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Abstract

Evidence implicating differential gene expression as a significant driver of evolutionary novelty continues to accumulate, but our understanding of the underlying sources of variation in expression, both environmental and genetic, is wanting. Heritability in particular may be underestimated when inferred from genetic mapping studies, the predominant "genetical genomics" approach to the study of expression variation. Such uncertainty represents a fundamental limitation to testing for adaptive evolution at the transcriptomic level. By studying the inheritance of expression levels in 10,495 genes (10,527 splice variants) in a threespine stickleback pedigree consisting of 563 individuals, half of which were subjected to a thermal treatment, we show that 74–98% of transcripts exhibit significant additive genetic variance. Dominance variance is also prevalent (41–99% of transcripts), and genetic sources of variation seem to play a more significant role in expression variance in the liver than a key environmental variable, temperature. Among-population comparisons suggest that the majority of differential expression in the liver is likely due to neutral divergence; however, we also show that signatures of directional selection may be more prevalent than those of stabilizing selection. This predominantly aligns with the neutral model of evolution for gene expression but also suggests that natural selection may still act on transcriptional variation in the wild. As genetic variation both within- and among-populations ultimately defines adaptive potential, these results indicate that broad adaptive potential may be found within the transcriptome.

Key words: Gasterosteus aculeatus, gene expression, quantitative genetics, Q_{ST}, transcriptome, microarray.

Introduction

Recent research indicates that sequence variants linked to phenotypic differences are predominantly found in noncoding, regulatory regions of the genome (Flint and Mackay 2009; Hindorff et al. 2009; Ku et al. 2010; Wray 2013). Similarly, there is mounting evidence that gene regulatory networks, and differential patterns of expression in genes may be a significant cause of phenotypic novelty (Erwin and Davidson 2009; Davidson and Erwin 2010), suggesting that variation in gene expression is likely an important source of evolutionary potential. Given the relative ease and large scale at which it can be quantified, transcription is commonly used as a proxy for gene expression (Schena et al. 1995; Lockhart and Winzeler 2000). Although transcription represents only the first of many steps leading to an ultimate/functional gene product, even small variation in mRNA abundance can have a significant impact on the amount of protein (Kaern et al. 2005; Bar-Even et al. 2006; Ghazalpour et al. 2011), with 12–78% of variation in protein abundance explained by transcriptional variation, depending upon taxa, cellular location, and protein

type (Greenbaum et al. 2003). Moreover, transcriptional variation can underlie complex phenotypic variation (Ayroles et al. 2009; Liao et al. 2010; Hines et al. 2012; Richards et al. 2012). Thus, despite its "proxy" status with respect to gene expression, transcriptional variation itself is also a likely source of evolutionary potential.

Transcriptional variation can also be viewed as a phenotype (Houle et al. 2010), and as with any phenotype, it is subject to varying degrees of genetic and environmental control, and its inherent variation may be shaped by natural selection (Whitehead and Crawford 2006b). It is generally held that transcriptional variation is additive, with an underlying polygenic architecture (Gilad et al. 2008; Kim and Gibson 2010). Indeed, results emerging from association studies in humans are consistent with the view that a large fraction of transcriptomic variation is heritable (Dixon et al. 2007; Powell et al. 2013). Line cross analyses of both cereal crops (Swanson-Wagner et al. 2006; Chelaifa et al. 2013) and diverse animal taxa (Rottscheidt and Harr 2007; Wayne et al. 2007; Debes et al. 2012; Gao et al. 2013) are also suggestive of widespread

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genetic variance for transcription, though few report estimates of variance and/or heritability (but see Wayne et al. 2004). In comparison, transcriptome-wide variance components analyses and/or pedigree-based studies into the quantitative genetics of transcriptional variance in model organisms have been limited to mice (Cui et al. 2006), yeast (Brem et al. 2002), *Caenorhabditis elegans* (Li et al. 2006), and *Drosophila* (Ayroles et al. 2009). For wild, outbred populations, potential for heritable variation in transcription has been inferred on the basis of high levels of within-population variance, but precise estimates of genetic variance are rarely available, and when attempted, most suffer from low statistical power due to small sample sizes (Whitehead and Crawford 2006b). Moreover, there may be considerable plasticity in patterns of expression (Aubin-Horth and Renn 2009), which if left uncontrolled, can equally lead to biased estimates of heritability by confounding environmental and additive genetic sources of variation (Visscher et al. 2008). This represents a fundamentally important problem in elucidating the evolutionary potential of transcriptomic variation as our ability to predict a response to changing selective pressures—a particularly compelling challenge in an age of rapid habitat/environmental change—is dependent upon the degree to which we can disentangle heritable and environmental contributions to trait variation (Gienapp et al. 2008; Hoffmann and Willi 2008; Hoffmann and Sgrò 2011). Clearly a more controlled experimental evaluation is required.

Our understanding of how selective processes have shaped transcriptional variation also remains a point of contention. To date the literature is dominated by two competing viewpoints, both empirically derived from comparative analyses across broad taxonomic groups. One posits that gene expression differences are effectively neutral (Oleksiak et al. 2002; Khaitovich et al. 2004, 2006; Whitehead and Crawford 2006a). The other suggests that stabilizing selection is the dominant force shaping the evolution of gene expression (Rifkin et al. 2003, 2005; Lemos et al. 2005; Gilad, Oshlack, and Rifkin 2006; Bedford and Hartl 2009), based on assumptions that energetic costs of transcription should favor an optimal level of expression (Wagner 2005, 2007), and that purifying selection should act to conserve transcript function (Brawand et al. 2011). Mutation accumulation experiments offer support for both views, demonstrating that significant expression divergence can emerge by random processes, but that purifying and/or stabilizing selection also acts upon expression variation in animals (Denver et al. 2005; Rifkin et al. 2005). However, a detailed analysis of mutation accumulation lines has revealed that not all genes are equally mutable, depending to a large extent on their regulatory properties and the transmutational target size (Landry et al. 2007). Similarly, it has been shown that genes expressed in multiple tissues—likely to be facultative, housekeeping genes—tend to be less divergent among species than tissue-specific genes (Khaitovich et al. 2005; Gilad, Oshlack, and Rifkin 2006). Moreover, divergence in *cis*-regulated expression may frequently be under positive selection (Emerson et al. 2010). Thus, although the debate regarding the predominant mode of evolution continues, it is becoming increasingly clear that other nonneutral evolutionary dynamics are at work on the transcriptome.

Complicating the study of transcriptomic evolution is the lack of a consensus model of its neutral evolution, stemming from discrepancies in data and assumptions regarding the rate of change in expression divergence (Khaitovich et al. 2004; Ogasawara and Okubo 2009). Equally problematic is the contradictory nature of its mutational variance: Transcription has been associated with both increased and decreased mutation rate—transcription-associated mutagenesis and transcription-coupled repair, respectively—and there is conflicting evidence as to whether levels of gene expression are positively or negatively correlated with mutation rate (Hanawalt and Spivak 2008; Kim and Jinks-Robertson 2012; Martincorena et al. 2012; Park et al. 2012). In the absence of a reliable/validated neutral model, comparison against a distribution of putative neutral divergence provides an expedient and informative alternative approach. This is the rationale underlying a well-studied quantitative approach to the problem of trait divergence (Spitze 1993; Leinonen et al. 2013), yet surprisingly few studies to date have actually applied this to transcriptional data (Roberge et al. 2007; Kohn et al. 2008).

In this article, we undertake a quantitative genetics approach to study the inheritance and evolution of transcriptional variation, addressing a number of issues earlier highlighted as central for resolving this topic (Gibson and Weir 2005; Gibson 2008). We do so in a model teleost fish, the threespine stickleback (*Gasterosteus aculeatus*). Teleosts such as the stickleback represent ideal candidates to decompose phenotypic variation into its genetic and environmental components. Almost all are obligate ectotherms; thus, metabolic/transcriptional variation may be induced by thermal treatment (Nikinmaa et al. 2013). Moreover, relatively large clutch sizes and external fertilization facilitate the creation of complex breeding designs with multiple related individuals which permit an accurate estimation of genetic variance components for focal traits.

The threespine stickleback is also a particularly interesting model to address many outstanding questions related to transcriptional variation and its role in the adaptive evolutionary process given that adaptive morphological variation associated with the species' expansion into novel habitats has been tied to differential expression of candidate genes and putative regulatory elements in its genome (Chan et al. 2010; Kitano et al. 2010; Jones et al. 2012). Adaptive divergence has also been linked to transcriptional differences in candidate genes for osmoregulation (McCairns and Bernatchez 2010), and in pigmentation differences between freshwater and marine populations (Greenwood et al. 2012). Moreover, examples are beginning to accumulate in which trends in transcription profiles are corroborated by functional assays. For example, differential expression of thyroid-stimulating hormone mRNA between stream-resident and marine sticklebacks reflects differences in lower plasma levels of thyroid hormone and reduced metabolic rate, hypothesized to underlie part of the physiological adaptation to permanent freshwater residency (Kitano et al. 2010). Additionally, transcript expression accurately reflects a functional reaction for

some components of hypoxia response (Leveelahti et al. 2011), and is reasonably predictive of gene expression/activity with respect to an oxidative stress response in steady-state systems (Nikinmaa et al. 2013).

Specifically, we address the three following questions: 1) How heritable is transcriptional variation; 2) to what extent does environmental variation affect transcript abundance, and are there genetically based differences in this plasticity of expression; and 3) to what extent has transcriptional variation been shaped by natural selection? To this end, we apply rigorous and statistically powerful quantitative genetic approaches to mRNA profiles of lab-reared threespine sticklebacks originally derived from wild source populations. To address the question of heritable variation in transcript expression—a critical element in evaluating the adaptive potential of any phenotype—we employ a half-sib breeding design of first generation fish ($n = 563$) representative of the putative ancestral, marine population, and quantify mRNA expression using an oligo microarray featuring approximately 15,000 transcripts. The experiment also includes an environmental manipulation (an acute rapid temperature increase) in order to facilitate an evaluation of plasticity in expression, as well as family-based variation in environmental sensitivity, a proxy for genotype–environment interaction (G×E). To test for signatures of selection on the transcriptome, we use the index of quantitative trait divergence (Q_{ST}) to compare expression variation within and among three discrete populations representing a range of diverse environmental/habitat conditions: One marine and two lakes of differing size and community complexity with pronounced latitudinal difference (supplementary fig. S1A, Supplementary Material online).

Results

Partitioning of Transcriptional Variance

The vast majority (10,384) of the 10,527 transcripts detected above background levels exhibited significant additive genetic variance (V_A; estimates presented in supplementary table S1, Supplementary Material online). Significant dominance variance (V_D) was also found in 10,499 transcripts. After correcting for potential "false positives" (i.e., Type I errors), estimated through phenotypic simulation (supplementary figs. S2A and B and appendix S1, Supplementary Material online), this would suggest significant genetic variation underlying the expression of at least 7,813 transcripts (74.2%)—heritability estimates for 8,593 transcripts (81.6%) exceeded the mean of possible false positive estimates ($h^2 > 0.094$). The proportion of total phenotypic variance explained by either additive genetic or dominance variance was quite similar: The mean estimate of narrow-sense heritability (h^2) for transcript abundance was 0.226 (0.070–0.596, 95% confidence intervals [CIs]; fig. 1A), whereas the mean proportion of variance explained by dominance variance (d^2) was 0.161 (0.078–0.361, 95% CIs; fig. 1B). Posterior density interval estimates overlapped for 10,155 transcripts, suggesting approximately equal contributions of dominance and additive genetic variance for most transcripts. In contrast, additive genetic effects were

significantly greater than dominance variance for 229 transcripts (fig. 1C); and for 115 transcripts, significant dominance variance was detected in the absence of significant additive genetic variance (fig. 1D).

Environmental (i.e., temperature) effects were less prevalent than genetic variance, irrespective of estimation method. When assessed as simple fixed effects, model coefficients describing average differences between temperature treatments were deemed significant for 4,253 transcripts (fig. 2A), following false discovery rate (FDR) correction for multiple comparisons (5,550 uncorrected). Note that in the two environment case, the coefficient describing this effect (β_{Env}) represents the mean difference in $\log_2$ transcript abundance between 17 and 23 °C treatments; thus, the mean fold change. Incorporation of variation among families when modeling temperature effects (random slopes; family × temperature) suggested 4,221 transcripts in which thermal environmental effects are likely mediated through G×E (fig. 2C). Comparing temperature-specific estimates of total phenotypic variance revealed 1,578 transcripts with significantly different estimates of V_P (i.e., nonoverlapping posterior density intervals [PDIs]) in each thermal environment (ΔV_P; fig. 2C). In total, 66.4% of transcripts showed at least one signal of thermal sensitivity, significantly less than the 74.2% (FDR corrected) with additive genetic variation for expression ($\chi^2_{[1]} = 154.8$; $P < 0.001$). Moreover, in only 47 cases (0.4%) did the absolute range of ΔV_P exceed the PDI estimates of either V_A or V_D (fig. 2D). Finally, for all transcripts with a significant response to the temperature treatment, absolute values of β_{Env} were significantly correlated with ΔV_P ($r = 0.347$; $P < 0.001$; fig. 2B).

Signatures of Selection on the Transcriptome

For 84.2% of transcripts, PDIs for the index of quantitative divergence (Q_{ST}) overlapped with those of neutral expectation. Estimates of neutrality were significantly higher based on the rate of divergence (Δ) of gene expression: 99.2% of transcripts ($\chi^2_{[1]} = 1,319$; $P < 0.001$). Signatures of directional selection—Q_{ST} estimates significantly greater than neutral expectation (fig. 3)—were inferred for 1,411 transcripts (15.8%). In contrast, estimation of Δ suggested only 71 transcripts (0.8%) under directional selection: 58 of these were also inferred to be under selection in the Q_{ST}-based analysis, but 13 were inferred neutral by Q_{ST}. Neither analysis detected a signal of stabilizing selection: Zero transcripts displayed an upper PDI or CI less than the lower ranges defining neutral expectation.

The per chromosome proportion of transcripts showing signals of selection differed significantly across the 21 linkage groups ($\chi^2_{[20]} = 68.8$; $P < 0.001$), ranging from 10.1% on chromosome VIII to 25.5% on chromosome XIX. 17.1% (156/912) of transcripts on unlinked scaffolds exhibited signals of selective divergence, a proportion not significantly different from that on known linkage groups ($\chi^2_{[1]} = 1.07$; $P = 0.302$). None of the ten mitochondrial transcripts present in the analysis showed signatures of selection.

We detected four putative peaks of high selective divergence, excluding those located on unlinked scaffolds (fig. 3).
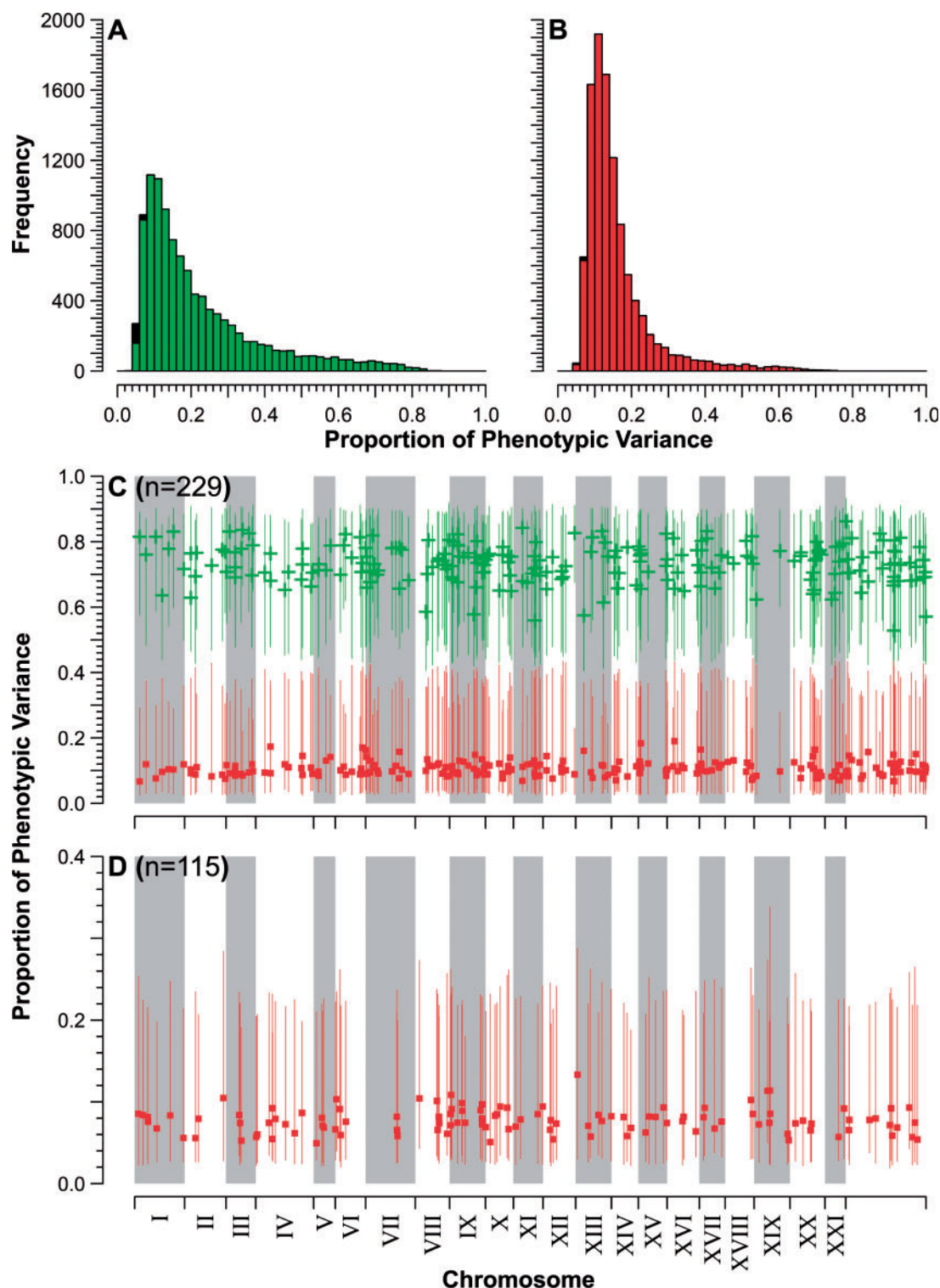
FIG. 1. Proportion of phenotypic variance explained by additive genetic and dominance variance. (A) Frequency distribution of narrow-sense heritability (h^2) estimates for all 10,527 transcripts. Nonsignificant estimates are represented in the black fraction, whereas statistically significant estimates are plotted in gray (green online). (B) Distribution of estimates for the dominance proportion of total phenotypic variance (d^2). Significant estimates are plotted in gray (red online), nonsignificant in black. (C) Chromosomal location of the 229 transcripts for which h^2 is significantly greater than d^2. Point estimates of h^2 are plotted as "+" (green online); d^2 are squares (red online). Vertical lines denote 95% PDIs. Mitochondria and unlinked scaffold are combined in the region to the right of chromosome XXI. (D) Chromosomal location of the 115 transcripts with putatively significant d^2 and nonsignificant h^2 estimates—note that these may represent "false positive" estimates on the basis of phenotypic simulations ($d^2 \leq 0.144$). Symbols and chromosomal arrangement as in (C).
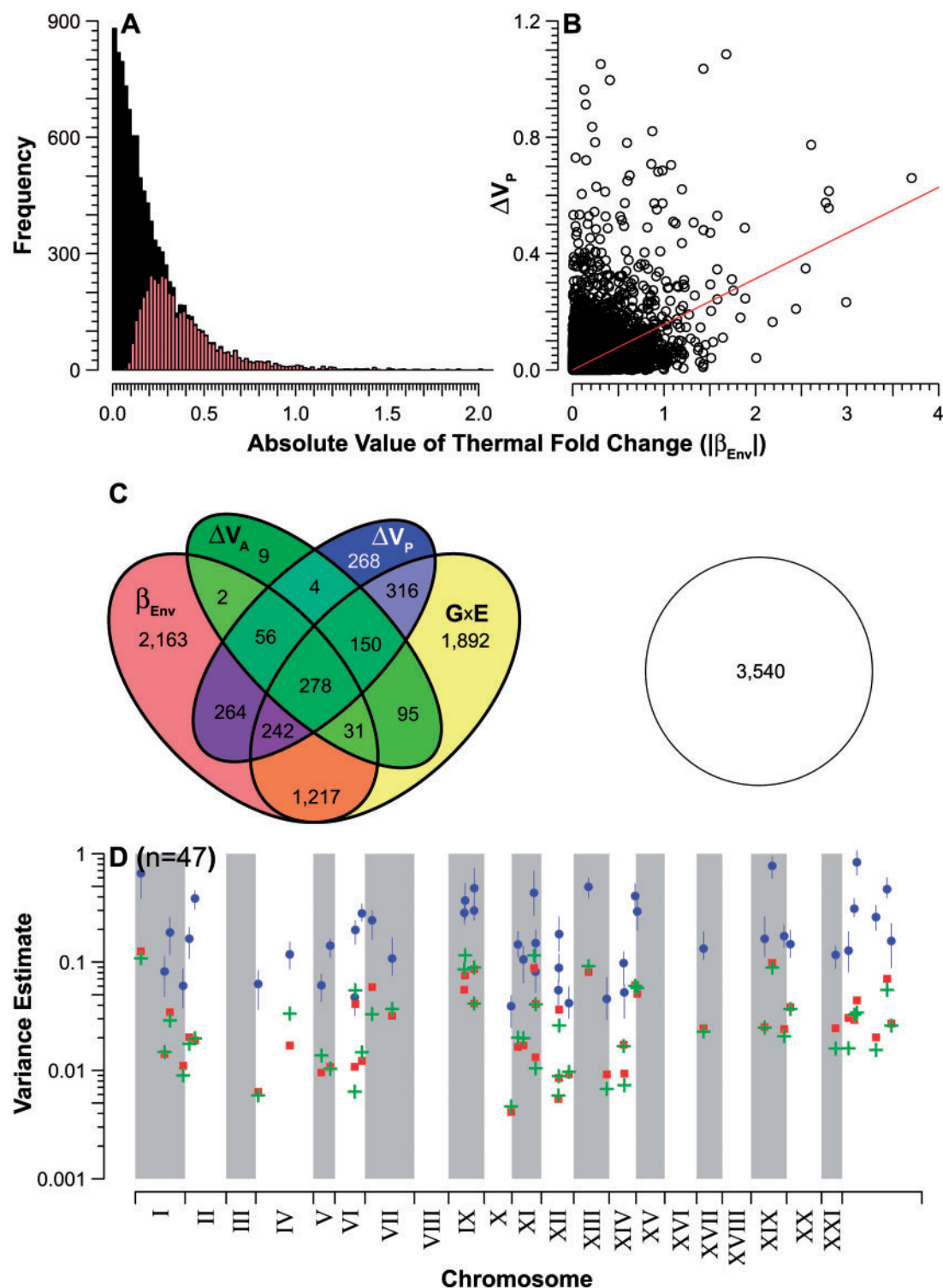
Fig. 2. Significant temperature effects on transcript expression. (*A*) Distribution of fixed-effects coefficients describing the effect of thermal treatment on mean transcript expression (βEnv). The gray fraction (colored online) denotes statistically significant values. (*B*) Relationship between βEnv and △VP, the difference in total phenotypic variance estimated in the two temperature treatments. In total, 6,987 transcripts with a temperature response are plotted. (*C*) Union of transcripts based on their significance with respect to various indicators of environmental effects. Indicators include βEnv, differences in temperature-specific estimates of additive genetic variance (△VA), G×E, and △VP. Overlapping regions denote multiple significant effects. The open circle represents the proportion of nonsignificant transcripts. (*D*) Chromosomal location of the 47 transcripts for which the magnitude of △VP exceeded interval estimates of both VA and VD. For clarity, only point estimates are provided for VA (+) and VD (squares); circles represent point estimates of △VP, bounded by 95% PDIs (vertical lines). Chromosomal arrangement as in figure 1.
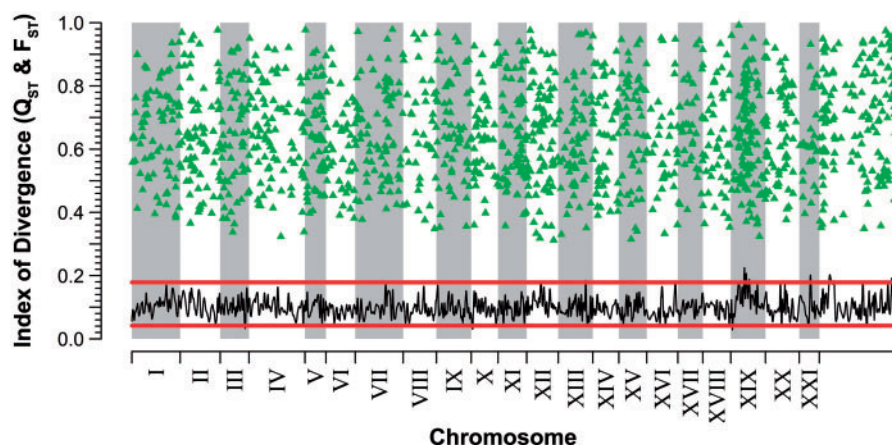
Fig. 3. Among-population divergence in transcript abundance, plotted as a function of genomic location. Loess smoothing (5-kb intervals) of lower 95% posterior density interval values indicate putative genomic regions rich in adaptive expression divergence. Triangles denote QST point estimates for transcripts which significantly exceed a baseline of neutral divergence, as defined by 17 microsatellite markers (range defined by horizontal lines; see Materials and Methods for details). For clarity, probes whose levels of expression divergence fell within the range of neutral differentiation are not shown. Chromosomal arrangement as in figures 1 and 2.

Three peaks were located on chromosome XIX: A 460-kb window 7.9 Mb from the beginning of the chromosome, a 500-kb window around position 9 Mb, and a 390-kb window at 10.7 Mb (supplementary fig. S3A, Supplementary Material online). The remaining peak mapped to a 485-kb window around position 6.5 Mb of chromosome XXI (supplementary fig. S3B, Supplementary Material online).

Discussion

Our results raise two interesting observations relevant to the evolution of transcriptional variation, namely: 1) The vast majority of transcripts exhibit significant genetic variance in expression, indicating that within-population transcriptional variation is heritable and thus potentially responsive to selection; and 2) although the majority of differences in transcript expression among populations are likely neutral, directional selection is also evident. Although observations here regarding the relative fraction of selected transcripts may be species and/or population specific, inferential discrepancies arising from different analytical frameworks may help bring new perspective on the broader debate regarding the roles of neutral evolution and stabilizing selection in transcriptomic evolution.

Pervasive Heritability—Evidence for Broad Adaptive Potential in the Transcriptome

Whether one considers all transcripts with evidence of significant V_A, or a more conservative FDR corrected number, results indicate that a majority of transcripts active in stickleback liver tissue exhibit heritable variation in expression. If the behavior of these transcripts is representative of all 29,245 splice variants in the stickleback genome, this would suggest that at least 74% of the stickleback transcriptome—perhaps as high as 98%—may have heritable variation underlying patterns of expression. This is substantially greater than initial expectations: In the first known synthesis of available data, Gibson and Weir (2005) concluded that irrespective of tissue or organism, 10–50% of transcripts would likely vary due to heritable differences. The reason for this discrepancy may lie in the fact that much of the work describing heritability of transcriptional variance is dominated by quantitative trait locus (QTL)-based estimation. For example, early eQTL studies of human lymphoblastoid cell lines suggested that only 31% of transcripts display heritable variation in expression (Monks et al. 2004), with similar results (28% of transcripts) observed in a an independent genome-wide association study (GWAS; Dixon et al. 2007). Although QTL and related GWAS approaches may provide significant insights into the genetic architecture underlying expression variation, associated estimates of heritability may suffer from both overestimating the proportion of variance explained by individual loci, and a failure to capture all additive genetic variance due to the statistical exclusion of minor-effect loci (Petretto et al. 2006; Manolio et al. 2009). Yet in the context of transcriptional variation, an additional "missing heritability" problem may be an inability to correctly identify the number of transcripts with heritable variation. In a recent study of gene expression in whole blood, Powell et al. (2013) demonstrate a marked reduction in the number of heritable transcripts when estimated based on an eQTL approach (21%) compared with a pedigree-based analysis of same data (64%)—in fact QTL could be mapped to only 30% of the probes for which V_A was judged significant under a robust pedigree and analytical framework.

Our findings with respect to the number of transcripts with heritable variation in expression are, however, comparable with those from similar pedigree-based analyses. Heritable variation has been reported in 60% of transcripts expressed in the livers of inbred mouse strains (Cui et al. 2006), and for 42% and 63–82% of transcripts for human adipose tissue and blood, respectively (Price et al. 2011; Powell et al. 2013). And though constraints imposed by their study system preclude the estimation of additive genetic variance, Ayroles et al.

(2009) nevertheless reveal broad-sense heritability for 68% of transcripts expressed during development in inbred *Drosophila* lines. Together, these results suggest that it is likely the majority fraction of the transcriptome for which patterns of differential expression have some genetic basis.

Although it is generally held that transcriptional variation is additive (Gilad et al. 2008; Kim and Gibson 2010), nonadditive sources of genetic variance have been somewhat neglected in the empirical literature. Plant-based research is one exception to this trend, perhaps because these systems are more amenable to the types of complex breeding designs which facilitate estimation of nonadditive effects and/or the viability of mixed-ploidy hybrids (Riddle et al. 2010). For example, line cross analyses between inbred strains of maize have revealed an abundance of transcripts exhibiting dominance variance for expression, though results are equivocal as to whether V_A or V_D is more prevalent: examples can be found supporting equal proportions of transcripts with both (Pea et al. 2008; Li et al. 2009), as well as only a minority fraction (10–30%) exhibiting V_D (Swanson-Wagner et al. 2006; Stupar et al. 2007). Similar analyses of inter-specific wheat hybrids suggest that significantly fewer transcripts exhibit V_D compared to those with significant V_A (Qi et al. 2012; Chelaifa et al. 2013). Although low numbers of significant estimates imply a potential limitation of statistical power, one study of *Arabidopsis* nevertheless reveals 3% of transcripts with dominance variance in expression, compared with 8% with V_A (Zhang et al. 2008). For vertebrates, we are aware of only four studies reporting the presence of V_D for transcription. A line cross analysis of inter-specific Takifugu hybrids suggests the occurrence of V_D, though details are insufficient to comment on its prevalence (Gao et al. 2013). Similarly, a cross between wild and domesticated Atlantic salmon revealed that a third of differentially expressed transcripts may have significant V_D, compared to 66% with significant V_A (Debes et al. 2012). Dominance variance is also far less prevalent than V_A in mice, irrespective of tissue type (Rottscheidt and Harr 2007), and in one human study, only 5–32% of probes expressed in whole blood show significant dominance variance (Powell et al. 2013). That up to 99% of stickleback transcripts show evidence of significant V_D is surprising, and likely indicative of abundant false positive estimates. Unlike for V_A, we are unable to simulate phenotypes with "known" V_D using the experimental pedigree; thus, a formal power analysis cannot be performed. However, we attempted to estimate the FDR by simulating over a range of known h^2, with d^2 (V_D) implicitly zero. Deviance Information Criterion (DIC)-based selection favored a model including V_D in nearly all (1,009) 1,100 simulations, and mean d^2 of these false estimates was 0.144 (supplementary fig. S2D, Supplementary Material online). This would suggest that any "significant" V_D estimate for which $d^2 \le 0.144$ should be regarded as suspect. Only 4,323 transcripts with putatively significant V_D exceed this threshold (41.1%), suggesting a far more conservative estimate for the proportion of the transcriptome with dominance variance for expression.

The Genetic Basis of Transcriptional Variance

The range of heritability estimates observed for stickleback liver transcripts is remarkably similar to that reported in diverse study systems: A mean of point estimates of 0.226 (median = 0.169; fig. 1A). Cui et al. (2006) report a median transcript heritability of 0.22 in mouse liver, with 75% of transcripts having a heritability greater than 0.01. In stickleback liver, the minimum point estimate for all transcripts with significant heritability was 0.041, with a minimum lower 95%PDI estimate of 0.018—although based on phenotypic simulations, estimates less than 0.094 should be viewed with some skepticism. A median heritability of 0.35 for transcript expression has been reported for human lymphoblastoid cell lines, though this estimate may be upwardly biased given that heritability was estimated only for differentially expressed transcripts (Monks et al. 2004). Other human studies show that average transcript heritability differs across tissues: 0.37 in blood and only 0.24 in adipose (Price et al. 2011)—95% quantiles of nonzero estimates from this study overlap (blood = 0.018–0.497; adipose = 0.042–0.539), both with each other and with those reported here for stickleback liver transcripts (0.070–0.596).

Surprisingly, the proportion of variance described by dominance variance (d^2) was similar to h^2. When the same probes in human blood exhibit both significant V_A and V_D, the dominance fraction of phenotypic variance is much less than h^2 (Powell et al. 2013). In contrast, less than 3% of stickleback liver transcripts had heritability significantly larger than the dominance fraction (fig. 1C), although this is significantly greater than the fraction of probes with only dominance variance (fig. 1D; $\chi^2_{[1]} = 37.7$; $P < 0.001$). This may be due to a possible conflation of maternal effects and dominance variance in half-sib breeding designs (Lynch and Walsh 1998), or perhaps more likely due to the wide/conservative parameter ranges described by the 95%PDIs of the estimates. If only point estimates are considered, 906 stickleback transcripts (8.6%) exhibit $h^2 > 0.5$, a significantly larger fraction than the 213 transcripts (2.0%) for which d^2 is greater than 0.5 ($\chi^2_{[1]} = 451$; $P < 0.001$). This may be indicative of a greater role for *cis*-regulation of gene expression (Lemos et al. 2008), although this remains to be confirmed at the genomic level. It is interesting to note, however, that transcripts with only dominance variance, thus putatively under *trans*-regulatory control, are largely categorized by genes involved in cellular organization and function, whereas transcripts with h^2 significantly greater than d^2 seem to represent genes of relatively higher function (supplementary table S2, Supplementary Material online). This is a pattern consistent with the view of *cis*-regulatory sequences contributing important genetic variation for adaptation through their influence on morphology, physiology, and behavior (Wray 2007). We reiterate, however, that at present this conjecture is speculative, though we suggest that it certainly warrants future consideration.

Although the majority of transcripts appear to have genetic variation for expression, less than 50% of the total phenotypic variation is typically explained by additive effects, and h^2 exceeds 0.5 for only 8.6% of transcripts. Our estimates are

not substantially different than those derived from human studies which estimate that only 5% of the transcriptome exhibits $h^2 > 0.5$ (Gilad et al. 2008). Dominance variance does not appear to capture the remaining unexplained phenotypic variance. Cumulatively, h^2 and d^2 explain on average only 38.3% of total phenotypic variance (0.154–0.778, 95% CIs; supplementary fig. S4A, Supplementary Material online). Although the PDIs of the combined estimates are also quite wide, even the upper limit only rarely captures 100% of the phenotypic variance (0.830 on average; supplementary fig. S4C, Supplementary Material online), suggesting that at least 17% of expression variance remains unexplained for most transcripts. Other nonadditive genetic effects (e.g., genetic maternal effects, epistasis) may contribute significantly to transcriptional variation. Maternal effects may be a less likely source of variance as they generally diminish over time, having their greatest influence on traits early in life (Roff 1997). Epistasis, however, is a frequently overlooked effect potentially leading to bias in estimates of transcriptional heritability (Haig 2010; MacLean 2010; Zuk et al. 2012). However, highly specialized breeding designs are required to accurately quantify these nonadditive genetic effects (Rosa et al. 2006). Constraints imposed by female fecundity necessitated use of a half-sib design for this study which precludes our ability to address these issues reliably, though we strongly encourage future research in this direction.

Environmental Variance

Environmental effects are also a likely candidate to explain residual variance, and the ability to explicitly model critical environmental factors, in this case temperature, is a major advantage of experimental systems. In stickleback livers, 66.4% of transcripts showed some evidence of significant temperature sensitivity over a wide range of indicators. Moreover, 60.4% of these 6,987 transcripts exhibited evidence of G × E ($n = 4,221$; fig. 2C), suggesting a large potential influence of genotype-specific sensitivity to environmental effects. It is noteworthy that the among-family variation in the response to the type of temperature effects studied here is also suggestive of broad-sense heritable variation in plasticity for transcript abundance, a phenomenon reported in another model ectotherm, C. elegans (Li et al. 2006).

With only two treatments, we cannot explicitly estimate the phenotypic variance component due to temperature. However, our measure of phenotypic differences between environments, ΔV_P, may be a useful proxy in this regard: It correlates reasonably well with model coefficients describing temperature effects (fig. 2B), but has the advantage of being measured in units of variance. Moreover, when expressed as a ratio relative to total phenotypic variance, we observe the expected, albeit weak, negative correlation with the cumulative genetic proportion of phenotypic variance ($r = -0.087$; $P < 0.001$; supplementary fig. S4B, Supplementary Material online). Furthermore, when ΔV_P relative to total V_P is modeled as a function of genomic position, chromosomal "valleys" of low broad-sense genetic variance appear to coincide with many "peaks" of our proxy "$V_{E(temp)}$" metric (supplementary fig. S4D, Supplementary Material online). However, the grand

cumulative proportion of phenotypic variance, combining relative genetic and ΔV_P values, explains only a 0.496 proportion of total phenotypic variance in expression for an average transcript (supplementary fig. S4D, Supplementary Material online)—based on the CIs of all estimates (0.206–0.905), nearly 10% of expression variance remains unexplained.

Although reasonable to expect temperature to be a major contributor to expression variance in an ectotherm, this does not rule out other environmental factors. For example, in yeast lines transcription appears to exhibit greater environmental variation than that of genetic effects in response to varying nutritional inputs (Landry et al. 2006), and in Daphnia pulex, 72–85% of the transcriptome is differentially expressed in response to abiotic environmental stressors (Colbourne et al. 2011). Manipulating other environmental variables would undoubtedly also induce some degree of expression variance, though we are hard-pressed to suggest one which would likely produce greater effects than temperature, given its direct effects on the metabolism and rate of transcription in ectotherms. Thus, in this context, it is striking that genetic effects appear to capture a significantly greater proportion of expression variance.

Neutrality and Directional Selection Reconsidered

Both Q_{ST} results and divergence rate estimates (Δ) reveal that for the majority of transcripts, among-population differences in expression are reflective of neutral processes. Moreover, neither analysis could detect a significant signal of stabilizing selection. Together, these observations provide a case which challenges the view of stabilizing and/or purifying selection as the predominant mode of evolution of the transcriptome, and aligns with a neutral model of transcriptome evolution.

To some extent, the failure to detect stabilizing selection may be related to the tendency for dominance variance to upwardly bias estimates of Q_{ST}, potentially leading to false signals of neutrality (reviewed in Leinonen et al. 2013). However, the same metric (Δ; Lemos et al. 2005) used to infer a majority fraction of transcripts under stabilizing selection across a diverse collection of taxa also points to neutral divergence between focal stickleback populations. This in turn points to a problem frequently encountered in studies employing metrics of the rate of evolution: A neglect to consider uncertainty in parameter estimates (Hendry and Kinnison 1999). In our application, we employ a simple bootstrapping procedure to express uncertainty in terms of CIs, and explicitly use this uncertainty when inferring significance of neutrality or selection. Others argue that selection of "conservative" estimates for parameters defining neutrality is sufficient (Lemos et al. 2005; Fay and Wittkopp 2008); however, if the magnitude of differences between upper and lower CIs exceeds that of the neutral region, point estimates may have a high probability of providing false signals. This certainly appears to be the case with these data, wherein the CIs for point estimates of Δ indicative of stabilizing selection range from

approximately 30 to 100 times greater than the neutral range (supplementary fig. S5C, Supplementary Material online). Moreover, many transcripts identified to be under stabilizing selection by the average value of Δ have Q_{ST} values well above neutrality (supplementary fig. S5B and D, Supplementary Material online), values extremely unlikely for a trait under stabilizing selection.

It is perhaps noteworthy that most empirical studies which conclude that stabilizing and/or purifying selection is the predominant mode of transcriptional evolution are based on interspecific comparisons (Lemos et al. 2005; Gilad, Oshlack, Smyth, et al. 2006; Bedford and Hartl 2009; Brawand et al. 2011), though see Nuzhdin et al. (2004) who demonstrate substantial adaptive/selected variation between *Drosophila simulans* and *D. melanogaster*. Yet simulations—the results of which closely match those of observed data—suggest that gene expression divergence is not a simple linear function of generation time: Initially it occurs rapidly, but tends toward an asymptote (Ogasawara and Okubo 2009). Paradoxically then, the rate of change of gene expression divergence when compared among extremely phylogenetically distant taxa would be less than that among more recently diverged groups. To some extent this is reflected by comparing trends between intra- and interspecific contrasts, the former typified by greater rates of drift and directional selection, though the largest fraction of transcripts show signatures of stabilizing selection (Lemos et al. 2005). The implications of this are 2-fold: 1) Comparisons across broader taxonomic groups may be most likely to reveal signatures of stabilizing selection; and 2) the generations immediately following—perhaps even during—divergence, may represent a dynamic phase when mutation, drift, and selection may have the most profound effects on expression divergence.

Adaptive differentiation between populations of the same species would certainly fit the description of a dynamic phase in a taxon's history. The populations used in this study show adaptive phenotypic and genetic divergence typical for this species (Cano et al. 2006). Moreover, divergence has occurred relatively recently, likely within the past approximately 8,000 years following retreat of Pleistocene ice sheets (Mäkinen and Merilä 2008). And although the majority of transcriptional divergence is neutral, a relatively high proportion of transcripts (15.8%) show signatures consistent with divergent selection. Irrespective of the actual mechanisms and selection pressures which underlie this divergence, it is clear that some degree of adaptive differentiation has occurred at the level of the transcriptome.

It should be noted, however, that transcript abundance differs among tissues, and that tissue-specific (i.e., specialized) expression patterns are more likely to be subject to positive selection (Brawand et al. 2011). Whether liver tissue represents a potentially biased sample—either over- or underrepresentative of potential adaptive differences among habitats sampled—remains open for debate. However, killifish populations adapted to a natural temperature cline also show expression divergence in the liver, but in these populations, the majority of transcripts showing signatures of natural selection are under stabilizing selection (Whitehead and Crawford 2006a). This, and the multifaceted functional role of the liver, leads us to believe tissue-related bias should be minimal.

Another potential source of concern is the discrepancy between Q_{ST} and Δ in the number of transcripts showing signals of directional selection. This is unlikely due to the use of only two populations in the rate divergence analysis: Our previous work clearly shows that among-population differences in both expression and enzymatic activity are largely driven by the Lake Vättern population (Nikinmaa et al. 2013), a population which was included in the analysis due to its unambiguous evolutionary relationship with the Baltic Sea population. Rather, we would suggest that differences between methods may be reflective of difficulties in analysis of variance (ANOVA)-based comparisons which do not correct for nonindependent phylogenetic associations: Simulations show that failure to reject neutrality, even under strong selective pressures may be common for such tests (Eng et al. 2009). We would argue that Q_{ST}-based analyses implicitly include a phylogenetic correction in that neutrality is inferred from the same types of genetic data which would be used to generate the phylogenetic covariance matrices in admixture modeling (Eng et al. 2009). Moreover, the wide PDIs bounding Q_{ST} estimates represent a conservative comparison against neutral parameter space (supplementary fig. S5D, Supplementary Material online). Consequently, we are confident that results of Q_{ST} are truly reflective of recent evolutionary history in this system.

Further support for our inference of selection can be gleaned from its genomic locations, and the nature of its putative functional significance. For example, we show a peak of high selective divergence mapping to a 485-kb window around position 6.5 Mb of chromosome XXI (fig. 3 and supplementary fig. S3B, Supplementary Material online). This also corresponds to a chromosomal inversion associated with global patterns of marine–freshwater divergence in the species (Jones et al. 2012). Additionally, we show several peaks on chromosome XIX (fig. 3 and supplementary fig. S3B, Supplementary Material online), the stickleback sex chromosome. Interestingly, sex differentiation is one of the higher-ranked functional clusters identified in an enrichment analysis of putatively selected transcripts. On their own, such analyses are merely suggestive of further avenues of research, and are not confirmatory of functional significance. Nevertheless, they form one line of evidence which is consistent with selection on transcriptional variation playing a role in the process of adaptive differentiation in this system.

Finally, we do not wish to espouse a pan-selectionist view of transcriptional divergence—far from it given that neutral divergence was predominant. Nevertheless, our results show that a relatively larger-than-expected proportion of the transcriptome has diverged in response to directional selection. Taken together, evidence of within population heritable variation and selective variation among populations provide convincing supporting for the contention of broad adaptive potential within the stickleback transcriptome.

Materials and Methods

Broodstock and Husbandry

Crosses for among-Population Comparisons

Broodstock were obtained by sampling mature sticklebacks from three wild, Fennoscandian populations: Lake Pulmanki (Pulmankijärvi; PUL) in Finnish Lapland; the Baltic Sea in the vicinity of Helsinki, Finland (HEL); and Lake Vättern (VAT) from south-central Sweden (supplementary fig. S1A, Supplementary Material online). Full-sib F_1 families were created by crossing parental fish at the sampling sites. Fertilized eggs were transported to the laboratory at the University of Helsinki. Offspring were initially maintained at $17 \pm 1\,^{\circ}C$, with a photoperiod of 18 h light and 6 h darkness. Six months after hatching, environmental conditions were gradually changed to simulate wintering conditions (24 h darkness; water temperature $9 \pm 1\,^{\circ}C$). After 5 months, fish were stimulated into breeding condition by gradually reverting environmental conditions to an 18:6 h light/dark photoperiod, and water temperature to $17 \pm 1\,^{\circ}C$.

Mating pairs were chosen randomly, and new crosses were made to obtain F_2 offspring from within each population. At the time of the experiments, F_2 offspring were approximately 20 months old. Although sexually mature, all experimental fish were reproductively inactive. Twelve F_2 fish from each population ($N = 36$) were randomly selected and assigned to separate, screened containers in one of two identical tanks (six fish per population per tank). Fish were acclimated overnight. One tank was maintained as a control at $17\,^{\circ}C$. At the beginning of the next photoperiod, water in the second tank was heated approximately $1\,^{\circ}C$ per hour for 6 h to a final temperature of $23\,^{\circ}C$. After 1 h at final temperature, each fish was euthanized in a lethal concentration of MS-222 in water, and its liver removed and immediately frozen in liquid nitrogen for storage at $-80\,^{\circ}C$.

Crosses for Quantitative Genetics

Thirty mature males and 60 gravid females were collected from the Baltic Sea for use as broodstock in a paternal, half-sib design (HEL; supplementary fig. S1B, Supplementary Material online). A more detailed description of crossing protocols has been described elsewhere (Leinonen et al. 2011), but in brief, each male was artificially crossed (in vitro, Barber and Arnott 2000) with two separate females, producing 30 unique half-sib blocks (supplementary fig. S1B, Supplementary Material online), 60 families in total. Groups of 15 individuals from each of the 60 families were randomly assigned to separate 10-l tanks. Fish were reared for 6 months at $17 \pm 1\,^{\circ}C$ and 12:12 h light/dark photoperiod.

After 6 months, two individuals from each family were randomly selected and arbitrarily assigned to control or treatment. Fish were placed in the tanks in the evening and allowed to acclimate overnight. The treatment fish were subjected to the same temperature treatment as described above and sampled. This was conducted for five consecutive days, so that five treated and five control fish were sampled for each of the 60 families ($N = 563$, see Sample Preparation). Analysis of power and bias was performed by simulating

phenotypic data, based on the final pedigree and over a range of known heritabilities (Morrissey et al. 2007), and iterative estimation with the same analytical method used on experimental data (supplementary fig. S2 and appendix S1, Supplementary Material online).

Sample Preparation

Total RNA was isolated from liver tissue by means of Tri Reagent (Sigma, St Louis, MO and Ambion, Austin, TX), following manufacturer's protocols. RNA was treated with DNase (Promega, Madison, WI) and reisolated using Tri Reagent. RNA concentration was quantified using a Nanodrop ND-1000 (Thermo Scientific, Waltham, MA), and RNA quality was assessed using an Experion automated electrophoresis system (Bio-Rad, Hercules, CA). RNA was extracted from 35 of 36 individuals used for population comparison (one PUL control missing), and all 600 individuals of known pedigree comprising the quantitative genetics data set. RNA labeling, hybridizations, and scanning were performed by Agilent certified commercial service providers: The Finnish Microarray and Sequencing Centre (Turku, Finland; array 1), and the University Health Network Microarray Centre (Toronto, Canada; array 2). Thirty-seven individuals were removed from the quantitative genetic analyses due to either missing tissue, bad quality RNA, or bad array hybridization. The final data set contained 563 individuals in total, 283 controls (158 females and 125 males) and 280 thermally treated (152 females and 128 males).

Microarray Design and Data Normalization

Two microarrays, based on user-defined 60-bp oligonucleotide probes, were designed using the custom gene expression platform from Agilent (Santa Clara, CA). The first—used for among-population comparisons (i.e., Q_{ST} estimation)—was a $4 \times 44K$ array consisting of 43,605 probes representing 19,274 unique genes, including most known splice-variants (27,723 transcripts; Leder et al. 2009), with most transcripts replicated at least twice. Full technical details are provided in previously published work (Leder et al. 2009, 2010), but in brief, total RNA (400 ng) was amplified and Cy3 labeled. cRNA was examined with the Nanodrop ND-1000 and the Experion Automated Electrophoresis System to assess the concentration and quality of labeling. Each sample (1.65 μg) was hybridized to its array at $65\,^{\circ}C$ overnight (17 h). Arrays were scanned with Agilent Technologies Scanner (model G2505B). Spot intensities and other quality control features were extracted with Agilent's Feature Extraction Software (v9.5.3.1), and array quality was assessed through the use of Agilent control features, as well as spike-in controls.

For the second array, the probe set was reduced by removing several replicate probes, as well as many of the probes that did not appear to hybridize to liver RNA in the first experiment, in order to fit on the newer $8 \times 15K$ array format. RNA quality was determined using the Agilent Bioanalyzer 2100, and only RNA with integrity numbers greater than 7.0 were used for labeling. RNA (500 μg) was labeled (Cy3 or Cy5) using the Agilent QuickAmp Kit for two-color microarray-based

gene expression analysis. Equal numbers of individuals within family groups (control and temperature-treated; males and females) were assigned to each dye, and alternately labeled. Control and treated individuals were selected at random for hybridization to a given array (Agilent Hi-RPM kit). Images of the arrays were acquired using a G2565BA DNA Scanner, and image analysis was performed using Agilent's Feature Extraction Software (v9.5; protocol GE2-v5_95_Feb07 with default settings).

Postprocessed signals were standardized across arrays using a supervised normalization approach, implemented in the package "snm" for R/Bioconductor (Mecham et al. 2010), which has been shown to remove technical artifacts without altering the underlying variance structure of expression data (Qin et al. 2013)—the latter being essential for variance components analysis, as undertaken in this study (for an expanded explanation of the normalization procedure, see supplementary appendix S1, Supplementary Material online). For estimating quantitative genetic parameters, the entire second data set (i.e., all probes present on the second microarray) was normalized across arrays/slides: "Biological variables" included sex, family, and temperature treatment; "adjustment variables" included dye, array, and batch (i.e., slide). Following normalization, data were screened for potential outliers. Individual intensity values more than 2 SDs from their family-by-treatment mean were flagged and removed. Any probes with missing values for an entire family, or for more than 56 individuals (i.e., 10% of data), were removed from the final data sets. For transcripts with two or more replicate probes, we calculated pairwise correlations between probe sets. Replicate probes with a correlation coefficient less than 0.9 were discarded, and replicates which remained were averaged by transcript for each individual. Raw and normalized data, in addition to R scripts describing the normalization procedure, are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3098.

For among-population comparisons, nonnormalized transcripts common to both arrays were merged into a single data set for a broader normalization across studies/array designs, thereby providing standardized data sufficient for estimating both within- and among-population components of genetic variance for inferring selection (see Final Data and Analyses and/or supplementary appendix S1, Supplementary Material online). Biological variables included population, family, and temperature treatment; adjustment variables included dye, array, batch (i.e., slide), and array design; previously identified outliers were removed from the final data set. Data and R scripts are also archived on ArrayExpress (accession E-MTAB-3099).

Microsatellite Data

Details of genotyping are outlined elsewhere (Cano et al. 2006, 2008). Briefly, DNA was extracted from pectoral fin tissue using fine silica 96-well filter plates. Seventeen putatively neutral microsatellite loci, that is, markers approximately 3 cM or more from a mapped QTL region, were genotyped for 28 individuals in each of the three populations (HEL, VAT, and PUL); see Cano et al. (2006) for a list of loci. Forward primers were labeled with fluorescent dyes (FAM, TET, and HEX), and a GTTT-tail was added to the 5′-end of the reverse primers to enhance 3′-adenylation (Brownstein et al. 1996). Polymerase chain reaction products were diluted (1:50), mixed with ROX size standard, and run on a MegaBACE 1000 capillary sequencer. Fragment Profiler (v1.2) was used to score genotypes, with manual corrections as needed.

We evaluated the data set through outlier analysis to ensure that all markers fell within a simulated range of neutral expectation (Antao et al. 2008). As the index of quantitative divergence (Q_{ST}) has been shown to behave similarly to a single-locus estimate of F_{ST}, it should be compared against a distribution of single-locus estimates, rather than the CI of mean F_{ST} (Whitlock 2008). We defined a conservative parameter space indicating neutral differentiation based on the full range (minimum and maximum) of F_{ST} values observed in all 17 neutral markers.

Final Data and Analyses
Quantitative Genetics

Expression levels for 10,527 transcripts (approximately 36% of the stickleback transcriptome), comprising 10,495 predicted/projected genes (Ensembl Stickleback Genome version 65.1, updated May 2010), were retained for analysis after normalization and quality control checks. We decomposed expression variation into additive genetic (V_A) and dominance (V_D) variance using a linear mixed-effects model (i.e., the "animal model"). Fixed effects included dye (Cy3 or Cy5), sex, temperature treatment (17 or 23 °C), and their interaction terms; random effects were assigned by the relationship matrix (i.e., pedigree) for the estimation of V_A and V_D. We employed a Bayesian implementation of the animal model in the R packages "MCMCglmm" and "nadiv" (Hadfield 2010; Wolak 2012). Variance components were estimated by MCMC (Markov chain Monte Carlo) sampling of their posterior distributions, after first removing effects of fixed model terms (temperature, sex, and dye), with parameter estimates based on the posterior mode, and bounded by 95% PDIs. We ran a series of three nested models to test the posterior significance of both V_A and V_D: A simple "null" model included only fixed effects; a second model included random variation attributable to the individual (i.e., "animal") level weighted by the half-sib pedigree structure (V_A); and a final model also including dominance, obtained by inversion of the pedigree matrix to obtain a "fraternity matrix" from which V_D can be estimated (Ovaskainen et al. 2008)—matrix inversion was implemented using the "nadiv" package (Wolak 2012). Models were run with an initial burn-in of 50,000 iterations, followed by an additional 200,000 iterations from which each 200th point on the Markov Chain were sampled. We used the DIC to infer significance of a given variance component by comparing the more "complex" model containing the term with a simpler model without: Significance of V_A was determined by comparison with the "null" model, and a "maximal" model

(V_A and V_D) was evaluated against both the V_A only model and the null model.

Within our analytical framework, likelihood ratio testing and associated p-values for the significance of random effects (i.e. variance estimates) are not possible. Nor can we conduct probability-based significance testing by profiling their posterior distributions, since variance estimates are constrained to be positive. This precludes the use of standard adjustments for multiple comparisons (e.g., FDR correction). Consequently, we used phenotypic simulations to determine the reliability of estimates through an evaluation of "local" false discoveries. We simulated 1,000 trait values with no additive genetic variance using the package "pedantics" (Morrissey et al. 2007), and compared the animal model DIC to a null model—selection of the animal model indicated a false positive results. We then calculated the mean heritability estimate for all false positives (0.094; $n = 247$) to define a threshold value indicative of a reliable estimate. Likewise, we simulated data under the assumption of no dominance variance over a range of known heritabilities ($h^2 = 0$–1 in 0.1 intervals; 100 simulations each). As with the transcriptional data, models including V_D estimates were compared with both a simpler animal model and a null model. For all false positives, we calculated the mean estimate of d^2 to define the significant threshold value (0.144; $n = 1,009$).

As only two temperature treatments were employed, we could not directly estimate environmental variance associated with thermal effects (herein simplified as $V_{E(temp)}$) as a random effect. However, we attempted to discern the relative contributions of environmental effects on phenotypic variance in a number of ways. First, we estimated both total phenotypic variance (V_P) and V_A separately in each "environment" (i.e., thermal treatment): We reasoned that if V_P was significantly different between environments, this could be attributable to a G×E and/or to nongenetic effects—given the thermal treatment imposed on an ectothermic organism, it stands to reason that an environmental effect associated with temperature ($V_{E(temp)}$) would be a likely source of this nongenetic variation. We estimated the relative magnitude of this effect as the difference between parameter estimates (i.e., 1,000 sampling points from the Markov Chains) in each thermal treatment. Significant differences in phenotypic (ΔV_P) and/or additive variance (ΔV_A) were assigned if the 95% PDIs of the difference profiles excluded zero. To simplify comparisons with estimates of V_A and V_P, we converted ΔV_P estimates to their absolute values, reversing lower and upper PDIs for negative estimates, and assuming a lower estimate of zero for nonsignificant differences. Finally, we evaluated the potential for G×E by testing for broad-sense genetic variation in expression reaction norms (family × temperature). We ran an additional series of mixed-effects models (dye, sex, and temperature as fixed effects): A minimal model included random variation among families only in the intercept, and a second model which also included random variation among families in the coefficient describing temperature (i.e., environmental) effects (random slopes; family × temperature). Significance of G×E was evaluated by comparison of model DICs. Relative effect sizes of temperature, sex, and their interaction were

estimated from the posterior distributions of their coefficients; P values were also profiled from the posterior distributions. Correction for multiple comparisons was performed using a local FDR procedure implemented in the R package "fdrtool" (Strimmer 2008).

Comparison among Populations

Following normalization and quality control 8,904 transcripts, representing 8,882 predicted/projected genes (Ensembl Stickleback Genome version 65.1, updated May 2010) common to both microarrays were retained for analysis. To infer whether among-population differences were best explained by neutral or putative selective processes, we contrasted an index of quantitative genetic differentiation (Q_{ST}) for each probe with that of neutral genetic divergence (Spitze 1993; Leinonen et al. 2013). In this approach, an estimate of within population variation equivalent to V_A is required for robust inference. By combining array data (i.e., pooling and normalizing probes / transcripts common to both arrays), we were able to include a broader range of pedigrees, thereby permitting a more accurate and analytically comparable estimate of V_A for calculating Q_{ST}. Variance components (i.e., Q_{ST} parameters) were estimated through mixed-model analysis using MCMCglmm: Temperature and dye effects were partitioned out as fixed model terms, with variance among (σ^2_{GB}) and within populations (σ^2_{GW}; V_A) estimated as random effects. Confidence intervals (95% PDIs) were obtained by sampling the posterior distribution of 1,000 Q_{ST} estimates sampled from the Markov chain. Significance of Q_{ST} was inferred for those estimates with PDIs that did not overlap with the region of neutral differentiation, as defined by F_{ST} of neutral microsatellites. Additionally, we attempted to identify potential genomic regions of high selective divergence by modeling lower interval estimates ($PDI_{0.05}$) as a function of transcript position within the stickleback genome with loess regression over 5-kb intervals.

Finally, we contrasted inference from Q_{ST}-based analyses with that obtained by estimating rate of expression divergence. We used the divergence parameter (Δ) proposed by Lynch (1990) and adapted by Lemos et al. (2005) for application to mRNA expression data: Under this framework a parameter space between 10^{-4} and 10^{-2} represents neutral evolution, based on assumed rates of mutational variance. Estimates of Δ above and below neutrality are indicative of directional and stabilizing selection, respectively. As this metric requires an estimation of generation time between groups, we focused analyses on the two populations with the least ambiguous colonization histories: Baltic Sea and Lake Vättern (supplementary fig. S1, Supplementary Material online)—their stickleback populations share a recent genetic origin (Mäkinen et al. 2006), and the waterbodies themselves share a geological history. Following glacial retreat of the Fennoscandian peninsula, the area currently occupied by Lake Vättern was a bay of the Baltic Sea during its Yoldia Sea stage (approximately 10 ka BP), but by the end of the Ancylus Lake stage (approximately 8 ka BP), Lake Vättern was no longer contiguous with the Baltic (Stålberg 1939; Björck 1995). As sticklebacks breed only once per year,

with new recruits added annually, these dates were used to bound estimates of the numbers of generations since divergence. We generated five balanced replicate blocks within each population–temperature treatment by stratified bootstrapping to estimate standard mean-square error within and between populations in a nested ANOVA. Generation time was sampled from a uniform distribution of integers between 8,000 and 10,000. Confidence intervals of Δ were profiled by bootstrapping the entire estimation procedure 1,000 times. Selection was inferred on the basis of CI exclusion from the parameter space defining neutral evolution (10^{-4} to 10^{-2}).

Functional Annotation Analysis

A complete functional enrichment analysis is beyond the main scope of this article. Nevertheless, such analyses may provide preliminary interpretive value of current results, and more importantly, may be of utility for other researchers. As such, they are included as supplementary material, Supplementary Material online. We used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) to determine whether candidate transcript sets were significantly overrepresented by genes of particular functional categories (Huang et al. 2007). Although DAVID will accept several probe identifiers, Entrez Gene identifiers generally produce the most comprehensive annotation data (Maglott et al. 2005; Sherman et al. 2007). Thus, to facilitate analyses, stickleback genes were assigned an Entrez GeneID based on their human orthologs, determined using BioMart (Durinck et al. 2005; Smedley et al. 2009) and BLAST search. Of the 10,527 transcripts detected above background, 92% (9,661) were assigned an Entrez GeneID. Input for analysis consisted of a list of candidate transcripts contrasted with a customized "background" set including only those transcripts represented on the custom array and detected above background. Though DAVID can integrate nonredundant functional annotations across multiple databases, we focused on Gene Ontology (GO) annotations at the levels of "biological process" and "molecular function" (Harris et al. 2004). We further restricted output by grouping enriched terms into functional annotation clusters, using default settings (Huang et al. 2009). Finally, we excluded those clusters with an overall enrichment score less than 0.5, and for which the mean fold enrichment of constituent GO terms was significantly less than 1.

Supplementary Material

Supplementary appendix S1, figures S1–S5, and tables S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

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