Temperature-dependent gene regulatory divergence underlies local adaptation with gene flow in the Atlantic silverside.

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Abstract

Gene regulatory divergence is thought to play an important role in adaptation, yet its extent and underlying mechanisms remain largely elusive for local adaptation with gene flow. Local adaptation is widespread in marine species despite generally high connectivity and is often associated with tightly linked genomic architectures, such as chromosomal inversions. To investigate gene regulatory evolution under gene flow and the role of inversions associated with local adaptation to a steep thermal gradient, we generated RNA-seq data from Atlantic silversides (*Menidia menidia*) from two locally adapted populations and their F1 hybrids, reared under two temperatures. We found substantial divergence in gene expression and thermal plasticity between populations, with up to 31% of genes being differentially expressed. Reduced thermal plasticity, temperature-dependent gene misexpression and the disruption of co-expression networks in hybrids point towards a role of regulatory incompatibilities in local adaptation, particularly under colder temperatures. Chromosomal inversions show an accumulation of regulatory incompatibilities but are not consistently enriched for differentially expressed genes. Together, these results suggest that gene regulation can diverge substantially among populations despite gene flow, partly due to the accumulation of temperature-dependent regulatory incompatibilities within inversions.

Keywords: Local adaptation; Gene flow; Marine fish; Gene expression; Hybrid gene misexpression; Gene regulatory incompatibilities; Regulatory architecture
**Introduction**

Despite the lack of obvious barriers to gene flow in the oceans, it has become clear that local adaptation is widespread in marine species (D. O. Conover et al., 2006; Sanford & Kelly, 2011). Local adaptation with gene flow has repeatedly been associated with large divergent haplotype blocks linked to chromosomal inversions (Han et al., 2020; Kirubakaran et al., 2016; Tigano et al., 2021; Wilder et al., 2020). However, we still lack a detailed understanding of the molecular mechanisms associated with local adaptation under gene flow. While gene regulatory variation has been argued to be a major target of selection (Mack & Nachman, 2017; McGirr & Martin, 2020a; Pavey et al., 2010), the mechanisms underlying gene expression divergence and the role of inversions remain largely unknown.

In contrast to sequence variation, gene expression is less affected by recombination (Berdan et al., 2021). Therefore, gene expression analysis offers key opportunities to dissect the mechanisms of local adaptation despite the presence of large recombination-suppressing inversions. Generally, intraspecific adaptive divergence is thought to be facilitated by trans-regulatory changes, such as variation in transcription factors (Cutter & Bundus, 2020; Hart et al., 2018; Mack & Nachman, 2017; McGirr & Martin, 2020b). However, under strong gene flow and the presence of inversions, modular and tissue-specific cis-acting changes might be advantageous, as co-evolved cis-regulatory elements and target gene(s) remain linked and are less likely to be broken up by homogenising gene flow (Berdan et al., 2021; Crow et al., 2020; Gould et al., 2018; Said et al., 2018; Verta & Jones, 2019; Wittkopp et al., 2004; Wittkopp & Kalay, 2011). On the other hand, inversions can globally affect gene expression by harboring divergent trans-factors (Naseeb et al., 2016; Said et al., 2018). Thus, even if sequence divergence is concentrated within narrow genomic regions, trans-effects of inversions could drive regulatory changes of genes throughout the genome.
Cis- and trans-regulatory changes are not mutually exclusive and co-evolve to reach or maintain optimal expression levels (Fraser et al., 2010). This co-evolution can lead to the emergence of Dobzhansky-Muller hybrid incompatibilities between populations (Mack & Nachman, 2017; McGirr & Martin, 2020a; Signor & Nuzhdin, 2018). Local adaptation despite gene flow might suggest the evolution of regulatory incompatibilities (Mack & Nachman, 2017; Ortíz-Barrientos et al., 2007), which can manifest in ‘hybrid gene misexpression’; that is expression levels in hybrids are higher or lower than their parental expression range (C. R. Landry et al., 2007; Christian R. Landry et al., 2005; Mack & Nachman, 2017; Ortíz-Barrientos et al., 2007). Hybrid gene misexpression, and hence regulatory incompatibilities, can arise early in the divergence process (Barreto et al., 2015; McGirr & Martin, 2020a; Renaut et al., 2009) and incompatible regulatory alleles might particularly accumulate between non-recombining haplotypes within inversions (Berdan et al., 2021). However, gene regulatory variation can be highly context-dependent, and might only become visible in specific environments, tissues, sexes and/or life-stages (Berdan et al., 2021; Hu et al., 2022; Mugal et al., 2020; York et al., 2018). Overall, it remains unclear, if regulatory incompatibilities and hybrid misexpression arise under ongoing gene flow, and if so, how this is facilitated by inversions.

Atlantic silverside (Menidia menidia), a marine teleost, is an excellent system to investigate the gene regulatory variation, and the impact of inversions, underlying local adaptation. Atlantic silversides are distributed along the North American Atlantic coast and show a high degree of local adaptation across their range (D. O. Conover & Heins, 1987; Hice et al., 2012; Yamahira & Conover, 2002), despite gene flow (Lou et al., 2018; Mach et al., 2011; Wilder et al., 2020). Countergradient variation in growth rate is a major adaptation to differences in temperature, as are differences in other traits, such as fecundity, metabolic rate, swimming performance, and foraging behavior (David O. Conover et al., 2009; Hice et
al., 2012; Yamahira & Conover, 2002). Multiple inversions (0.4 to 12.5 Mb in length), which cumulatively cover about 16% of the entire genome, have been shown to segregate and show strong differentiation along this latitudinal cline, with otherwise minimal genetic background differentiation between populations (Akopyan et al., 2022; Tigano et al., 2021; Wilder et al., 2020). This suggests that chromosomal rearrangements that locally reduce recombination play a key role in maintaining co-adapted alleles despite gene flow (Akopyan et al., 2022).

Here, we use RNA-sequencing to investigate gene expression variation between locally adapted Atlantic silverside populations and use F1 hybrids to identify disruption in gene expression and map the genetic architecture of regulatory divergence. By comparing individuals across two rearing temperatures, resembling their local and non-local environments, we further investigate the temperature-dependence of gene expression variation. Lastly, we investigate the effect of inversions on gene expression variation. Our results support a highly environment-dependent role for gene expression divergence in local adaptation with gene flow, and a context-specific role of inversions through the accumulation of temperature-dependent regulatory incompatibilities.

**Methods**

**Common garden and plasticity experiment.** To investigate the role of gene expression in local adaptation in Atlantic silversides, we set up full reciprocal crosses by strip-spawning wild-caught males and females in batches (NY♀ x NY♂ (NY), NY♀ x GA♂ (NYxGA), GA♀ x NY♂ (GAXNY), and GA♀ x GA♂ (GA)), with each cross created from a mix of gametes from ~40 females and ~40 males. Parents were caught at spawning time at Jekyll Island, Georgia (GA; 31°03’N, 81°26’W) and Patchogue, New York (NY; 40°45’N, 73°00’W) in spring 2017. While the use of wild-caught parents can introduce maternal or
other environmental effects in offspring, raising large numbers of F1s over multiple
generations in the lab was logistically and financially not feasible for these experiments,
which is a common problem for non-model systems. Fertilized eggs were kept in 20 L rearing
containers placed in large temperature-controlled water baths at constant salinity (30 psu) and
photoperiod (15L:9D). This photoperiod was chosen to mimic the summer photoperiod
experienced by silverside during their larval growth period over the summer. We split the
fertilized eggs of each pure cross (NY and GA) into four batches and hatched and reared two
batches per cross at 20ºC and two batches at 26ºC. Hybrid crosses were each split into two
batches, with one batch for each crossing direction incubated at either 20ºC or 26ºC. The two
temperatures, 20ºC and 26ºC, were chosen to reflect the common rearing temperatures in the
wild at each of the parental spawning locations (NY and GA), respectively. Individuals were
reared to an approximate total length of 30 mm (Fig. S1), with the rearing durations (from 37-
87 days since fertilization (dpf); Table S1) differing between populations and temperature
regimes because of variation in growth rates between treatment groups (Fig. 1A) (Hice et al.,
2012; Yamahira & Conover, 2002). Sampling times were standardized by length rather than
by age (dpf) because fish of similar length will be at a similar developmental stage, while
highly varying growth rates across populations and temperatures would have caused vastly
different developmental stages had we standardized sampling by age. Importantly, we
selected the target sampling size of 30 mm because at this length all individuals will have
reached the juvenile stage and are outside the temperature-sensitive sex determination period
(8–21 mm; (David O. Conover & Fleisher, 1986)), which differs in its effect between
populations (D. O. Conover & Heins, 1987; Hice et al., 2012) (see Supplementary Methods
for more detail). Sampling logistics resulted in inevitable slight variation in individual length
at sampling (Fig. S1-2), but we accounted for that length variation in downstream analyses,
as described below. In the G AxNY hybrid cross (GA mother), most individuals died at 26ºC
and all at 20ºC, and thus we could not include this cross in the present study. We compared the total length at sampling between experimental groups graphically using effect size distributions (Ho et al., 2019) and statistically using ANOVAs Tukey Honest Significant Differences test on the ANOVA results (TukeyHSD function in R). We approximated individual growth rates for each sequenced individual by dividing the total length (in mm) at sampling by their age (in days from hatching to sampling), and compared growth rates across populations and temperatures, using analysis of variance (ANOVA) with the aov command in R, including population, temperature, and their interaction as terms. Furthermore, we performed a Tukey Honest Significant Differences test on the ANOVA results to post-hoc compare individual groups [population * treatment]. All animal care and euthanasia protocols were carried out in accordance with the University of Connecticut’s Institutional Animal Care and Use Committee (A17-043).

**RNA-seq & data processing.** Total RNA was extracted from whole larvae (n=42; Fig. 1A; Table S1) using the ZymoResearch Direct-zol Miniprep RNA plus kit, following homogenization in Trizol using a pestle, with on-column DNase I treatment. RNA quantity was determined using the HS Assay kit for the Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA) and quality was assessed using a Fragment Analyzer (Agilent, Santa Clara, CA) at the Cornell University Biotechnology Resource Centre. RIN values ranged from 5.3 to 8.3, with an average RIN of 6.9 (Fig. S3). RNA-seq libraries were prepared at BGI Genomics using the stranded Illumina TruSeq mRNA sequencing kit with Poly-A selection and each library was sequenced to an average of 37.1 M 150 bp paired-end reads (± 0.194 M s.d.) using an Illumina HiSeq 4000 sequencer at BGI. Raw sequencing data were aligned to the linkage-map anchored *Menidia menidia* reference genome (Akopyan et al., 2022; Tigano et al., 2021) using STAR (Dobin & Gingeras, 2015) with default settings (Table S2). The
chromosome-scale genome used for this analysis was improved by anchoring the Atlantic silverside reference genome v1 (Tigano et al., 2021) to a female GA linkage map (Akopyan et al., 2022) (see Supplementary methods for details). Duplicates were marked using the picard tools and read count tables for each annotated coding sequence (CDS) and each individual were generated using HTSeq-count for reads with a minimal alignment quality of 20 (-a 20).

**Gene expression analyses.** We performed initial gene expression analyses across all individuals using DESeq2 (Love et al., 2014), retaining all genes with a minimum of 42 counts per million (~1 cpm per individual) and the following model: ~ population + temperature + population * temperature + length; with populations being NY, GA or HYB (NYxGA), temperature either 20°C or 26°C. Principal components analyses were performed using pcaMethods on rlog-transformed read counts (Stacklies et al., 2007). We also tested for the effect of body length on gene expression to test if gene expression variation was driven by variation in individual size/developmental stage (see Supplementary text for detail). Furthermore, we tested for potential differences in sex ratios between groups by analyzing the expression of cyp19a (see Supplementary text for detail). Due to the strong effect of temperature on expression patterns (see Fig. 1C, Fig. S4), we identified differentially expressed genes separately for each rearing temperature with a false-discovery rate threshold of 0.05 and the following model: ~ length + population. We only included genes with a minimum count of 20 and 22 counts per million across all individuals at 20°C and 26°C, respectively. Genotype x environment interactions (GxE) by defining genes with evidence of GxE as those falling into one of the following two categories (Fig. 3A): 1) only one population, i.e. genotype, shows differential expression between temperatures (FDR < 0.05) or 2) both populations show differences between temperatures, but in opposite directions. We
also identified genes that showed conserved responses to temperature in both populations. Lastly, we determined the inheritance mode for each gene based on the difference in gene expression between the pure parental populations and the hybrids following the criteria in Coolon et al. (Coolon et al., 2014) (Fig. 3A, Supplementary methods). Genes that show higher (overdominant) or lower (underdominant) expression in hybrids compared to both parental populations (Fig. 3A) (FDR < 0.05), potentially harbor genetic regulatory incompatibilities between populations (Coolon et al., 2014; Groszmann et al., 2013; He et al., 2013; Mack & Nachman, 2017; McManus et al., 2010; Mugal et al., 2020). We combined genes with overdominant and underdominant expressions as ‘misexpressed’. 

**Allele-specific expression analysis.** To determine whether expression divergence between populations is primarily driven by changes in cis-regulatory (e.g. enhancers) or trans-regulatory elements (e.g. transcription factors), we mapped differences in regulatory modes between GA and NY using allele-specific expression (ASE) analyses in hybrids at each temperature. F1 hybrids carry a GA (southern) and NY (northern) copy of each chromosome within a shared trans environment (the hybrid cell), hence, any ASE bias at a particular gene can be attributed to the divergence in cis-regulatory elements, rather than trans-acting factors (Coolon et al., 2014; Wittkopp et al., 2004). If no ASE bias is present in hybrids but parental populations show divergent expression, trans-acting factors likely cause the regulatory divergence (Coolon et al., 2014; Wittkopp et al., 2004).

To reduce the impact of allele-specific mapping bias on our analyses, we first produced a reference genome with all fixed and nearly fixed SNPs between populations masked. We called SNPs from the genome-aligned RNA-seq data using GATK v.3.8 (McKenna et al., 2010) with the HaplotypeCaller pipeline, retaining bi-allelic SNPs with a
genotype quality above 30, minimum depth of 5x and maximum depth of 52, minor allele frequency above 5% and with less than 25% missing data. We determined allele frequencies in pure F1 individuals from GA and NY across temperatures using vcftools v.0.1.16 and used allele frequency differences (AFD) to identify fixed (AFD = 1) and nearly fixed (AFD > 0.99) sites between GA and NY following (Berner, 2019). We then used the ASeR pipeline (Combs & Fraser, 2018) (https://github.com/TheFraserLab/ASEr) to mask all sites that were heterozygous in more than 75% of hybrids and showed AFD values above 0.95th percentile of the empirical distribution (AFD > 0.392). While interspecies comparisons typically focus on only fixed genetic differences between species (Coolon et al., 2014; McManus et al., 2010), we also included strongly differentiated SNPs in our intraspecies comparison, as fixed sites are relatively rare due to the strong gene flow and differentiated SNPs are also functionally important. After remapping RNA-seq data for hybrid individuals to the masked reference genome, keeping only uniquely mapping reads, we produced allele-specific haplotype counts for highly-differentiated heterozygous SNPs using the phaser pipeline (Castel et al., 2016) (see Supplementary methods). We confirmed the absence of mapping biases by testing that the distribution of haplotype counts is centered around zero (Fig. S3).

We used a population-level approach to test for allele-specific expression (Wang et al., 2020) and classified genes by regulatory mode based on the criteria by Coolon et al. (Coolon et al., 2014) (Supplementary text). Compared to Coolon et al. (Coolon et al., 2014), the approach we followed from Wang et al. (Wang et al., 2020) makes use of biological replicates, as we have in our study, and analyzes allele-specific expression by fitting negative binomial generalized linear models and Wald statistical tests using DESeq2. We performed the ASE analysis independently for each temperature-regime. To test for the effect of temperature on cis- and trans-effect sizes, we compared the difference in log-fold-change from the ASE test
(cis-effect) and the transTest (trans-effect) between temperatures using a Kolmogorov-Smirnov test in R.

**Co-expression network analyses.** In general, genes do not act independently but rather interact within networks of co-expressed genes. The properties of co-expression networks can also evolve and low preservation of networks and/or their disruption in hybrids might point toward regulatory changes involved in local adaptation (Filteau et al., 2013). We used the R-package WGCNA (Langfelder & Horvath, 2008) to infer co-expression networks for the NY and GA populations and their F1 hybrids (NY x GA). Within pure NY and GA co-expression networks, we identified clusters of highly correlated genes, hereafter modules. We then tested for an effect of rearing temperature on module expression by using rank-transformed module-eigengenes as response variables in a linear mixed effects model. Individual fish length was used as a random covariate in the model to account for variation in size. *P*-values were corrected for multiple testing by False Discovery Rate correction (*Q*-value). Furthermore, we estimated the correlation between module eigengene and growth rate (mm per day) using Pearson correlation.

Module preservation analysis was used to test whether gene composition and among-gene connectivity properties of modules in each pure population network were maintained in the hybrid network. We used WGCNA to infer gene co-expression networks for each population and F1 hybrids (NY x GA). We compared pure GA and NY population networks to each other, and each to the hybrid network, to identify changes in network properties. We used each pure population network as a reference, then tested whether modules in the other pure population, and the hybrid network, were similar (preserved) or different. In each comparison, the mean and variance of seven common network-based statistics are calculated...
using a permutation procedure, in which genes are randomly assigned to modules 200 times. This creates a null distribution of network statistics, against which $P$-values and $Z$-transformed scores for each statistic are calculated. A composite preservation statistic ($Z_{\text{summary}}$) is calculated to summarize $Z$-scores across all preservation statistics following (Langfelder et al., 2011). Our interpretation of the results was based on guidelines by (Langfelder et al., 2011), in which simulated data suggest that $Z_{\text{summary}} < 10$ represents weak evidence of module preservation, while $Z_{\text{summary}} < 2$ suggests no preservation at all. Pure population modules that are weakly preserved in the hybrid network represent potential gene regulatory pathways that are misexpressed.

To determine the functional role of each temperature-related and growth-correlated gene co-expression module in GA and NY, we performed gene ontology (GO) overrepresentation analysis using topGO in R. We used all expressed genes after filtering as the background gene list. GO terms with $p$-values below 0.05 were retained as significant.

**Impact of inversions on gene regulation.** We tested if specific groups of genes (e.g. DEGs, misexpressed genes, co-expression modules) were enriched inside one or multiple inversions. First, we confirmed that known major inversions are polymorphic in our dataset by performing principal components analysis using SNPrelate (Zheng et al., 2012) based on SNPs within inversions (Akopyan et al., 2022; Tigano et al., 2021). We conservatively defined genes as being within an inversion if they started and ended within the inversion. For each known inversion that was polymorphic in our study individuals (inversions on chr.4, chr.7, chr.8, chr.18, chr.19 and chr.24), we tested if the proportion of genes of a particular group is higher inside a particular inversion compared to the proportion of genes in the focal group across the collinear genome using Fisher’s Exact Tests in R.
Results

Substantial divergence in gene expression and thermal plasticity across populations. In our lab crosses, northern NY individuals grew faster than southern GA individuals, as expected based on past studies (David O. Conover & Present, 1990; Hice et al., 2012), and growth rates were consistently higher at 26°C than at 20°C (Fig. 1B, Fig. S1). Intermediate hybrid growth rates suggested additive variation in growth (see details in Supplementary results; Fig. 1B).

Principal components analysis (PCA) of genome-wide expression patterns (n=18,517 genes) of unrelated individuals (see Supplementary material) revealed strong separation between GA and NY along PC1 (PVE_{PC1}=24%; Fig. 1C), with a substantial overlap of hybrids with the maternal NY population, potentially due to maternal or other environmental effects stemming from the use of wild-caught parents. More than a quarter of the genes were differentially expressed (DE) between GA and NY when compared within a temperature regime, with greater expression divergence at 26°C (5,649 DE genes or 30.6% of all expressed genes) compared to at 20°C (4,655 DE genes or 25.2% of all expressed genes; Fig. 1D, Table S3). Although we observed differences in total length and age between some of the experimental groups, we did not detect any significant length differences between our principal comparisons (between populations within temperatures and between temperatures within groups; Fig. S3 and Table S4). In line with this, there was no correlation between difference in length and age with the number of DE genes between groups (Figs. S5 and S6) and no substantial effect of length or age variation on differential gene expression (see Supplementary results for details). This suggests that this individual variation does not significantly impact gene expression comparisons, which is potentially explained by the fact...
that all individuals have reached the same developmental juvenile stage at these observed sizes.

Sex can have strong effects on gene expression, but the phenotypic sex of the sampled individuals could not be determined because of their young age and RNAlater preservation, and sex-linked genetic markers for this species do not exist due to their complicated sex determination mechanisms (see Supplementary text). Thus, we tested for the expression variation of a sex-linked gene (cyp19a) but did not detect strong evidence for a role of sex in driving expression differences between groups (Fig. S7; Supplementary text; (Duffy et al., 2010)). Furthermore, we tested if we selected closely-related individuals by chance despite the batch-spawning of many parents, as sex-determination mechanisms and sex-ratios can strongly differ between families in Atlantic silversides (Lagomarsino & Conover, 1993). However, individuals were not closely related, making it less likely that we only selected individuals from one sex for specific groups (see Supplementary material).
Figure 1: A) Common garden experimental design. Parental individuals were wild caught on Jekyll Island, Georgia (GA) and Patchogue, New York (NY). Fertilized eggs for each cross were reared under two different temperatures until larvae reached about 30mm of length. Hatching times and sampling times are given in Table S1. B) Temperature-dependent genetic
variance in size-at-age (proxy for growth) between individuals used for RNA-seq. The genetic variance of size-at-age was additive at 20°C, with HYB showing intermediate growth. However, size-at-age showed dominance (NY) and/or the influence of maternal effects at 26°C. ANOVA results are highlighted by asterisks: ***: p < 0.05; n.s.: p > 0.05. C) Principal component plots for PC1 vs PC2 and PC1 vs PC3 based on all expressed genes. Colours correspond to the crossing scheme in panel A, and temperatures are shown in open (26°C) or closed (20°C) circles. The ellipses give the 95% confidence interval of the data distribution for each cross for both temperatures combined. D) Venn diagram showing the number of unique and shared differentially expressed (DE) genes between NY and GA at 20°C and 26°C. See Fig. S8 for detailed MA-plots.

Within each population, temperature caused substantial differences in gene expression, but along different PC axes. PC2 ($\text{PVE}_{\text{PC2}}=13\%$) separated individuals from different temperature-treatments in GA, while PC3 (11%) and PC4 (8%) separated temperature-treatments in NY and HYB (Fig. 1C, Fig. S4), indicating that different subsets of genes responded to temperature in the different populations. Temperature-dependence in expression patterns between populations indicates the presence of strong genotype x environment interactions (GxE) (Fig. 2A; Fig. S9, Table S5). Thermal responses did indeed differ between populations, with more DE genes between temperatures in GA ($n_{\text{GA}} = 2371; 12.8\%$) than in NY ($n_{\text{NY}} = 1504; 8.1\%$) (Fig. 2B,C, Table S5). The majority of genes showed temperature-specific responses only in one population ($n_{\text{GA-only}} = 1736, 9.4\%; n_{\text{NY-only}} = 1235, 6.7\%$) and a few genes changed expression in opposite directions ($n_{\text{opposite}} = 38; 0.2\%$) (Fig. 2B-D, Table S5). Only 269 (1.5%) out of 3278 temperature-responsive genes changed expression in the same direction in GA and NY (Fig. 2B-D).
Figure 2: Genotype x environment interactions. A) Theoretical examples for genotype x environment interactions (GxE) for individual genes based on temperature-dependent expression profiles within populations. GxE can either include population-specific responses or opposite temperature responses between populations. B) Comparison of gene expression differences (log fold change; LFC) between temperature regimes (20ºC vs 26ºC) in GA and NY. Genes with evidence for GxE are coloured based on the colour scheme in Panel A. Genes with concordant temperature responses between populations, highlighted in orange, do not show any evidence for GxE. C) MA-plots highlight that temperature-responses in pure-bred parental F1 populations are stronger in both magnitude and number compared to their F1
hybrids. D) Venn diagram showing the large difference and weak overlap in the number of genes showing differential expression across temperatures in the different groups.

Reduced thermal plasticity in hybrids coupled with temperature-dependent misexpression. To identify potential physiological and gene regulatory incompatibilities in hybrids, we searched for dysregulation of thermal expression plasticity and gene expression patterns in hybrids compared to parental populations (Fig. S10).

Thermal gene expression response differed strongly between parental populations and F1 hybrids (HYB). In stark contrast to both parental populations, only 166 genes (0.9%) showed expression differences between temperatures in HYB (Fig. 2C and 2D, Table S5). Many genes differed in expression between HYB and GA in a temperature-dependent fashion (20°C = 7068 [38.2%]; 26°C = 3740 [20.2%]). We found fewer DE genes between HYB and NY at 20°C (n=3472 [18.8%]) and notably, only 24 DE genes (0.13%) at 26°C, in line with temperature-dependent maternal effects and/or NY ancestry dominance (Fig. 1B, C).

As expected for recent evolutionary divergences, most genes showed conserved inheritance in hybrids (i.e. no significant difference in expression between hybrids and both parental groups) at both temperatures (60.0% of all expressed genes at 26°C and 44.8% at 20°C; Fig. 3, Table S6). However, we also observed substantial hybrid gene misexpression at 20°C, with over 13.5% of all expressed genes having higher or lower expression (‘transgressive’) in hybrids compared to the parental populations at 20°C (Fig. 3B). In
contrast, only 0.07% of genes were misexpressed at 26°C (Fig. 3B), with most misexpressed genes at 20°C showing conserved expression patterns at 26°C (Fig. 3C).

To determine the selective forces underlying gene expression evolution between locally adapted populations, we compared DE genes between parental populations and misexpressed genes (McGirr & Martin, 2020a). We found that the majority of the misexpressed genes were not differentially expressed between parental populations (20°C: n=1934 [77.2% of all misexpressed genes]; 26°C: n = 8 [61.5%]), in line with stabilizing selection maintaining optimal expression levels in the parental populations despite divergence in gene regulatory mechanisms (Wray et al., 2003). Nevertheless, a substantial portion of the misexpressed genes were differentially expressed between NY and GA, particularly at 20°C (20°C: n=571 [22.8% of all misexpressed genes]; 26°C: n = 5 [38.5%]), indicating that expression levels of these genes are potentially under divergent selection (McGirr & Martin, 2020a; Pavey et al., 2010). Furthermore, of the 3009 genes with evidence for GxE interactions (population-specific temperature responses) in at least one of the three groups (Fig. 2B), 677 (24.5%) were misexpressed in hybrids in at least one temperature regime (670 at 20°C and 8 at 26°C, with one shared), which is more than expected by chance (HGT\textsubscript{20°C}: p = 3.17e-31; HGT\textsubscript{26°C}: p = 0.00065). Together, these results suggest that gene regulatory mechanisms have rapidly diverged in a temperature-dependent manner between populations despite ongoing gene flow.
Figure 3: Gene expression inheritance is dependent on temperature. A) Cartoon of expected expression patterns among populations under different types of gene expression inheritance. B) Comparison of gene expression differences between HYB and both parental populations (NY and GA) to identify inheritance modes for individual genes (see also Fig.
S6). The proportion of genes with specific inheritance modes is shown in lollipop plots on the right, with percentages next to each class (see Table S6 for details). Points in all plots are coloured based on the inferred inheritance mode. C) Alluvial plot highlighting changes in the number of genes for each inheritance mode between temperature regimes. This plot only includes genes expressed in both temperature regimes.

**Disruption of temperature-sensitive gene expression networks in hybrids.**

The gene co-expression network analysis with WGCNA clustered 18,526 genes into roughly equal numbers of modules within NY, GA, and hybrid networks (NY: 23 modules; GA: 28 modules; HYB: 22 modules; Table S7-8), with modules representing groups of genes that show correlated expression patterns and are potentially functionally related. Most modules were at least moderately preserved between NY and GA ($Z_{summary} > 10$), meaning they are similar in their network properties between populations (see Methods for details). However, seven (30.4%) and 13 (46.4%) modules in NY and GA, respectively, were not strongly preserved in the other population (Table S7-8), suggesting that the coexpression patterns of genes in these modules have evolved between populations. We found that five GA modules (Table S7) and eleven NY modules (Table S7) showed an effect of temperature on module eigengene expression, while three and five modules were significantly associated with growth rate (size-at-age) in GA and NY, respectively (Fig. 4A). Three temperature-sensitive modules in NY, including one growth-associated module (brown NY module), were only weakly preserved in GA (Table S8). Similarly, two temperature-sensitive modules in GA were only weakly preserved in NY, one of which (tan module) was also significantly correlated with growth rate (Table S7).
We next tested whether pure population modules were disrupted in hybrids, which would support the divergence and incompatibility of the underlying gene regulatory mechanisms between NY and GA observed on the single gene level (Fig. 3). The majority of parental population modules were not well preserved in hybrids (Fig. 4B,C). This was most pronounced in GA where 79% of modules were weakly ($Z_{summary} < 10$) or not-at-all preserved ($Z_{summary} < 2$; Fig. 4B; Table S8), suggesting a strong disruption of co-expression network architecture in hybrids. Parental population modules tended to be less preserved in hybrids than each pure population network in comparison to each other. For example, twice as many GA modules were preserved in the NY network than in the hybrid network (48% vs. 21%; Table S7), with the mean and median $Z_{summary}$ scores being consistently higher in parental than hybrid population comparisons (Fig. 4C). Similar to parental population comparisons, modules in the NY-hybrid comparison were better preserved than in the GA-hybrid comparison, although preservation was still generally low (Fig. 4; Table S7). Temperature-responsive co-expression modules were generally less preserved than modules not influenced by temperature, though this result was only significant in the GA vs hybrid comparison (Fig. 4B, see inserts). Of the growth-associated modules, only two (out of five) from the NY network were well-preserved in hybrids (Table S7), and none from the GA network were (Table S8). In line with hybrid gene misexpression and divergence in regulatory mechanisms, which we detected for central hub genes in co-expression modules (Supplementary results), these results support the presence of regulatory divergence between locally adapted silverside populations.
Figure 4. Disruption of gene expression networks.  A) Correlation between growth rate and module eigengene expression for two temperature-responsive modules, the Brown module in GA and the Turquoise module in NY. B) Module preservation composite $Z_{\text{summary}}$ scores for each of two comparisons between NY and GA networks and the hybrids. Each colored point represents a module identified in the pure GA network (A) or pure NY network (B). $Z_{\text{summary}}$ scores are plotted against the size of each module in numbers of genes (see ...
Table S7 and S8). Insets are the boxplots of $Z_{\text{summary}}$ scores in each comparison for modules responsive to temperature (Temp.) or not responsive to temperature, as identified in ANCOVA (Table S7 and S8). C) Network preservation, given as $Z$-summary score, for each co-expression network comparison shown as boxplots, with the distribution of values shown as violin plots. HYB networks are generally less well preserved than pure networks. The grey, dashed line shows a $Z$-summary score of 10, below which modules are considered weakly preserved. Mean values for each comparison are also given.

**Temperature-dependent contribution of inversions to regulatory divergence.** To explore the impact of inversions on gene expression variation, we examined whether divergently regulated genes were enriched within inversions. If inversions play a predominant role in gene regulatory divergence, we expect enrichment for DE genes and misexpressed genes within inversions segregating in our dataset, namely on chromosome 4 (inv4), chr.7 (inv7), chr.8 (inv8), chr.18 (inv18), chr.19 (inv19) and chr.24 (inv24) (Fig. S11-S12). We found only 527 (11.3%) and 661 (11.7%) DE genes, and 248 (10.2%) and 5 (41.6%) misexpressed genes inside segregating inversions at 20ºC and 26ºC, respectively. However, when we tested if the proportion of DE and misexpressed genes was higher within individual inversions compared to the collinear genome, we found that DE genes were significantly enriched within inv4 and inv18 at 20ºC, and within inv18 at 26ºC (Table 1). Misexpressed genes were enriched within all inversions at 20ºC, and within inv4, inv8, and inv18 at 26ºC (Table 1). Furthermore, inv18 and inv24, the two inversions that show the strongest differentiation in allele frequencies between our populations (Akopyan et al., 2022), were also enriched for genes belonging to temperature-dependent GA and NY gene modules. Genes belonging to the “tan” NY-module and “darkorange” GA-module were enriched inside inv18, and genes from the “darkgreen” NY-module and the growth-related “brown” GA-module were enriched inside inv24. Three
of the four modules were only weakly preserved in the GA-NY and/or pure-hybrid comparison (Table S7-8), suggesting that the enrichment within the most strongly divergent inversions on chromosomes 18 and 24 is potentially associated with the disruption of these networks, perhaps in a temperature-dependent manner.

**Table 1: Inversion enrichment results.** Shown are odds ratio (OR) and p-values from Fisher’s exact tests, comparing the proportion of DE genes or misexpressed genes within inversions compared to the proportion of DE or misexpressed genes in the collinear genome. Some inversions were not tested for misexpressed genes at 26°C due to the absence of misexpressed genes in those inversions.

<table>
<thead>
<tr>
<th></th>
<th>inv4</th>
<th>inv7</th>
<th>inv8</th>
<th>inv18</th>
<th>inv19</th>
<th>inv24</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE at 20°C</td>
<td>OR = 1.27</td>
<td>OR = 0.57</td>
<td>OR = 1.19</td>
<td>OR = 1.52</td>
<td>OR = 0.57</td>
<td>OR = 1.23</td>
</tr>
<tr>
<td></td>
<td>p = 0.01813</td>
<td>p = 0.07037</td>
<td>p = 0.05237</td>
<td>p = 0.001218</td>
<td>p = 0.2187</td>
<td>p = 0.07341</td>
</tr>
<tr>
<td>DE at 26°C</td>
<td>OR = 1.11</td>
<td>OR = 0.95</td>
<td>OR = 1.05</td>
<td>OR = 1.31</td>
<td>OR = 1.07</td>
<td>OR = 1.22</td>
</tr>
<tr>
<td></td>
<td>p = 0.3005</td>
<td>p = 0.9039</td>
<td>p = 0.6109</td>
<td>p = 0.03302</td>
<td>p = 0.8693</td>
<td>p = 0.07272</td>
</tr>
<tr>
<td>Mis-GEx at</td>
<td>OR = 2.20</td>
<td>OR = 4.21</td>
<td>OR = 2.80</td>
<td>OR = 2.85</td>
<td>OR = 3.56</td>
<td>OR = 3.28</td>
</tr>
<tr>
<td>20°C</td>
<td>p = 0.03035</td>
<td>p = 5.174e-06</td>
<td>p &lt; 2.2e-16</td>
<td>p = 2.784e-08</td>
<td>p = 3.56</td>
<td>p = 3.398e-14</td>
</tr>
</tbody>
</table>
Temperature-dependent gene regulatory architectures. Overall, 820 genes (4.4% of all expressed genes) with allele counts were available for allele-specific expression (ASE) analyses (see Supplementary results for more details). Of these, we found evidence for ASE at 134 genes (FDR < 5%) and 58 genes (FDR < 5%) at 20°C and 26°C, respectively (Fig. 5, Fig. S13-S14). Expression differences between GA and NY were more often associated with all-trans differences compared to cis-regulatory divergence (all-cis) at both temperatures (Fig. 5A), with 8.4% (52 out of 619 genes) and 15.8% (102 out of 644 genes) of genes being all-trans regulated at 20°C and 26°C, respectively (Table S9). In comparison, only 21 genes (3.4%) and 16 genes (2.5%) were all-cis regulated at 20°C and 26°C, respectively (Fig. 5, Fig. S14). This was lower than the proportion of all-trans regulated genes (26°C: \(X^2=80.91, \text{df}=1, p<2.2e-16; \) 20°C: \(X^2=14.1, \text{df}=1, p=8.629e-05\) (Table S9).

We found that the proportion of genes consistently all-cis regulated across temperatures (6.1% of all-cis regulated genes were all-cis regulated at both 20°C and 26°C) was lower compared to the proportion of consistently all-trans regulated genes (24.8% of all-trans regulated genes were all-trans regulated at both 20°C and 26°C) (Fig. 5C). Thus, we tested if cis- or trans-effects change more strongly between temperatures. In general, differences in trans-effects were stronger than differences in cis-effects (KS test: D=0.19033, p=4.042e-13; Fig. S15), suggesting that the effect sizes of trans-changes are more
temperature-dependent, despite the overall smaller proportion of changes in all-trans genes between temperature treatments.

Overall, trans-regulatory changes seemed to be prevalent in Atlantic silversides. However, it is difficult to draw broad conclusions from the small number of genes we could make inferences from here, and more detailed analyses of regulatory architectures are needed to test these results.
Figure 5. Temperature-dependent gene regulatory architectures. A) Illustrations highlighting how regulatory architectures can diverge in cis and trans, and how this might affect expression levels in hybrids, and importantly how environmental differences might
lead to different inferred regulatory modes, e.g. conserved at 20°C but cis- or trans-regulatory divergence at 26°C. B) Scatterplots show differences in expression between parental populations versus expression differences between maternal and paternal alleles in hybrids at 20°C (top) and 26°C (bottom). Dots are coloured by their inferred regulatory mode and represent individual genes. Expected expression patterns for all-cis and all-trans regulated genes are highlighted by solid lines/arrows. C) Changes in the number of genes for each inferred regulatory mode between temperature regimes are illustrated in this alluvial plot. Genes that are not expressed under one temperature regime are shown in grey.

Discussion

Despite our growing understanding of the genomic changes underlying local adaptation with gene flow, the underlying molecular mechanisms, and the contribution of putatively locally adaptive inversions to gene regulatory evolution remain largely unknown. Here, we test for divergent gene expression mechanisms between locally adapted populations of a marine fish that harbors multiple divergent inversions. Overall, differential gene expression and differences in temperature-responses are substantial between locally adapted populations. Misexpression and disruption of co-expression modules is pervasive at colder rearing temperatures, yet less pronounced at warmer temperatures, pointing to a role of temperature-dependent regulatory incompatibilities in local adaptation. Misexpressed genes are strongly enriched within chromosomal inversions, suggesting that these structural variants have accumulated incompatible alleles. Lastly, we found that divergence in gene expression appears to be more strongly driven by trans- than cis-regulatory divergence, although regulatory architectures seem to be partly temperature-dependent, especially trans-regulatory variation. Overall, our results suggest an important role of gene regulatory divergence and
regulatory incompatibilities, partly driven through polymorphic inversions, in local adaptation under gene flow in a marine fish.

**Strong divergent genome-wide expression and temperature responses between locally adapted populations.** Gene expression is highly divergent between locally adapted Atlantic silverside populations despite gene flow between them (Wilder et al., 2020), with over 30% of all genes being differentially expressed between populations. Such levels of expression divergence are extremely high compared to estimates from other studies focused on intraspecific comparisons, which largely range from 0.45% to a maximum of 20% genes being differentially expressed (Barreto et al., 2015; Fischer et al., 2021; Hanson et al., 2017; Jacobs & Elmer, 2021; Juneja et al., 2016; Mack et al., 2018; Velotta et al., 2017). They are more comparable to strong interspecific divergences between *Drosophila* spp. (25-35% of genes; (Coolon et al., 2014)), highlighting strong regulatory differences between Atlantic silverside populations. We note though that in contrast to several of the above-mentioned studies, our analyses measured expression across whole juvenile organisms rather than individual tissues, which could lead to a higher proportion of differentially expressed genes, although it has been suggested that combining different cell- and tissue-types leads to a reduction in power for detecting DE genes (Hunnicutt et al., 2022). The lack of tissue-specificity hinders us from determining if expression differences are driven by differences in tissue composition or divergent expression within tissue types, and if so, which tissue is driving such differences (Hunnicutt et al., 2022; Price et al., 2022). Despite this, our results show that there are putatively functional differences between populations, either through differences in tissue composition or differential regulation. Tissue-specific and/or cell-type specific analyses in Atlantic silversides will be needed to better understand the molecular mechanisms and extent of divergence across different organs. Furthermore, experimental
groups differed in age, as we controlled for length rather than age due to differences in growth rate between populations and temperatures (Fig. 1A; (David O. Conover & Present, 1990; Hice et al., 2012)), and also showed slight differences in length due to logistical limitations on sampling. However, differences in age and length between experimental groups were not correlated with the strength of overall differential expression, and therefore likely do not play a driving role in the strong differential expression observed in this system (Fig. S5 and S6).

We used wild-caught parents for the crosses due to the logistical constraints of raising many adults in captivity for several generations, which means that maternal and other environmental effects could impact the extent of expression divergence. We detected evidence for maternal effects in growth and gene expression in our dataset but could not fully discern their impact without a full reciprocal design (made impossible due to excessive mortality among offspring from one direction of the cross). However, gene expression inheritance patterns suggest that maternal inheritance of expression is relatively common (~13 - 19% of all expressed genes; Fig. 3B), although the mechanisms underlying this are not known in Atlantic silversides. In general, maternal effects might be adaptive in the context of local adaptation (Dey et al., 2016; Galloway, 2005), but detailed experimental tests with reciprocal hybrid crosses would be needed to discern that.

The strong divergence in thermal plasticity between populations suggests local adaptation in temperature-response between NY and GA on the molecular level (David O. Conover & Present, 1990; Hice et al., 2012), which has also been observed in other systems occupying divergent thermal habitats (Campbell-Staton et al., 2021). The stronger divergence in gene expression between temperature regimes in GA compared to NY suggests that thermal plasticity is stronger in southern silverside populations inhabiting warm environments with less pronounced seasonal variation. This pattern is opposite to Atlantic
cod, where cold-adapted populations seem more sensitive to temperature changes (Hutchings et al., 2007). The stark molecular divergence in temperature-responsive pathways is further supported by the low preservation of many temperature-responsive gene co-expression networks between NY and GA (Fig. 4; Table S7-8). The weak thermal plasticity and low preservation of temperature-responsive co-expression modules in F1 hybrids suggests the disruption of temperature-responsive pathways in hybrids, as observed in other fish (Oomen et al., 2021; Payne et al., 2022). If this disruption of temperature-responsive pathways leads to lower fitness in wild hybrids, it might provide a potential explanation for the maintenance of local adaptation despite ongoing gene flow. So far we have only focused on gene expression, but other regulatory processes, such as alternative splicing, might also play important roles in thermal adaptation in silversides, and could compensate for disrupted expression patterns (Healy & Schulte, 2019; Verta & Jacobs, 2022). In contrast, there is the possibility that hybrids show higher thermal tolerance than parental populations (Pereira et al., 2014) and further experimental studies are needed to determine the physiological effects of hybridization in this system.

In contrast to the gene-by-gene analysis, we found more temperature-sensitive modules in NY compared to GA. These contrasting patterns could either be due to mechanistic differences (e.g. distribution of GxE genes across modules) or analytical differences between network analyses and gene-by-gene analyses. More work will be needed to understand the underlying reasons for this, and to better understand differences in temperature-sensitivity between populations.

Lastly, differences in expression between groups could also stem from differences in sex ratios between groups, as temperature affects sex determination in silversides (D. O. Conover & Heins, 1987; David O. Conover & Fleisher, 1986). Although we were not able to determine the phenotypic sex for the sampled individuals, expression of a sex-linked
aromatase gene (cyp19a) suggests that there are no significant differences in sex-ratios between temperatures within populations (Fig. S7), and that variation in sex ratios is not the main driver of the observed differences in population differences or thermal plasticity.

**Maintenance of local adaptation through hybrid gene misexpression.** Regulatory incompatibilities between populations or species can lead to the misexpression of genes in hybrids, even at early stages of divergence (Barreto et al., 2015; McGirr & Martin, 2020a; Moran et al., 2021; Mugal et al., 2020; Renaut et al., 2009). If the resulting misexpression has negative fitness effects, such regulatory incompatibilities can contribute to local adaptation (Filteau et al., 2013; McGirr & Martin, 2020a). Atlantic silversides show surprisingly strong misexpression in hybrids (13.5% of all expressed genes) at lower temperatures (20°C) compared to other examples of intraspecific clinal adaptation, such as in the copepod *Tigriopus californicus* (hybrid misexpression in 1.2% of genes (Barreto et al., 2015)), and even slightly higher than levels of misexpression in crosses of young species pairs, e.g. lake whitefish (10%) (Dion-Côté et al., 2014) or Caribbean pupfish (up to 9.3%) (McGirr & Martin, 2020a). While misexpression in hybrids could result from aberrant development (Mack & Nachman, 2017), we did not detect any stark developmental differences (e.g. in hatching time) between pure and hybrid crosses reared at the same temperature, supporting the role of regulatory incompatibilities. Similar to the differential expression results and misexpression results in other systems (Barreto et al., 2015; McGirr & Martin, 2020a), hybrid gene misexpression in Atlantic silversides could potentially be biased by differences in tissue composition between groups, and in tissue- and cell-type specific analyses are needed to investigate gene dysregulation in hybrids on a cell-type specific level. However, since we are investigating differences between highly connected populations, large differences in tissue composition are unlikely.
Interestingly, the very low proportion of misexpressed genes at 26°C highlights how misexpression can be highly condition-dependent, similar to gene expression (Fig. 3; (Bittner et al., 2021; Gould et al., 2018), and that misexpression is contributing more strongly to local adaptation under more challenging environmental conditions, in this case lower temperatures.

Most misexpressed genes are thought to be under stabilizing selection in parental populations (Moran et al., 2021; Wray et al., 2003), resulting in similar expression levels between populations. Yet, we found several hundred misexpressed genes that were differentially expressed between GA and NY populations in Atlantic silversides, suggesting that their expression levels are under divergent selection and putatively involved in local adaptation (Kulmuni & Westram, 2017; McGirr & Martin, 2020a; Pavey et al., 2010). These genes are potentially involved in the divergence of locally adaptive phenotypes. Future work should focus on determining the molecular mechanisms of hybrid gene misexpression on the tissue level and determine the phenotypic and fitness effects of misexpression to better understand their contribution to local adaptation.

Furthermore, the low preservation of co-expression modules, including temperature-responsive and growth-correlated modules (Fig. 4, Table S7-8), could partially be explained by potential genetic incompatibilities and the resulting misexpression of central regulatory genes within modules (Supplementary text). The disruption of growth-related modules in hybrids could be related to the observed difference in growth rate in hybrids compared to the parental populations, particularly intermediate growth rates at 20°C. This difference in growth rate has potentially negative fitness effects in their parental habitats, particular in northern habitats, as growth rates show countergradient variation, with selection for higher intrinsic growth rates in northern populations to compensate for shorter growing seasons (need to reach large size before winter to survive), and potential selection on lower growth rates in the south as fast growth can have negative effects on swimming abilities and predator escape.
Thus, while intermediate growth rates may be adaptive at central locations of the thermal cline, it might be maladaptive in either parental habitat.

**Inversions and local adaptation under gene flow.** In species with highly concentrated genomic architectures, e.g. due to inversions, one might expect that differentially regulated genes are located within inversions due to the accumulation and maintenance of locally adaptive genetic variation through reduced recombination (Mérot et al., 2020; Stevison et al., 2011). In some systems, inversions are enriched for DE genes (Berdan et al., 2021; Cassone et al., 2011; Fuller et al., 2016; Marquès-Bonet et al., 2004), suggesting that inversion-linked *cis*-regulatory variation is the main cause for divergent gene expression (Berdan et al., 2021; Crow et al., 2020). However, in the Atlantic silverside, most DE genes were located outside inversions. This contrasts with patterns of genetic differentiation observed between NY and GA that show most pronounced differentiation within inversions, particularly *inv18* and *inv24* (Fig. S11; Wilder et al. 2020; Akopyan et al. 2022). Thus, inversions may not only have *cis*-effects on expression but could have more widespread genome-wide effects on expression through *trans*-specific effects, e.g. through the differential expression of transcription factors linked to inversions (Naseeb et al., 2016; Said et al., 2018). However, inversions also show evidence for *cis*-effects on expression, as supported by the enrichment for DE genes (*inv4, inv18*) and genes from growth- and temperature-associated co-expression modules (*inv18* and *inv24*). In particular, *inv24* has been previously associated with body size evolution in Atlantic silversides (Therkildsen et al., 2019). Together, this suggests that *inv18* and *inv24* harbor adaptive *cis*-regulatory genetic variation underlying locally adaptive traits.
In addition to driving differential expression, inversions can potentially contribute to population divergence by reducing hybrid fitness through the accumulation of genetic incompatibilities that lead to hybrid gene misexpression and the disruption of regulatory networks (Feder et al., 2014; Mugal et al., 2020; Navarro & Barton, 2003). In Atlantic silversides, all inversions are enriched for misexpressed genes, especially at 20°C. Thus, inversions likely contribute to local adaptation under gene flow via accumulation of incompatibilities that lead to dysregulation in hybrids, and potentially reducing their fitness in their parental habitats.

**Regulatory mechanisms are temperature dependent.** In contrast to protein-coding changes, gene regulatory variation offers the possibility to minimize fitness trade-off across environments by adjusting expression levels between divergent habitats, leading to potential conditional neutrality (Bono et al., 2017; Gould et al., 2018; Wadgymar et al., 2017). While we could not determine in this study if differences in gene expression divergence between temperatures led to differences in fitness across environments, we found evidence for GxE for thousands of genes in the Atlantic silverside. Furthermore, the difference in misexpressed genes in hybrids between environments, with nearly no misexpression at 26°C but pervasive misexpression at 20°C, suggests that genetic incompatibilities and fitness effects might be largely invisible at 26°C. This fits observed growth patterns in hybrids, which are similar to the maternal NY population at 26°C (Fig. 1B), suggesting no strong negative effects on growth at higher temperatures, compared to the intermediate growth rate at 20°C. Since we only used unidirectional crosses (NY x GA) in this experiment, due to high mortality of our GA x NY cross, we cannot disentangle maternal from other effects. In general, colder temperatures are thought to be more challenging for Atlantic silversides, and genetic incompatibilities and the resulting fitness effects might be more prevalent in suboptimal
habitats (Bomblies et al., 2007). This asymmetry in misexpression might shape the outcome of hybridization between populations, e.g. potentially less negative fitness impacts at warmer temperatures.

In line with theoretical expectations (Cutter & Bundus, 2020) and empirical studies in other systems (Ballinger et al., 2023; Signor & Nuzhdin, 2018; Snoek et al., 2017), we found that \textit{trans} effects tend to be more temperature-dependent than \textit{cis} effects. In combination with earlier studies (Ballinger et al., 2023; Signor & Nuzhdin, 2018; Snoek et al., 2017), this suggests that \textit{trans}-regulation might play a key role in rapid temperature responses and temperature adaptation (Ballinger et al., 2023). While the proportion of consistently \textit{all-trans} regulated genes between temperatures is smaller than for \textit{all-cis} genes, this could potentially be explained by additional temperature-dependent \textit{trans}-effects, e.g. ‘\textit{cis} x \textit{trans}’ interactions rather than distinct changes of \textit{all-cis} or \textit{all-trans} effects. It is important to keep in mind that we were only able to investigate a small proportion of genes and investigated expression across whole larvae rather than individual tissues or cells, which could potentially bias the results and make it more difficult to extrapolate the results. Despite that, these results are a starting point for understanding the evolution of gene regulatory architectures underlying local adaptation. Overall, the temperature-dependence of gene regulatory architectures, particularly the temperature-dependence of \textit{trans}-effects, highlights the need to study gene regulatory mechanisms across relevant contexts, e.g. developmental stages, environments, and genotypes/ divergent populations, to better understand the gene regulatory mechanisms associated with local adaptation.
Limitations and Conclusions. Together, this work highlights that regulatory mechanisms and thermal responses can rapidly diverge between locally adapted populations in the face of gene flow. Widespread misexpression and the disruption of gene co-expression networks in hybrids under certain environmental conditions suggest the presence of regulatory incompatibilities, with the accumulation of such incompatibilities within inversions. This work is only based on unidirectional crosses, whole-body expression patterns and a single developmental time point, limiting our ability to discern maternal effects and identify tissue-specific patterns and functions of regulatory changes. Nonetheless, this study suggests a prominent role of gene regulatory divergence in local adaptation with gene flow and indicates how inversions can shape the molecular mechanisms underlying local adaptation.
Data availability

All raw data are available on NCBI in the SRA under the BioProject PRJNA694674, with SRA accession SRR13523227 to SRR13523268. The linkage-map anchored reference genome, the corresponding genome annotation and gene ontology annotation are available on Dryad: https://doi.org/10.5061/dryad.76hdr7t3s.

Author contributions


Funding

This study was funded through National Science Foundation grants to NOT (OCE-1756316) and HB (OCE-1756751)

Conflict of Interest. The authors declare no conflict of interest. Editorial processing of the manuscript was done independently of N.O.T., who is an Associate Editor of Evolution.

Acknowledgements

Thanks to Nicolas Lou, James Harrington, and Chris Murray for assistance with rearing and sampling, and Harmony Borchardt-Wier for assistance with lab work.
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