4$, OLM $P = 0.34$, LCM $P = 0.33$), justifying the use of GDD instead of Julian calendar (see Supporting Information 1).

Two fish per population per replicate (four fish per tank) were sampled for the microarray experiment, for a total of 44 fish. Their heads were removed posterior to the operculum, the pelvic girdle and internal organs were removed, and the remaining muscle, bone and epidermis were cut into small (<0.5 cm all around) pieces and preserved in RNALater (Ambion, Inc). Samples were frozen at −20 °C until RNA could be extracted.

Mortality levels during the experiment were 12% and varied among populations [HLF, 1% (two of 140); CLF, 8% (five of 60); OLM, 18% (11 of 60); LCM, 42% (17 of 40)]. The high mortality in marine populations should arguably be noted as a potential bias, but should not be in a direction that compromises our conclusions. If mortality was due to salinity-temperature-related stress, the marine survivors should be those better able to plastically adjust their transcript levels to acclimate to these temperatures (Gracey *et al.* 2004); thus, mortality during the experiment should underestimate the differences observed between marine and freshwater populations. Mortalities were replaced with nonexperimental stickleback and were excluded from the analysis.

Microarrays

The threespine stickleback 8 × 60 K microarray was designed as an extension of a previously published and validated stickleback array (Leder *et al.* 2009). The microarray design was submitted to Agilent Technologies using Agilent eArray v.7.7. Each array contained 308 negative controls (3xSLv1), 350 Agilent spike-ins for quality control, 612 positive controls and 60 343 experimental features representing 19 959 genes and 20 021 transcripts (88% of known, projected and novel protein-coding genes in stickleback – Flicek *et al.* 2014). Hereafter, we refer to these 19 959 genes and 62 alternative splice variants as 'genes'. 19 881 genes were replicated three times on each array; an additional 40 genes were

replicated ten times, as per Agilent protocols. In total, each array contained 61 662 control and feature spots.

RNA extraction and amplification

RNA extraction (February 2011) and reverse transcription/amplification (June 2011) were conducted at the Institut de Biologie Intégrative et des Systèmes (IBIS; Université Laval, Québec, QC, Canada). Briefly, 0.07 g of white muscle tissue, with dermal and skeletal tissue, per fish was homogenized, and total RNA was isolated using the Trizol (Invitrogen)–chloroform method (Chomczynski & Sacchi 1987). Total RNA was suspended in RNA Secure (Ambion, Inc). The quality of each sample was measured on the Thermo Scientific NanoDrop 2000c UV Spectrophotometer (for RNA abundance and contamination) and the Bio-Rad Experion (for RNA degradation). Only samples with A260/A280 ~2 (average 2.0, SD 0.03) and A260/A230 ratios >1.8 were retained for analysis, and every individual yielded high-quality RNA (see Table S2.1, Supporting information). Suspended RNA was preserved at −80 °C. Processing occurred over several days, with representatives from all populations and all treatments processed each day (as per Magni *et al.* 2011).

In June 2011, reverse transcription and amplification of RNA was performed. Total RNA was converted to cDNA and then cRNA according to Agilent's One-Color Microarray-Based Gene Expression protocol, using Agilent's Low Input Quick Amp Labeling Kits. Excess cyanine-3 was removed with Qiagen's RNeasy mini kits. Labelled cRNA was quantified using the NanoDrop; samples with cRNA yield >0.825 μg and specific activities >6 pmol cyanine-3 per μg cRNA were used for the microarrays (Table S2.2, Supporting information).

Labelled cRNA was hybridized onto the microarray by Genome Québec (McGill University, Montréal, QC, Canada). For each sample, 600 ng of cyanine-3-labelled linearly amplified cRNA was used, as per the Agilent Gene Expression Hybridization Kit protocol. Six 8 × 60 K arrays were loaded with cRNA sample (one individual per array for 44 arrays) and allowed to hybridize in a SureHyb chamber at 65 °C for 17 h. Slides were then washed in Agilent Gene Expression Wash Buffers 1 (treated with Triton X-102) and 2 to remove excess sample. High-resolution 3-micron images were taken of the slides using an Agilent Technologies Scanner G2505C.

Microarray statistical analyses

Microarray images were processed using AGILENT FEATURE EXTRACTION v. 10.7.3.1. Gridded features were located using the 'cookie-cutter' method (Agilent Technologies, Inc. 2009). Median pixel intensity within the cookie was used as a measure of feature intensity. Any features flagged as population outliers (features with signal intensities that differed significantly from those of replicate features, as calculated using a *Q*-test) and/or nonuniform outliers (features with signal variances that exceeded the estimated variance multiplied by a chi-squared confidence interval) during within-array normalization were excluded from analyses (Agilent Technologies, Inc. 2009). Background was estimated as the average of all negative controls (i.e. features designed to be unable to bind to labelled cRNA). Data underwent both spatial and multiplicative detrending. Based on the Feature Extraction quality control reports, two arrays (one sample each of OLM at 22 °C and LCM at 22 °C) were discarded for failing to meet robust quality measures.

Processed background-subtracted median intensities for all probes were inputted into the Linear Model for Microarrays (LIMMA, Smyth 2004, 2005) software package in R (Ihaka & Gentleman 1996). Between-array normalization was performed using the quantile method on $\log_2$-tranformed signal intensities (Fig. S2.1, Supporting information). High-intensity features that were flagged in Feature Extraction as having intensity values >2.6 SD above the background signal SD (Agilent Technologies, Inc. 2009) were subsequently retained for further analysis, if they passed this threshold in ≥85% of the 7 or 22 °C treatment arrays. Features that did not meet this threshold were filtered from all arrays, reducing the total number of genes to 14 208. Following filtering, control spots were removed from the analysis, and replicate probes within an array were averaged.

The processed data were fit to a mixed effects model in MATLAB (The MathWorks Inc 2012) using the function *anovan*. An initial analysis ruled out tank effect, and so the full model included origin (marine, freshwater) and temperature (7, 22 °C) as fixed effects, with population (HLF, CLF, OLM, LCM) as a random effect nested within origin. Contrasts were then performed to determine the number of genes influenced by temperature in marine vs. freshwater stickleback. False discovery rate was controlled following Storey (2003), and the significance level for any test was set at $q \leq 0.05$. Overall gene expression differences were visualized using FLEXARRAY v.1.6.1 (Blazejczyk *et al.* 2007). A proportional Venn diagram was generated using BIOVENN (Hulsen *et al.* 2008). The data were reanalysed in LIMMA (Smyth 2004, 2005) to produce population-level data (see Supporting Information 1).

Gene enrichment analysis

Gene ontology identification (GOID) accessions from the stickleback genome were downloaded using the ENSEMBL GENOME BROWSER (Flicek *et al.* 2014). Seven thousand three

hundred and sixty of the differentially expressed transcripts had GOIDs we could use in further analyses. The relative contribution of various functional categories was visualized using the CATEGORIZER tool (Hu *et al.* 2008).

To explore whether certain functional categories were over-represented in the data, GOID accessions for differentially expressed gene sets were compared with the reference microarray gene set (all genes from the microarray with GOID accessions, 13 932 genes) using GOEAST (Zheng & Wang 2008). Adrian Alexa's improved weighted scoring algorithm was used to decorrelate related accessions, and the data were analysed using Fisher's exact test. The significance level of enrichment was set at the recommended 0.01, with a minimum of five associated genes required in the gene set. The gene sets that were analysed included all genes up-regulated at 7 °C for each origin separately, and all genes up-regulated at 22 °C for each origin separately. Hierarchical gene ontology graphs were constructed using GOEAST and uploaded to Dryad (doi:10.5061/dryad.5q65k).

Candidate genes

Jones *et al.* (2012) performed genome scans of 21 stickleback from multiple marine–freshwater pairs sampled from across their global distribution and looked for genomic regions that consistently showed genetic divergence between marine and freshwater populations. Gene sequence positions for differentially expressed genes found in the current study were downloaded from Ensembl. The positions of genes scored as being plastic in one or both origins were compared with the positions of regions showing marine–freshwater divergence. If there was full or partial overlap in sequence positions, that gene was considered a candidate for plasticity evolution.

Results

Temperature-induced gene expression plasticity in marine and freshwater stickleback

The full model revealed that, although several hundred genes showed significant origin or interaction terms, the majority of genes were significant only for the temperature term (n = 9479 genes; Table 1; Fig. S2.2, Supporting information). When contrasts were performed within origin, transcript abundance was significantly affected by temperature in marine and/or freshwater stickleback for 10 699 (75%) of the 14 208 genes represented in the filtered data set. Of these, 6380 genes were up-regulated at 7 °C, and 4319 genes were up-regulated at 22 °C.

Table 1 Number of genes with significant origin, temperature, origin and temperature, or interaction terms. q denotes P-value after false discovery rate. Null indicates a lack of significance for all terms and interactions

Model term	No. of genes $q < 0.05$	No. of genes $q < 0.1$
Null	3773	2322
Origin	188	206
Temperature	9479	10 039
Origin + temperature	436	786
Origin * temperature (interaction term)	332	855

Both freshwater and marine stickleback were plastic for the same 5745 genes, but freshwater stickleback exhibited plasticity for an additional 3347 genes, while marine stickleback were plastic for an additional 1607 genes (Fig. 2). Thus, freshwater stickleback had significantly more genes respond to the temperature treatment than did marine stickleback (χ^2 = 611, d.f. = 1, $P < 0.001$).

Gene enrichment

Freshwater and marine stickleback gene expression was characterized largely by differences in the rankings of the GO Slim categories, and not the categories themselves (Figs S2.3, S2.4, S2.4, Tables S2.3, S2.4, S2.5, Supporting information).

Cold temperatures up-regulated 48 and 54 enriched gene classes in freshwater and marine stickleback, respectively (Table 2, gene enrichment file in Dryad). Enriched in both origins were classes involved in

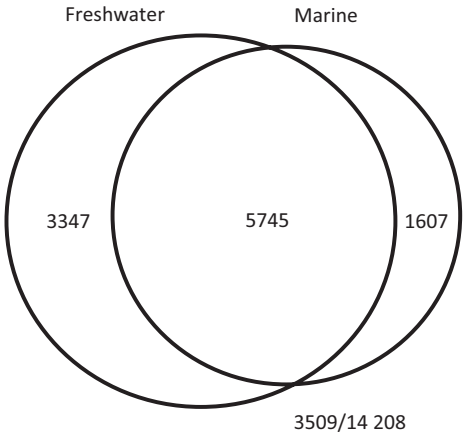

Fig. 2 Venn diagram showing the number of genes with plastic expression from separate marine and freshwater contrasts. The #/14 208 indicates the number of genes without plastic expression/total.

protein catabolism, translational and transcriptional regulation, and aerobic respiration within mitochondria (including genes SDHC, COX1, OGDHb, IDH3A, CS, SDHB, ACO2). Of the latter, the gene set for freshwater stickleback was further enriched for gene classes involved in cellular respiration, the electron transport chain, ATP proton transport, mitochondrial membrane and proton-transporting two-sector ATPase complex. Of the sixty unique genes found in these categories, thirty were plastic in freshwater stickleback only, including PPARGC1β, PPARAa, NRF-1 and COXIV. The gene set for marine stickleback was enriched for genes involved in ribonucleoprotein production; this class was not enriched in freshwater stickleback.

Warm temperatures up-regulated 22 and 10 enriched gene classes in freshwater and marine stickleback, respectively (Table 2, gene enrichment file in Dryad). Protein ubiquitination was enriched in both origins, with the same 24 genes involved in marine and freshwater stickleback. For biological processes, the marine stickleback expression data set was further enriched for DNA-dependent transcriptional regulation and hexose biosynthetic process, while the freshwater stickleback expression data set was enriched for protein folding, translation, protein ADP ribosylation, oxidation–reduction process and nucleosome assembly.

Candidate genes

From Jones *et al.* (2012), we identified 21 chromosomal regions that overlapped with 15 temperature-induced genes on our array (Table 3). Eight of these genes were plastic for both origins: FILIP1L, CPEB4, NXT2, RPAP3, PNPLA3, SLC25a15b and two novel transcripts. Although all eight loci overlapped with regions of marine–freshwater divergence, no divergence-related SNPs from these genes are known, and they are likely outlier regions of divergence for reasons other than temperature-related plasticity.

One chromosome region from Jones *et al.* (2012) overlapped with a gene up-regulated at 22 °C in marine stickleback only: IGFBP2a. No divergence-related coding SNP is known for this gene. One chromosome region overlapped with a gene up-regulated at 7 °C in freshwater stickleback only: PPARAa. This gene was considered by Jones *et al.* (2012) to be a strong candidate under selection for marine–freshwater divergence. No divergence-related SNPs for this gene are known. Five chromosomal regions overlapped with genes up-regulated at 22 °C in freshwater stickleback only: INHa; SPEG, which has two known SNPs; OBSL1; a novel gene located near GARP on linkage group IV, with one known SNP; and NLRC5, which has four known SNPs.

Discussion

We explored the evolutionary role for gene expression plasticity in adaptation to novel environments by rearing threespine stickleback from two marine and two freshwater populations at two different temperatures (7 and 22 °C). These freshwater populations are derived from common marine ancestors. The contemporary marine form resides in environments that are presumably similar in thermal regime to the ancestral condition, while the freshwater form resides in thermally variable environments and are presumably adapted to these new conditions (Barrett *et al.* 2011). Estimates of gene expression plasticity via transcript abundance using an unbiased genome-wide approach revealed, first, that a large number of genes showed similar temperature responsiveness in marine and freshwater stickleback. This was evident despite rearing marine stickleback under freshwater salinities. Second, there were differences in plasticity between marine and freshwater stickleback, with freshwater stickleback having significantly more genes exhibiting plastic expression. Collectively, these results support the Baldwin effect. Here, we discuss the possible mechanisms underlying these patterns and some alternative hypotheses to explain the potential role of gene expression plasticity in adaptation.

Plasticity and the lack of evolution

Baldwin (1896, 1902) hypothesized that plasticity may play an important role by facilitating individual survival in new environments. Such plasticity could be expressed and functional in both the old and new environments or be newly expressed in the new environment (Schlichting 2008). By facilitating survival in the face of strong directional selection, this plasticity could result in stabilizing selection and a lack of evolutionary change (Ghalambor *et al.* 2007). Although several studies have validated the significance of plasticity for survival (Yeh & Price 2004; Engel & Tollrian 2009; Vedder *et al.* 2013), experimental evidence for the role of gene expression plasticity in the colonization of new environments, using whole genome approaches, can help resolve the role of plasticity during adaptive divergence (Pavey *et al.* 2010).

Over fifty-seven hundred genes were plastic in both marine and freshwater stickleback. The majority of these were similarly plastic in both origins, in terms of direction and slope (for instance, note the low origin x temperature interaction, Table 1). That is, plasticity for these genes does not appear to have evolved since colonization of the freshwater environment. This similarity in plasticity was found under freshwater conditions, raising two possibilities: either plasticity for these genes

Table 2 Gene ontology of significantly enriched genes involved in biological processes, for marine or freshwater threespine stickleback, up-regulated at 7 or 22 °C. For identification of genes, see gene enrichment file in Dryad. Level = the longest number of paths connecting gene ontology accession term to root of hierarchical tree

Term	Accession	Level	No. genes in gene class	No. genes on microarray	Log odds	*P*-value
Marine stickleback, 7 °C						
RNA processing	GO:0006396	7	61	113	1.351642	6.98E-13
Translation	GO:0006412	6	86	228	0.834458	1.85E-08
GTP metabolic process	GO:0046039	10	83	230	0.770633	1.74E-07
Protein transport	GO:0015031	5	127	391	0.618743	2.05E-07
Cellular macromolecular complex assembly	GO:0034622	5	46	101	1.106434	2.10E-07
DNA replication	GO:0006260	7	20	33	1.518617	2.17E-07
Ribonucleoprotein complex biogenesis	GO:0022613	3	19	28	1.681656	4.27E-07
Folic acid-containing compound metabolic process	GO:0006760	9	10	14	1.755657	9.75E-05
Glutamine metabolic process	GO:0006541	9	9	12	1.826046	9.78E-05
Protein folding	GO:0006457	6	34	84	0.936229	0.000122
Nucleocytoplasmic transport	GO:0006913	7	51	144	0.743584	0.000132
Aerobic respiration	GO:0009060	6	11	19	1.452588	0.000509
tRNA metabolic process	GO:0006399	8	37	88	0.991105	0.000918
Mismatch repair	GO:0006298	8	6	8	1.826046	0.001673
Small GTPase-mediated signal transduction	GO:0007264	6	78	264	0.482092	0.002053
Ubiquitin-dependent protein catabolic process	GO:0006511	9	28	80	0.72651	0.002844
Protein methylation	GO:0006479	8	9	15	1.504118	0.003447
Vesicle-mediated transport	GO:0016192	4	39	114	0.693596	0.004968
Cellular amino acid biosynthetic process	GO:0008652	8	32	50	1.597227	0.005676
RNA modification	GO:0009451	7	12	21	1.433728	0.006183
Photosynthesis	GO:0015979	3	4	5	1.919155	0.008294
Chlorophyll biosynthetic process	GO:0015995	7	4	5	1.919155	0.008294
GPI anchor biosynthetic process	GO:0006506	9	6	10	1.504118	0.008462
Glycerol-3-phosphate metabolic process	GO:0006072	6	6	10	1.504118	0.008462
Proteolysis involved in cellular protein catabolic process	GO:0051603	7	36	94	0.85642	0.009236
Freshwater stickleback, 7 °C						
RNA processing	GO:0006396	7	65	113	1.017383	2.14E-09
GTP metabolic process	GO:0046039	10	101	230	0.627916	4.79E-07
Intracellular protein transport	GO:0006886	6	136	330	0.536335	5.05E-07
Cellular macromolecular complex assembly	GO:0034622	5	51	101	0.829408	4.69E-06
Nucleocytoplasmic transport	GO:0006913	7	66	144	0.689664	1.05E-05
Ubiquitin-dependent protein catabolic process	GO:0006511	9	39	80	0.778669	8.77E-05
Glutamine metabolic process	GO:0006541	9	10	12	1.55216	0.000124
ATP hydrolysis coupled proton transport	GO:0015991	10	16	25	1.171338	0.000224
Cellular respiration	GO:0045333	5	20	32	1.137123	0.000293
Protein catabolic process	GO:0030163	5	61	113	0.925753	0.000306
DNA replication	GO:0006260	7	19	33	1.018728	0.000565
Electron transport chain	GO:0022900	4	12	16	1.400157	0.001034
ATP synthesis coupled proton transport	GO:0015986	12	10	14	1.329768	0.001039
Folic acid-containing compound biosynthetic process	GO:0009396	10	8	10	1.493266	0.001066
Small GTPase-mediated signal transduction	GO:0007264	6	103	264	0.457301	0.001286
Proteolysis involved in cellular protein catabolic process	GO:0051603	7	49	94	0.875315	0.001868
Vesicle-mediated transport	GO:0016192	4	46	114	0.505866	0.004262
Phosphorus metabolic process	GO:0006793	3	441	1211	0.357846	0.005387
tRNA metabolic process	GO:0006399	8	41	88	0.713315	0.006842
Chromatin organization	GO:0006325	5	34	75	0.673839	0.008261
Translational initiation	GO:0006413	7	10	17	1.04966	0.008357
Regulation of anion transport	GO:0044070	6	6	8	1.400157	0.00844
Marine stickleback, 22 °C						
Protein ubiquitination	GO:0016567	9	28	95	0.970191	8.98E-05

Table 2 *Continued*

Term	Accession	Level	No. genes in gene class	No. genes on microarray	Log odds	*P*-value
Regulation of transcription, DNA dependent	GO:0006355	8	145	829	0.217373	0.004895
Hexose biosynthetic process	GO:0019319	7	4	7	1.925337	0.009583
Freshwater stickleback, 22 °C						
Protein folding	GO:0006457	6	27	84	0.956883	0.000151
Translation	GO:0006412	6	61	228	0.69216	0.000237
Protein ubiquitination	GO:0016567	9	28	95	0.831812	0.000691
Protein ADP ribosylation	GO:0006471	8	4	6	2.00935	0.007225
Oxidation–reduction process	GO:0055114	3	106	532	0.26695	0.007926
Nucleosome assembly	GO:0006334	10	17	59	0.799132	0.008399

Table 3 Genes with plastic expression also found within outlier regions of divergence in Jones *et al.* (2012)

Origin	Temperature up-regulated	Gene abbr.	Gene name	LG	Known gene function
Marine	22 °C	IGFBP2a	Insulin-like growth factor binding protein 2a	I	Negative regulator of cell proliferation
Freshwater	7 °C	PPARAa	Peroxisome proliferator-activated receptor alpha a	IV	Regulator of fatty acid uptake, intracellular binding, mitochondrial β-oxidation, peroxisomal fatty acid oxidation, ketogenesis, triglyceride turnover, gluconeogenesis, bile synthesis/secretion
Freshwater	22 °C	INHa	Inhibin, alpha	I	Growth/differentiation factor, hormone, FSH inhibitor
Freshwater	22 °C	SPEG	SPEG	I	Myocyte cytoskeletal development
Freshwater	22 °C	OBSL1	Obscurin-like 1	I	Cytoskeletal connections found in z disc and M bands
Freshwater	22 °C	Novel	Novel gene	IV	Unknown
Freshwater	22 °C	NLRC5	NOD-like receptor family CARD domain containing 5	XIX	Involved in immunity

'Origin' indicates the marine or freshwater origin that exhibited gene expression plasticity. LG, linkage group. The eight genes with plastic expression in both origins are not included here, for reasons outlined in the text.

was insensitive to freshwater salinities or this plasticity was expressed for the first time upon encountering freshwater salinities. Either way, these expression profiles were retained in the freshwater stickleback. Although we could not directly assess the role of these genes during colonization, many of them have been implicated in cold-temperature adaptation in fish. Included in this group were genes involved in transcriptional regulation, which compensate for cold-produced RNA secondary structures (Gracey *et al.* 2004; Rebl *et al.* 2013), protein catabolism, particularly through ubiquitination (Rebl *et al.* 2013; Smith *et al.* 2013), temperature-shock responses (HSP60, HSP70, HSP90, HSP40 – Renaut *et al.* 2006; Kyprianou *et al.* 2010; Carrasco *et al.* 2011) and global gene expression temperature sensing (HMGB1 – Gracey *et al.* 2004; Podrabsky & Somero 2004). This suggests that gene expression plasticity for these genes evolved in the marine environment to withstand changing temperatures. Plasticity was apparently not disrupted during colonization of freshwater, and the functions of these genes were thereby maintained without need of further evolution. The fitness effects of these gene expression patterns, and their expression in the marine environment, remain to be determined, but genes with known temperature-related functions are ideal candidates for future study of the first stage of the Baldwin effect.

The evolution of plasticity

Pre-existing plasticity may allow individuals to persist despite environmental stressors, but is unlikely to produce a maximal fit between total phenotype and environment (Ghalambor *et al.* 2007). This allows room for the subsequent evolution of plasticity, in terms of reaction norm height and/or slope (Baldwin 1896). Such evolution can include genetic changes at loci affecting nonplastic traits, as well as at loci that decrease or create and enhance plasticity (Crispo 2007; Morris & Rogers 2014). In this experiment, stickleback of freshwater origin exhibited more phenotypic plasticity (in terms of number of genes with plastic expression) than stick-

leback of marine origin. This supports the prediction that plasticity has evolved in parallel in freshwater populations (see also Supporting Information 1).

Although it is highly likely that many of the genes expressed in freshwater populations were being expressed as passive responses to other induced genes, freshwater temperature regimes impose significant selection pressures on marine stickleback (Barrett *et al.* 2011), and therefore, the observed pattern of greater plasticity in freshwater stickleback is likely adaptive. For example, OLM stickleback have a critical thermal minimum between 4 and 5 °C and have been shown to evolve cold tolerance 2.5 °C lower than their ancestors, three generations after being introduced to freshwater ponds with greater seasonal temperature extremes (Barrett *et al.* 2011). The targets of selection were then unknown; this study highlights some of the candidates.

Candidate loci for selection include those genes significantly enriched or up-regulated at 7 °C in freshwater stickleback only, as a subset of these loci may explain the lower critical thermal minima of freshwater stickleback. Under cold temperatures, stickleback increase the production of mitochondria within their muscle tissue, an adaptive response that compensates for reduced oxygen diffusion and metabolic reaction rates (Guderley 2004). The genes and enzymes associated with mitochondrial biogenesis and activity under different temperatures have been well characterized in threespine stickleback (Vézina & Guderley 1991; Orczewska *et al.* 2010; Kammer *et al.* 2011) and are largely consistent with our findings. Five gene classes and a variety of genes involved in mitochondrial biogenesis or activity were up-regulated at 7 °C only in freshwater stickleback, including PPARGC1β, PPARAa, NRF-1 and COX-IV, highlighting some functionally significant potential sources of adaptive divergence.

We compared our results to the genome scan of Jones *et al.* (2012). Several genes that were differentially expressed with temperature only in marine or freshwater stickleback were located within these outlier regions of genetic divergence, regions whose phenotypic effects were repeatedly the targets of selection. Several genes that responded to temperature in freshwater stickleback only (e.g. SPEG, the novel gene near GARP, NLRC5 and PPARAa) were known to contain SNPs or exhibit gene expression differences between marine and freshwater stickleback (Jones *et al.* 2012). Of these, SPEG is up-regulated at cooler temperatures in human tissue (Goto *et al.* 2011), the opposite of the pattern observed here. The function of NLRC5 in relation to temperature is unknown, but it has been implicated in antiviral responses in fish (Neerincx *et al.* 2010; Gao *et al.* 2012). PPARAa is associated with mitochondrial activity (and is known in other vertebrates to be implicated in energy balance regulation through its regulatory effects on hundreds of targets – Rakhshandehroo *et al.* 2010), a function enriched in freshwater stickleback only. These would all make suitable first candidates for identifying the mutations leading to gene expression evolution in stickleback.

Caveats

In this study, we have statistically treated OLM and LCM stickleback as if they represented two populations. Although recent work suggests that Alaskan stickleback separated by large geographical distances constitute a single population (Hohenlohe *et al.* 2010) and that there is no genetic divergence among British Columbian anadromous populations (Withler & McPhail 1985; Taylor & McPhail 1999), the use of next-generation sequencing has revealed local adaptation and/or genetic differentiation in Baltic and Oregon coast populations (Catchen *et al.* 2013; DeFaveri *et al.* 2013a,b). The marine stickleback used in this study did somewhat differ in gene expression patterns (Supporting Information 1). Overall, more advanced population genomic approaches are required to elucidate this question. For the above reasons, however, we maintain that sufficient evidence exists to statistically treat these marine groups as replicate populations, with replication necessary for making inferences about the ancestral condition.

Microarrays are subject to numerous sources of error, but the reliability of the data can be improved with different filtering methods (e.g. Pozhitkov *et al.* 2014). We analysed our data using multiple models and filtering methods (Supporting Information 1), but the overall patterns never changed. Filtering removed several thousand genes from the analysis, but this most dramatically affected the freshwater origin, reducing their number by nearly 1500 genes. This suggests that our method was conservative and removed genes that were constitutively noninduced by temperature in the marine stickleback, but which were induced by temperature in freshwater stickleback. The fact that the patterns held despite this filtering suggests the importance of gene expression plasticity in the evolution of freshwater stickleback.

Although marine stickleback can be raised at slightly above freshwater salinities without apparent harm (Heuts 1947), it is likely that the marine stickleback were not exhibiting the gene expression profiles that they would under natural salinities. Freshwater stickleback are the result of evolution at, in part, low salinities, and so identifying the differences between marine and freshwater stickleback raised at a common low salinity was necessary for making inferences about stickleback evolution. Low salinity may suppress

plasticity in marine stickleback; if so, it is all the more interesting to ask how this plasticity came to be in freshwater stickleback. Our study could not differentiate between genes that were only induced by temperature under low salinities, vs. those only induced by temperature under natural salinities; measuring gene expression under natural salinities would be worth further investigation, as it would uncover genes involved in individual survival that were expressed for the first time in freshwater conditions.

It is important to remember that temperature-induced gene expression does not necessarily have functional significance (Dalziel *et al.* 2009). Gene expression plasticity for a particular gene may be a passive response to other adaptive changes (Aubin-Horth & Renn 2009) or may be overridden at the level of protein abundance (Diz *et al.* 2012). Similarly, a lack of gene expression plasticity for a gene does not indicate a lack of temperature-related functionality. Lactate dehydrogenase A is constitutively expressed in muscle but transcript abundance is not affected by temperature (Vézina & Guderley 1991; Guderley *et al.* 1994), a finding confirmed in this study. Yet temperature-specific adaptations in the coding regions of this gene are known in other fish species (Johns & Somero 2004; Petricorena & Somero 2007). For the purposes of this experiment, it is the overall patterns that speak towards adaptive significance: plasticity has evolved in parallel in multiple freshwater populations, and this for gene groups with known temperature-related functions (see also Supporting Information 1).

Conclusion and future directions

There have been increasing calls to address the role of gene expression plasticity in adaptation and ecological speciation (Landry *et al.* 2007; Pavey *et al.* 2010; Thibert-Plante & Hendry 2011; Andrew *et al.* 2013). Experiments that can test these predictions and identify the underlying candidate genes offer a tangible first step towards explaining the role of phenotypic plasticity in adaptive evolution (McCairns & Bernatchez 2010; Morris & Rogers 2014). In our experiment, we have shown that temperature affects gene expression, that it affects certain genes similarly in both ancestral and derived populations, that plasticity has evolved in the derived populations and that at least some of these changes, due to their parallel nature and known functions, are likely adaptive. Overall, these results are consistent with the hypothesis that gene expression plasticity can evolve to meet the challenges of a novel environment. These results also provided candidate loci for future research into plasticity's role in promoting adaptive divergence by facilitating survival in new environments.

The next steps could experimentally test how fitness may be maintained by plasticity. Definitive tests demonstrating how underlying gene expression facilitates individual survival are still needed (Pavey *et al.* 2010). Our results suggest that such tests could be carried out using experimental evolution. Under controlled stress conditions, in association with temperature, we can potentially measure which genes are differentially expressed, while controlling for variation in ecologically relevant alternative alleles in different environments. Identifying these mutations still represents a considerable challenge, but given recent advances in vertebrate knockouts (e.g. Varshney *et al.* 2013), once these mutations are identified, candidate genes could be knocked out. By exposing both mutant and control treatments to the temperature stresses experienced when colonizing freshwater environments, the prediction that survival would be weaker for the mutant treatment can be tested directly. This experiment could even be conducted in the field, reinforcing that the integration of an ecological genomics framework in studies of phenotypic plasticity is a promising approach to elucidate the causal links between genes and the environment (Andrew *et al.* 2013; Morris & Rogers 2014).

Acknowledgements

The authors wish to thank the assistance of L. Harder, J. Theodor, D. Schluter, S. Pavey, K. Shortt, C. Di Poi, C. Coats, the LESARC staff, Semiahmoo First Nations, the Rogers, Aubin-Horth, Schluter, Landry and Bernatchez laboratories, and Génome Québec. The authors would also like to thank Diethard Tautz and three anonymous reviewers whose comments and suggestions improved the manuscript. MRJM held a National Sciences and Engineering Research Council of Canada (NSERC) Vanier Scholarship, NSERC Canadian Graduate Scholarship, Alberta Innovates Graduate Student Scholarship and Alberta Conservation Association Grant in Biodiversity during the course of this experiment. NAH is funded by NSERC Discovery and Canada Foundation for Innovation Leaders Opportunity Fund. SMR is funded by NSERC Discovery (RT735287) and an Alberta Innovates Technology Futures New Faculty Award (RT722016). RDHB is funded by NSERC Discovery, the Canada Foundation for Innovation Leaders Opportunity Fund and a Canada Research Chair. Use of animals was approved by CCAC (Rogers-Bio9R-41). Wild caught fish were captured under Department of Fisheries and Ocean Permit XR-315-2011 and BC Ministry of Environment NA-SU-PE-10-63485.

References

Agilent Technologies, Inc. (2009) *Agilent Feature Extraction Software (v10.7) Reference Guide*, 7th edn. G4460-90026, 308 pp. Agilent Technologies, Inc., Santa Clara, California.

Agrawal AA (2001) Phenotypic plasticity in the interactions and evolution of species. *Science*, **294**, 321–326.

Ahn D, Gibson G (1999) Axial variation in the threespine stickleback: genetic and environmental factors. *Evolution and Development*, **1**, 100–112.

Amarasekare P, Savage V (2012) A framework for elucidating the temperature-dependence of fitness. *American Naturalist*, **179**, 178–191.

Andrew RL, Bernatchez L, Bonin A *et al.* (2013) A road map for molecular ecology. *Molecular Ecology*, **22**, 2605–2626.

Aubin-Horth N, Renn S (2009) Genomic reaction norms: using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Molecular Ecology*, **18**, 3763–3780.

Aubret F, Shine R, Bonnett X (2004) Adaptive developmental plasticity in snakes. *Nature*, **431**, 261–262.

Baldwin JM (1896) A new factor in evolution. *The American Naturalist*, **30**, 441–451.

Baldwin JM (1902) *Development and Evolution*. The MacMillan Company, New York, New York.

Barrett RDH, Rogers SM, Schluter D (2008) Natural selection on a major armor gene in threespine stickleback. *Science*, **322**, 255–257.

Barrett RDH, Paccard A, Healy T *et al.* (2011) Rapid evolution of cold tolerance in stickleback. *Proceedings of the Royal Society of London B: Biological Sciences*, **278**, 233–238.

Bell MA (1976) Evolution of phenotypic diversity in the *Gasterosteus aculeatus* superspecies on the Pacific coast of North America. *Systematic Zoology*, **25**, 211–227.

Bell MA (1977) Late Miocene marine threespine stickleback, *Gasterosteus aculeatus aculeatus*, and its zoogeographic and evolutionary significance. *Copeia*, **2**, 277–282.

Bell MA (1995) Intraspecific systematics of *Gasterosteus aculeatus* populations: implications for behavioral ecology. *Behaviour*, **132**, 1131–1152.

Bell MA (2001) Lateral plate evolution in the threespine stickleback: getting nowhere fast. *Genetica*, **112–113**, 445–461.

Bell MA, Aguirre WE (2013) Contemporary evolution, allelic recycling, and adaptive radiation of the threespine stickleback. *Evolutionary Ecology Research*, **15**, 377–411.

Bell MA, Foster SA (1994) Introduction to the evolutionary biology of the threespine stickleback. In *The Evolutionary Biology of the Threespine Stickleback* (eds Bell MA, Foster SF), pp. 1–27. Oxford University Press, New York, New York.

Bell MA, Aguirre WE, Buck NJ (2004) Twelve years of contemporary armor evolution in a threespine stickleback population. *Evolution*, **58**, 814–824.

Blazejczyk M, Miron M, Nadon R (2007). *FlexArray: A Statistical Data Analysis Software for Gene Expression Microarrays*. Genome Quebec, Montreal, Quebec, Canada. Available from http://genomequebec.mcgill.ca/FlexArray.

Bradshaw AD (1965) Evolutionary significance of phenotypic plasticity in plants. *Advances in Genetics*, **13**, 115–155.

Carrasco MA, Tan JC, Duman JG (2011) A cross-species compendium of proteins/gene products related to cold stress identified by bioinformatic approaches. *Journal of Insect Physiology*, **57**, 1127–1135.

Catchen J, Bassham S, Wilson T *et al.* (2013) The population structure and recent colonization history of Oregon threespine stickleback determined using restriction site-associated DNA-sequencing. *Molecular Ecology*, **22**, 2864–2883.

Chan YF, Marks ME, Jones FC *et al.* (2010) Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a *Pitx1* enhancer. *Science*, **327**, 302–305.

Chevin L-M, Lande R (2010) When do adaptive plasticity and genetic evolution prevent extinction of a density-regulated population? *Evolution*, **64–4**, 1143–1150.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, **162**, 156–159.

Clague J, Harper JR, Hebda RJ, Howes DE (1982) Late Quaternary sea levels and crustal movements, coastal British Columbia. *Canadian Journal of Earth Sciences*, **19**, 597–618.

Colosimo PF, Hosemann KE, Balabhadra S *et al.* (2005) Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. *Science*, **307**, 1928–1933.

Crispo E (2007) The Baldwin Effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution*, **61–11**, 2469–2479.

Dalziel AC, Rogers SM, Schulte PM (2009) Linking genotypes to phenotypes and fitness: how mechanistic biology can inform molecular ecology. *Molecular Ecology*, **18**, 4997–5017.

Day T, Pritchard J, Schluter D (1994) A comparison of two sticklebacks. *Evolution*, **48**, 1723–1734.

DeFaveri J, Jonsson PR, Merilä J (2013a) Heterogeneous genomic differentiation in marine threespine sticklebacks: adaptation along an environmental gradient. *Evolution*, **67–9**, 2530–2546.

DeFaveri J, Shikano T, Shimada Y, Merilä J (2013b) High degree of genetic differentiation in marine three-spined sticklebacks (*Gasterosteus aculeatus*). *Molecular Ecology*, **22**, 4811–4828.

Diz AP, Martínez-Fernández M, Rolán-Alvarez E (2012) Proteomics in evolutionary ecology: linking the genotype with the phenotype. *Molecular Ecology*, **21**, 1060–1080.

Engel K, Tollrian R (2009) Inducible defences as key adaptations for the successful invasion of *Daphnia lumholtzi* in North America? *Proceedings of the Royal Society B*, **276**, 1865–1873.

Fitzpatrick BM (2012) Underappreciated consequences of phenotypic plasticity for ecological speciation. *International Journal of Ecology*, **2012**, 256017.

Flicek P, Amode MR, Barrell D *et al.* (2014) Ensembl 2014. *Nucleic Acids Research*, **42**, D749–D755.

Furin CG, von Hippel FA, Bell MA (2012) Partial reproductive isolation of a recently derived resident-freshwater population of threespine stickleback (*Gasterosteus aculeatus*) from its putative anadromous ancestor. *Evolution*, **66**, 3277–3286.

Gao L, He C, Liu X *et al.* (2012) The innate immune-related genes in catfish. *International Journal of Molecular Science*, **13**, 14172–14202.

Ghalambor CK, McKay JK, Carroll SP, Reznick DN (2007) Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, **21**, 394–407.

Goto K, Oda H, Kondo H *et al.* (2011) Responses of muscle mass, strength and gene transcripts to long-term heat stress in healthy human subjects. *European Journal of Applied Physiology*, **111**, 17–27.

Gracey AY, Fraser EJ, Li W *et al.* (2004) Coping with cold: an integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. *Proceedings of the National Academy of Sciences*, **101**, 16970–16975.

Grant PR, Grant BR, Smith JN, Abbott IJ, Abbott LK (1976) Darwin's finches: population variation and natural selec-

tion. *Proceedings of the National Academy of Sciences*, **73**, 257–261.
Grether GF (2005) Environmental change, phenotypic plasticity, and genetic compensation. *The American Naturalist*, **166**, E115–E123.
Guderley H (2004) Locomotor performance and muscle metabolic capacities: impact of temperature and energetic status. *Comparative Biochemistry and Physiology B*, **139**, 371–382.
Guderley H, Lavoie BA, Dubois N (1994) The interaction among age, thermal acclimation and growth rate in determining muscle metabolic capacities and tissue masses in the threespine stickleback, *Gasterosteus aculeatus*. *Fish Physiology and Biochemistry*, **13**, 419–431.
Hayward SAL, Murray PA, Gracey AY, Cossins AR (2007) Beyond the lipid hypothesis: mechanisms underlying phenotypic plasticity in inducible cold tolerance. *Advances in Experimental Medicine and Biology*, **594**, 132–142.
Heuts MJ (1947) Experimental studies on adaptive evolution in *Gasterosteus aculeatus* L. *Evolution*, **1**, 89–102.
Hoffmann AA, Sgrò CM (2011) Climate change and evolutionary adaptation. *Nature*, **470**, 479–485.
Hohenlohe PA, Bassham S, Etter PD, Stiffler N, Johnson EA, Cresko WA (2010) Population genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. *PLoS Genetics*, **6**, e1000862.
Hu Z-L, Bao J, Reecy JM (2008) CateGOrizer: a web-based program to batch analyze gene ontology classification categories. *Online Journal of Bioinformatics*, **9**, 108–112.
Hulsen T, de Vlieg J, Alkema W (2008) BioVenn—a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC Genomics*, **9**, 488.
Hutchinson I, James TS, Reimer PJ, Bornhold BD, Clague JJ (2004) Marine and limnetic radiocarbon reservoir corrections for studies of late- and postglacial environments in Georgia Basin and Puget Lowland, British Columbia, Canada and Washington, USA. *Quaternary Research*, **61**, 193–203.
Ihaka R, Gentleman R (1996) R: a language for data analysis and graphics. *Journal of Computational and Graphical Statistics*, **5**, 299–314.
Johns GC, Somero GN (2004) Evolutionary convergence in adaptation of proteins to temperature: A_4-lactate dehydrogenases of Pacific damselfishes (*Chromis* spp.). *Molecular Biology and Evolution*, **21**, 314–320.
Jones F, Grabherr M, Chan Y *et al.* (2012) The genomic basis of adaptive evolution in threespine sticklebacks. *Nature*, **484**, 55–61.
Kammer AR, Orczewska JI, O'Brien KM (2011) Oxidative stress is transient and tissue specific during cold acclimation of threespine stickleback. *The Journal of Experimental Biology*, **214**, 1248–1256.
Kovach-Orr C, Fussmann G (2013) Evolutionary and plastic rescue in multitrophic model communities. *Philosophical Transactions of the Royal Society of London B*, **368**, 20120084.
Kristjánsson BK, Skúlason S, Noakes DLG (2002) Rapid divergence in a recently isolated population of threespine stickleback (*Gasterosteus aculeatus* L.). *Evolutionary Ecology Research*, **4**, 1–14.
Kyprianou T, Pörtner HO, Anestis A, Kostoglou B, Feidantsis K, Michaelidis B (2010) Metabolic and molecular stress responses of gilthead sea bream *Sparus aurata* during exposure to low ambient temperature: an analysis of mechanisms underlying the winter syndrome. *Journal of Comparative Physiology B*, **180**, 1005–1018.
Landry CR, Lemos B, Rifkin SA, Dickinson WJ, Hartl DL (2007) Genetic properties influencing the evolvability of gene expression. *Science*, **317**, 118–121.
Latta LC, Weider LJ, Colbourne JK, Pfrender ME (2012) The evolution of salinity tolerance in *Daphnia*: a functional genomics approach. *Ecology Letters*, **15**, 794–802.
Leder E, Merila J, Primmer CR (2009) A flexible whole-genome microarray for transcriptomics in three-spine stickleback (*Gasterosteus aculeatus*). *BMC Genomics*, **10**, 426.
Lewontin RC (1974) The analysis of variance and the analysis of causes. *American Journal of Human Genetics*, **26**, 400–411.
Lindsey C (1962) Experimental study of meristic variation in a population of threespine sticklebacks, *Gasterosteus aculeatus*. *Canadian Journal of Zoology*, **40**, 271–312.
Magni P, Simeone A, Healy S, Isacchi A, Bosotti R (2011) Summarizing probe intensities of Affymetrix GeneChip 3′ expression arrays taking into account day-to-day variability. *IEEE/ACM Transactions on Computational Biology and Bioinformatics*, **8**, 1425–1430.
The MathWorks Inc (2012) *MATLAB and Statistics Toolbox Release*. The MathWorks Inc, Natick, MA.
McCairns RJS, Bernatchez L (2010) Adaptive divergence between freshwater and marine sticklebacks: insights into the role of phenotypic plasticity from an integrated analysis of candidate gene expression. *Evolution*, **64–4**, 1029–1047.
Moczek AP, Sultan S, Foster S *et al.* (2011) The role of developmental plasticity in evolutionary innovation. *Proceedings of the Royal Society B*, **278**, 2705–2713.
Morris MRJ, Rogers SM (2013) Overcoming maladaptive plasticity through plastic compensation. *Current Zoology*, **59**, 526–536.
Morris MRJ, Rogers SM (2014) Integrating phenotypic plasticity within an ecological genomics framework: recent insights from the genomics, evolution, ecology, and fitness of plasticity. In *Ecological Genomics* (eds Landry C, Aubin-Horth N), pp. 73–105. Springer, New York, New York.
Narum SR, Campbell NR, Meyer KA, Miller MR, Hardy RW (2013) Thermal adaptation and acclimation of ectotherms from differing aquatic climates. *Molecular Ecology*, **22**, 3090–3097.
Neerincx A, Lautz K, Menning M *et al.* (2010) A role for the human nucleotide-binding domain, leucine-rich repeat-containing family member NLRC5 in antiviral responses. *The Journal of Biological Chemistry*, **285**, 26223–26232.
Neuheimer A, Taggart C (2007) The growing degree-day and fish size-at-age: the overlooked metric. *Canadian Journal of Fisheries and Aquatic Sciences*, **64**, 375–385.
Orczewska JI, Hartleben G, O'Brien KM (2010) The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation. *American Journal of Physiology—Regulatory, Integrative and Comparative Physiology*, **299**, R352–R364.
Parsons KJ, Robinson BW (2006) Replicated evolution of integrated plastic responses during early adaptive divergence. *Evolution*, **60**, 801–813.
Pavey S, Collin H, Nosil P, Rogers SM (2010) The role of gene expression in ecological speciation. *Annals of the New York Academy of Sciences*, **1206**, 110–129.

Petricorena Z, Somero G (2007) Biochemical adaptations of notothenioid fishes: comparisons between cold temperate South American and New Zealand species and Antarctic species. *Comparative Biochemistry and Physiology A*, **147**, 799–807.

Pfennig DW, McGee M (2010) Resource polyphenism increases species richness: a test of the hypothesis. *Philosophical Transactions of the Royal Society B*, **365**, 577–591.

Pigliucci M, Murren CJ, Schlichting CD (2006) Phenotypic plasticity and evolution by genetic assimilation. *The Journal of Experimental Biology*, **209**, 2362–2367.

Podrabsky JE, Somero GN (2004) Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. *The Journal of Experimental Biology*, **207**, 2237–2254.

Powers DA, Lauerman T, Crawford D, DiMichele L (1991) Genetic mechanisms for adapting to a changing environment. *Annual Review of Genetics*, **25**, 629–659.

Pozhitkov AE, Noble PA, Bryk J, Tautz D (2014) A revised design for microarray experiments to account for experimental noise and uncertainty of probe response. *PLoS One*, **9**, e91295.

Rakhshandehroo M, Knoch B, Müller M, Kersten S (2010) Peroxisome-proliferator-activated receptor alpha target genes. *PPAR Research*, **2010**, 612089.

Rebl A, Verleih M, Köbis JM *et al.* (2013) Transcriptome profiling of gill tissue in regionally bred and globally farmed rainbow trout strains reveals different strategies for coping with thermal stress. *Marine Biotechnology*, **15**, 445–460.

Reed TE, Grøtan V, Jenouvrier S, Saether B-E, Visser ME (2013) Population growth in a wild bird is buffered against phonological mismatch. *Science*, **340**, 488–491.

Renaut J, Hausman J, Wisniewski ME (2006) Proteomics and low-temperature studies: bridging the gap between gene expression and metabolism. *Physiologia Plantarum*, **126**, 97–109.

Rogers SM, Tamkee P, Summers B *et al.* (2012) Genetic signature of adaptive peak shift in threespine stickleback. *Evolution*, **66–8**, 2439–2450.

Rogers SM, Mee JA, Bowles E (2013) The consequences of genomic architecture on ecological speciation in postglacial fishes. *Current Zoology*, **59**, 53–71.

Rundle HD, Nagel L, Boughman JW, Schluter D (2000) Natural selection and parallel speciation in sympatric sticklebacks. *Science*, **287**, 306–308.

Schlichting CD (2008) Hidden reaction norms, cryptic genetic variation, and evolvability. *Annals of the New York Academy of Science*, **1133**, 187–203.

Schluter D, Conte GL (2009) Genetics and ecological speciation. *Proceedings of the National Academy of Sciences (USA)*, **106**, 9955–9962.

Schulte PM (2014) What is environmental stress? Insights from fish living in a variable environment. *The Journal of Experimental Biology*, **217**, 23–34.

Schwander T, Leimar O (2011) Genes as leaders and followers in evolution. *Trends in Ecology and Evolution*, **26**, 143–151.

Simpson GG (1953) The Baldwin effect. *Evolution*, **7**, 110–117.

Smith S, Bernatchez L, Beheregaray LB (2013) RNA-seq analysis reveals extensive transcriptional plasticity to temperature stress in a freshwater fish species. *BMC Genomics*, **14**, 375.

Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology*, **3**, 3.

Smyth GK (2005) Limma: linear models for microarray data. In: *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*(eds Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W). Springer, New York, New York.

Somero GN (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *The Journal of Experimental Biology*, **213**, 912–920.

Storey JD (2003) The positive false discovery rate: a Bayesian interpretation and the q-value. *The Annals of Statistics*, **31**, 2013–2035.

Svanbäck R, Schluter D (2012) Niche specialization influences adaptive phenotypic plasticity in the threespine stickleback. *The American Naturalist*, **180**, 50–59.

Taylor EB, McPhail JD (1999) Evolutionary history of an adaptive radiation in species pairs of threespine stickleback (*Gasterosteus*): insights from mitochondrial DNA. *Biological Journal of the Linnean Society*, **66**, 271–291.

Thibert-Plante X, Hendry AP (2011) The consequences of phenotypic plasticity for ecological speciation. *Journal of Evolutionary Biology*, **24**, 326–342.

Tomanek L (2008) The importance of physiological limits in determining biogeographical range shifts due to global climate change: the heat-shock response. *Physiological and Biochemical Zoology*, **81**, 709–717.

Torres-Dowdall J, Handelsman C, Reznick D, Ghalambor C (2012) Local adaptation and the evolution of phenotypic plasticity in Trinidadian guppies (*Poecilia reticulata*). *Evolution*, **66**, 3432–3443.

Varshney GK, Lu J, Gildea D *et al.* (2013) A large-scale zebrafish gene knockout resource for the genome-wide study of gene function. *Genome Research*, **23**, 727–735.

Vedder O, Bouwhuis S, Sheldon BC (2013) Quantitative assessment of the importance of phenotypic plasticity in adaptation to climate change in wild bird populations. *PLoS Biology*, **11**, e1001605.

Vézina D, Guderley H (1991) Anatomic and enzymatic responses of the three-spined stickleback, *Gasterostues aculeatus* to thermal acclimation and acclimatization. *The Journal of Experimental Zoology*, **258**, 277–287.

Waddington CH (1953) Genetic assimilation of an acquired character. *Evolution*, **7**, 118–126.

Walker JA, Bell MA (2000) Net evolutionary trajectories of body shape evolution within a microgeographic radiation of threespine stickleback (*Gasterosteus aculeatus*). *Journal of Zoology, London*, **252**, 293–302.

West-Eberhard M (2003) *Developmental Plasticity and Evolution*. Oxford University Press, New York, New York.

Withler RE, McPhail JD (1985) Genetic variability in freshwater and anadromous sticklebacks (*Gasterosteus aculeatus*) of southern British Columbia. *Canadian Journal of Zoology*, **63**, 528–533.

Woltereck R (1909) Weitere experimentelle untersüchungen über artveränderung, speziell über das wesen quantitativer artunterschiede bei daphniden. *Verhandlungen der Deutschen Zoologischen Gesellschaft*, **19**, 110–172.

Wund MA, Baker JA, Clancy B, Golub JL, Foster SA (2008) A test of the 'flexible stem' model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the threespine stickleback radiation. *The American Naturalist*, **172**, 449–462.

Yampolsky LY, Glazko GV, Fry JD (2012) Evolution of gene expression and expression plasticity in long-term experimental populations of *Drosophila melanogaster* maintained under constant and variable ethanol stress. *Molecular Ecology*, **21**, 4287–4299.

Yeh PJ, Price TD (2004) Adaptive phenotypic plasticity and the successful colonization of a novel environment. *The American Naturalist*, **164**, 531–542.

Zheng Q, Wang XJ (2008) GOEAST: a web-based software toolkit for Gene Ontology enrichment analysis. *Nucleic Acid Research*, **36**(Suppl. 2), W358–W363.

M.R.J.M. designed and carried out the experiment and analyses and wrote the MS. R.R. conducted analyses in MATLAB and helped write and edit the MS. E.H.L. constructed the microarray and helped write and edit the MS. R.D.H.B. captured and raised the wild stickleback, reared the F1s, provided feedback on the experimental design and helped write and edit the MS. N.A.-H. provided funding for the microarrays, helped design and implement the experiment and helped write and edit the MS. S.M.R. captured and raised the wild stickleback, reared the F1s, helped design and implement the experiment, provided funding and helped write and edit the MS.

Data accessibility

Files in Dryad (25 files total), doi:10.5061/dryad.5q65k. MATLAB Statistics for Gene Expression Data (the MATLAB output file). Code Used for MATLAB. Normalized and Filtered Gene Expression Data (the LIMMA output file used to generate data for MATLAB). LIMMA Supplemental Results (LIMMA-generated data from supplemental model). LIMMA Targets File. Transcript ID (identifying Ensembl transcripts with feature IDs on microarray). Feature Extraction Output for Microarray Slides (6 files). Summary of Significantly Enriched Gene Classes. Hierarchical Gene Ontology Graphs (12 files).

Supporting information

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

Appendix S1 Methods and results.

Fig. S2.1 Microarray quality.

Fig. S2.2 Plots of the first two components of a Principal Components Analysis of the 7 and 22 °C microarrays for 20 021 genes, showing the effects of marine/freshwater origin and temperature.

Fig. S2.3 Graphical representation of the top 13 functional categories, for those genes up-regulated at (top) 7 °C and (bottom) 22 °C, in both marine and freshwater stickleback.

Fig. S2.4 Graphical representation of the top 13 functional categories, for those genes up-regulated at 7 °C in (top) marine stickleback and (bottom) freshwater stickleback.

Fig. S2.5 Graphical representation of the top 13 functional categories, for those genes up-regulated at 22 °C in (top) marine stickleback and (bottom) freshwater stickleback.

Table S2.1 Quality of total RNA.

Table S2.2 mRNA quality and cyanine-3 specific activity.

Table S2.3 Functional categories for those genes showing differential expression in both marine and freshwater stickleback, up-regulated at 7 or 22 °C.

Table S2.4 Functional categories for those genes showing differential expression only in marine or freshwater stickleback, up-regulated at 7 °C.

Table S2.5 Functional categories for those genes showing differential expression only in marine or freshwater stickleback, up-regulated at 22 °C.