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Tolerance traits related to climate change resilience are independent and polygenic

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Abstract

The resilience of organisms to climate change through adaptive evolution is dependent on the extent of genetically based variation in key phenotypic traits and the nature of genetic associations between them. For aquatic animals, upper thermal tolerance and hypoxia tolerance are likely to be a important determinants of sensitivity to climate change. To determine the genetic basis of these traits and to detect associations between them, we compared naturally occurring populations of two subspecies of Atlantic killifish, *Fundulus heteroclitus*, that differ in both thermal and hypoxia tolerance. Multilocus association mapping demonstrated that 47 and 35 single nucleotide polymorphisms (SNPs) explained 43.4% and 51.9% of variation in thermal and hypoxia tolerance, respectively, suggesting that genetic mechanisms underlie a substantial proportion of variation in each trait. However, no explanatory SNPs were shared between traits, and upper thermal tolerance varied approximately linearly with latitude, whereas hypoxia tolerance exhibited a steep phenotypic break across the contact zone between the subspecies. These results suggest that upper thermal tolerance and hypoxia tolerance are neither phenotypically correlated nor genetically associated, and thus that rates of adaptive change in these traits can be independently fine-tuned by natural selection. This modularity of important traits can underpin the evolvability of organisms to complex future environmental change.

KEYWORDS
association, genotype-phenotype, hypoxia tolerance, oxygen, temperature, thermal tolerance

1 | INTRODUCTION

The impacts of climate change on species and populations will in part be determined by their capacity to adapt to rapidly changing environmental conditions (Barrett & Hendry, 2012) which will likely require changes in multiple traits (Crain, Kroeker, & Halpern, 2008). Thus, understanding the genetic basis of key traits is of critical importance because rates of adaptation will depend upon both the availability of suitable genetic variation (Barrett & Schluter, 2008) and the underlying genetic architecture of the traits (Bay et al., 2017; Radwan & Babik, 2012; Shaw & Etterson, 2012; Visser, 2008). In addition, because associations among traits can influence rates of adaptation (Agrawal & Stinchcombe, 2009; Hansen & Houle, 2008), trait correlations and genotype–phenotype associations should be taken into account when forecasting organismal responses to climate change (Hoffmann & Sgrò, 2011).

Two key physiological traits that may shape the responses of aquatic ectotherms to climate change are upper thermal tolerance and hypoxia tolerance. Occurrences of high temperatures and low oxygen tensions are expected to increase in aquatic environments over the coming century (Díaz & Rosenberg, 2008; Harley et al., 2006), and both abiotic stressors are thought to be important determinants of the biogeographic distributions of aquatic organisms

(Chapman, Chapman, Nordlie, & Rosenberger, 2002; Deutsch, Ferrel, Seibel, Pörtner, & Huey, 2015; Sunday, Bates, & Dulvy, 2011). Consequently, the genetic mechanisms that underlie variation in upper thermal tolerance and hypoxia tolerance as well as any genetic associations or phenotypic correlations between these traits may have substantial impacts on the rate of, and capacity for, adaptive or plastic responses to climate change in aquatic ecosystems.

There is some evidence for a positive correlation between upper thermal tolerance and hypoxia tolerance in fish (Anttila et al., 2013; Smale & Rabeni, 1995), and similar physiological mechanisms are thought to play roles in determining tolerance limits for each stressor. For example, variation in the capacity of organisms to maintain oxygen supply and energy balance likely plays a role in establishing differences in tolerance of both high temperatures (Pörtner, 2001) and low oxygen levels (Mandic, Todgham, & Richards, 2009), and differences in mitochondrial performance have also been suggested to contribute to variation in upper thermal tolerance (Iftikar & Hickey, 2013) and hypoxia tolerance (Lau, Mandic, & Richards, 2017). However, the potential for an association between these tolerance traits has not been rigorously tested, and the genetic bases for variation in these traits remain poorly understood.

In this study, we utilize naturally occurring populations of Atlantic killifish, *Fundulus heteroclitus*, to assess phenotypic correlations and genetic associations between upper thermal tolerance and hypoxia tolerance. This topminnow inhabits intertidal saltmarshes from Nova Scotia, Canada to northern Florida, USA, and is differentiated into two subspecies: *F. h. macrolepidotus* ("northern") found from Nova Scotia to New Jersey, USA, and *F. h. heteroclitus* ("southern") found from New Jersey to Florida (Morin & Able, 1983). Southern killifish have better tolerance of high temperatures (Fangue, Hofmeister, & Schulte, 2006) and low oxygen levels (McBryan, Healy, Haakons, & Schulte, 2016) than do northern killifish, a pattern that is consistent with potential functional links between these traits, but could also be due to independent divergence or irreversible plasticity as a result of different rearing environments.

The northern and southern subspecies of Atlantic killifish meet and interbreed in a relatively small region along the coast of New Jersey (Powers & Schulte, 1998), which has resulted in clinal distributions of genetic variation along the coast (González-Vilaseñor & Powers, 1990; McKenzie, Dhillon, & Schulte, 2015; Powers & Place, 1978; Strand, Williams, Oleksiak, & Sotka, 2012). In particular, fixed differences in mitochondrial genotype exist between the subspecies (Whitehead, 2009), and clinal variation in mitochondrial genotype is extremely steep (González-Vilaseñor & Powers, 1990; McKenzie et al., 2015; Strand et al., 2012). The presence of admixed populations at the center of the contact zone between the subspecies facilitates experiments that test trait correlations and genotype–phenotype associations in fish with mixed northern and southern genetic backgrounds that developed in a common location. We use this system to test the hypothesis that there is a phenotypic correlation and genetic association between upper thermal tolerance (assessed as critical thermal maximum, CT_{max}) and hypoxia tolerance (assessed as time to loss of equilibrium in hypoxia, LOE_{hyp}) by examining phenotypic variation in both traits within and among populations, identifying genetic variation that is associated with this trait variation, and determining whether these traits are correlated at the level of the phenotype or associated at the level of the genotype. Together, this work provides insights into the genetic basis of two key traits that are likely to play an important role in the responses of aquatic ecotherms to climate change.

2 | MATERIALS AND METHODS

2.1 | Phenotyping and genotyping

Adult killifish were collected from five locations along the Atlantic coast of North America (GA, S.NJ, C.NJ, N.NJ, NH; Figure 1a, Supporting Information Table S1) in September 2012 and June 2014 (Supporting Information Table S2). Note that collection effort was unbalanced among populations to provide a larger sample size for genotype–phenotype association tests which were performed only in the C.NJ population (C.NJ n = 318, all others n = 49–73; Supporting Information Table S2). Mean water temperature and oxygen partial pressures through summer and fall of 2014 at monitoring stations near our collection sites are provided in Supporting Information Table S3 for reference. Fish were shipped overnight to the University of British Columbia where individuals were uniquely marked with subepidermal fluorescent elastomer tags (Northwest Marine Technology, Shaw Island, WA), and separated by population in 200 L fiberglass tanks in a single recirculating system. Holding conditions were 15°C, 12L:12D and 20 ppt, which was achieved by dissolving Instant Ocean® Sea Salt (Instant Ocean, Spectrum Brands, Blacksburg, VA) in dechlorinated City of Vancouver tap water. Fish were fed Nutrafin® Max Tropical Fish Flakes (Hagen, Mansfield, MA) to satiation once daily except for the 24 hr periods prior to any phenotyping trial. Acclimation conditions were maintained for at least one month prior to experiments and were restored for at least one month between upper thermal tolerance and hypoxia tolerance trials. Preliminary trials indicated that the order of measurement did not affect values obtained for either trait. Animal care and experimental procedures were in accordance with approved University of British Columbia animal care protocol A11–0,372.

Upper thermal tolerance was measured by critical thermal maximum (CT_{max}) as in Fangue et al. (2006). For each trial, 30 fish were transferred to a 130 L testing tank at 15°C and 20 ppt. After 10 min, 55°C 20 ppt water was gradually introduced to the testing tank to increase the water temperature at a rate of 0.3 ± 0.03°C/min. The tank was well aerated to maintain oxygen concentrations and to avoid thermal stratification of the water. Fish were monitored until loss of equilibrium was observed (i.e., when fish were unable to maintain normal dorsoventral orientation). CT_{max} was recorded as the temperature at which a fish lost equilibrium, then the fish was placed individually into a recovery tank containing water at 15°C and 20 ppt. At the end of each trial, fish were inspected to determine tag ID, weighed, and returned to their acclimation tanks. Survival assessed one week following CT_{max} trials was >98%.

Hypoxia tolerance was measured by time to loss of equilibrium at 0.2 kPa O_2 (LOE_{hyp}) similar to McBryan et al. (2016). For each trial, eight fish were transferred individually into 473 ml plastic containers with screw-on lids and mesh sides that were submerged in a 50 L testing tank with a false bottom 5 cm above the bottom of the tank. 15°C 20 ppt water was circulated within the testing tank using two Mag-Drive model 1.5 pumps (Danner Manufacturing Inc., Islandia, NY) placed below the false bottom, which minimized oxygen stratification throughout the tank without subjecting the fish to a substantial direct water current. The surface of the testing tank was covered with a layer of bubble wrap to reduce exchange of oxygen with the air, and fish were denied access to the surface of the water by their containers. After 10 min, nitrogen gas was bubbled into the tank, and oxygen partial pressure was monitored continuously using a NeoFox oxygen probe (Ocean Optics, Dunedin, FL). Oxygen partial pressure was reduced to 0.2 kPa (1% air saturation) over 30 min, then fish were monitored until they lost equilibrium. Loss of equilibrium was observed when a fish no longer responded to gentle movement of its holding container. LOE_{hyp} was recorded as the time the fish was exposed to 0.2 kPa O_2, then the fish was inspected to determine tag ID and transferred to a recovery tank containing aerated 15°C 20 ppt water. At the end of each trial, fish were weighed and then returned to their acclimation tanks. Survival assessed one week following LOE_{hyp} trials was >98%.

Fin clips from each fish were used for restriction site-associated DNA sequencing (RADseq; n: GA = 25, S.NJ = 46, C.NJ = 317, N.NJ = 44 and NH = 45), and were digested with Proteinase K (4.2 mg/ml) at 56°C overnight. Tissue digests were cleaned up using Agencourt Ampure XP beads (Beckman Coulter, Inc., Carlsbad, CA), and genomic DNA was eluted using low EDTA Tris-EDTA (TE) buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5). Libraries were prepared as in Ali et al. (2016). Briefly, 250 ng of each DNA sample was digested with SbfI restriction enzyme, and unique eight base pair barcodes were annealed to each sample with 2 μL of 50 mM indexed SbfI biotinylated RAD adapters. RADseq libraries were prepared by pooling samples from 96 individuals with populations approximately equally represented across libraries. A Bioruptor NGS sonicator (Diagenode, Inc., Denville, NJ) was used to fragment libraries to 200–500 bp (approximately nine cycles of 30 s on and 90 s off), which was confirmed by gel electrophoresis in a 2% sodium borate gel. Off-target DNA was removed with Dynabeads M-280 streptavidin magnetic beads (Life Technologies, Carlsbad, CA), and RAD target DNA was eluted in 55.5 μL of low EDTA TE buffer. Libraries were prepared for sequencing with NEBNext Ultra DNA Library Prep Kits for Illumina (New England Biolabs, Inc., Ipswich, MA). Initial libraries were preliminarily sequenced with 150-bp paired-end sequencing in a single lane of an Illumina HiSeq 4,000 (Illumina, Inc., San Diego, CA) at the University of California Davis Genome Center. The resulting read counts per individual were used to create normalized libraries for final sequencing. Normalization was performed by creating new libraries with adjusted input amounts of barcoded DNA from each individual. These libraries were then fragmented, purified and prepared for sequencing as described above. Final libraries were multiplexed and sequenced in two lanes. Reads were demultiplexed by Illumina and RAD barcodes requiring exact matches for both barcodes and partial restriction sites. If both a forward and reverse read contained a barcode, then the read was removed from the dataset.

Demultiplexed reads were mapped to the *Fundulus heteroclitus* reference genome 3.0.2 (Reid et al., 2016; National Center for Biotechnology Information [NCBI] GenBank assembly accession GCA_000826765.1) with BWA-MEM v0.7.12 (Li & Durbin, 2009). Identification of PCR duplicates was performed with SAMBLASTER v0.1.22 (Faust & Hall, 2014). Duplicated and discordant reads were removed, as well as any read with a mapping quality of <30. Genetic variants were called using Freebayes v0.9.21–19-gc003 cl (Garrison & Marth, 2012), and were filtered using VCFtools v0.1.15 (Danecek et al., 2011). Variants with base quality scores ≤20 were discarded, and 22 individuals with an average coverage <50% of the average coverage across all individuals were removed. No individuals had average coverage values >100X. Remaining variants were filtered to bi-allelic single nucleotide polymorphisms (SNPs) with minor allele frequencies ≥0.02. Sites with <10X coverage in at least 95% of individuals were removed, and genotype calls within sites that were based on coverage <10X were set to unknown. Putatively paralogous sites were identified with HDplot (McKinney, Waples, Seeb, & Seeb, 2017) using the C.NJ individuals, because the sample size for this population was relatively large and mixing genetic data across populations can interfere with the ability to detect paralogous loci. HDplot identifies putative paralogs by excess heterozygosity (*H*) and allele read depth deviations in heterozygous individuals (*D*). Filtering cutoffs for *H* and *D* have not been identified for *F. heteroclitus*, and vary somewhat among species (McKinney et al., 2017). Consequently, we removed sites using moderately lenient values of $H \geq 0.75$, $D > 10$ or $D < -10$ to reduce the number of loci misidentified as paralogous. Filtering resulted in 77,084 high quality SNPs for use in association mapping and selection analyses (*n* after filtering: GA = 25, S.NJ = 44, C.NJ = 305, N.NJ = 36 and NH = 45). SNPs were converted to their locations on a genetic map for *F. heteroclitus* (unpublished data), which assembles the majority of the genome to chromosomes. Mitochondrial genotypes were not identified by our RADseq approach. Therefore, we determined mitochondrial genotype using genotype-specific differences in polymerase chain reaction (PCR) amplification of the mitochondrial-encoded nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit V gene (Supporting Information Data S1).

2.2 | Data analysis and statistics

Statistical analyses were conducted in R v3.4.0 (R Core Team, 2017), and for all analyses alpha was set at 0.05. Potential effects of collection year on CT_{max} and LOE_{hyp} were tested with Student's *t* tests and Wilcoxon signed-rank tests, respectively, using data from the two populations that were collected in both 2012 and 2014 (GA and NH). No effects of collection year were detected ($p \geq 0.23$), and therefore, data from both years were combined for further analyses. Potential effects of mass on either tolerance trait were assessed by ANCOVA using data

from all populations. Mass was not a significant factor affecting variation in CT_{max} (mass $p = 0.73$; population $p < 8.8 \times 10^{-3}$; interaction $p = 0.10$), and was excluded from further analyses. However, there was a significant interaction between mass and population for LOE_{hyp} (interaction $p = 0.01$; population $p < 3.3 \times 10^{-14}$; mass $p = 0.17$). Therefore, we calculated mass-corrected LOE_{hyp} values using residuals from the ANCOVA model. Estimated effects of mass were modest, and did not have a substantial effect on statistical comparisons among groups (Supporting Information Table S2). Thus, uncorrected values are presented in Figure 1, but mass was included as a covariate in all genotype–phenotype association analyses for LOE_{hyp}.

Differences in CT_{max} among populations were tested by one-way ANOVA followed by Tukey post hoc tests, and differences in LOE_{hyp} among populations were tested by Kruskal–Wallis ANOVA followed by Nemenyi post hoc tests. Correlation between the traits in fish from an admixed population (C.NJ) was tested using Spearman's rank correlation coefficient, and the effects of mitochondrial genotype on CT_{max} and LOE_{hyp} were assessed using Wilcoxon signed-rank tests.

We assessed genotype–phenotype associations using data from the C.NJ population. Performing these analyses in a single population from a region of genetic admixture between the subspecies minimizes potential developmental effects on phenotypic variation that could interfere with association tests in wild-caught fish from different localities. Because using only the C.NJ population resulted in small changes in minor allele frequencies, we refiltered the SNPs for a minor allele frequency of at least 0.02 among the C.NJ individuals. This resulted in slightly fewer SNPs in these analyses compared to the 77,084 described above: 73,432 for CT_{max} and 73,398 for LOE_{hyp} (difference between the traits is due to one mortality between measurements).

Site-by-site associations were assessed using efficient mixed-model association (EMMA) tests in TASSEL v5.2.38 (Bradbury et al., 2007; Zhang et al., 2010). Population structure was estimated using a combination of ADMIXTURE v1.23 (Alexander, Novembre, & Lange, 2009) and STRUCTURE v2.3 (Pritchard, Stephens, & Donnelly, 2000). Preliminary analyses with ADMIXTURE determined that the best-fitting model for all populations in our study used $K = 4$, as this resulted in the lowest cross-validation error. This K value was used for final analyses in STRUCTURE (Supporting Information Figure S1a). The full model with all populations revealed that the GA and NH populations were dramatically divergent from the NJ populations and each other, which can increase false positives from genome-wide selection scans (Hoban et al., 2016). Therefore, we reran the analysis with only the three NJ populations (Supporting Information Figure S1b). In this case, the best-fitting model used $K = 2$. Admixture proportions for the C.NJ fish from this model were used as a covariate in the TASSEL analyses. A kinship matrix calculated in TASSEL using the Centered_IBS option was also included as a covariate. For LOE_{hyp}, mass was included as an additional covariate, and both LOE_{hyp} and mass values were log-transformed to linearize any potential allometric effects of body mass. Association tests were conducted without P3D calculation simplification, and false-discovery rate corrections of *p*-values were performed using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995).

Polygenic associations were assessed with random forest models (Goldstein, Hubbard, Cutler, & Barcellos, 2010) using the *randomForest* v4.6.12 R package (Liaw & Wiener, 2002). These models require no missing data across samples, and therefore, we imputed missing genotypes for the C.NJ fish using *Beagle* v4.1 (Browning & Browning, 2016). Mitochondrial genotype was included as a potential explanatory genetic marker, LOE_{hyp} data were corrected for variation in mass using ANCOVA residuals as if the mass of each fish was 4 g (close to the median mass), and effects of population structure were corrected as described in Zhao et al. (2012) using admixture proportions determined with STRUCTURE (above). Models were developed using an iterative purging approach similar to those of Holliday, Wang, and Aitken (2012) and Brieuc, Ono, Drinan, and Naish (2015; summarized in Supporting Information Figure S2). Details of this procedure are provided in the Supporting Information Data S1. Once the markers included in the most explanatory models were identified, the estimated importance ranks among markers and predictive capabilities of the models were refined by running 100 random forest models with 10,000 trees each for each trait with the corresponding set of markers (Chen & Ishwaran, 2012; Laporte et al., 2015). Explanatory markers for CT_{max} or LOE_{hyp} were then ranked based on average importance score (Supporting Information Tables S4 and S5, respectively), and the percentage of variation in CT_{max} or LOE_{hyp} was averaged across the 100 models.

SNPs under selection across the NJ contact zone between the northern and southern subspecies were identified with *pcadapt* v3.0.4 (Luu, Bazin, & Blum, 2017), which is thought to be an appropriate analytical approach for data from admixed individuals (Luu et al., 2017), using SNP data from the southern and northern NJ populations (S.NJ and N.NJ). False-discovery rate was controlled using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). SNPs demonstrating evidence of selection that were also associated with either CT_{max} or LOE_{hyp} were identified by comparison of the *pcadapt* and association mapping results. Similarities between allele frequency variation at trait-associated SNPs and variation in either CT_{max} or LOE_{hyp} among populations were identified by correlations between average CT_{max} or LOE_{hyp} and allele frequency; candidate SNPs for explaining trait variation across populations were identified as those with correlations for which $R^2 \geq 0.67$. Genes located nearby SNPs associated with CT_{max} or LOE_{hyp}, or demonstrating significant evidence of selection were identified using the R package *ChIPpeakAnno* v3.10.2 (Zhu et al., 2010).

3 | RESULTS

3.1 | Phenotypic variation in upper thermal tolerance and hypoxia tolerance

Upper thermal tolerance ($p < 2.2 \times 10^{-16}$) and hypoxia tolerance ($p < 2.2 \times 10^{-16}$) differed significantly among killifish populations (Figure 1c,e). Fish from the most southern population in the study

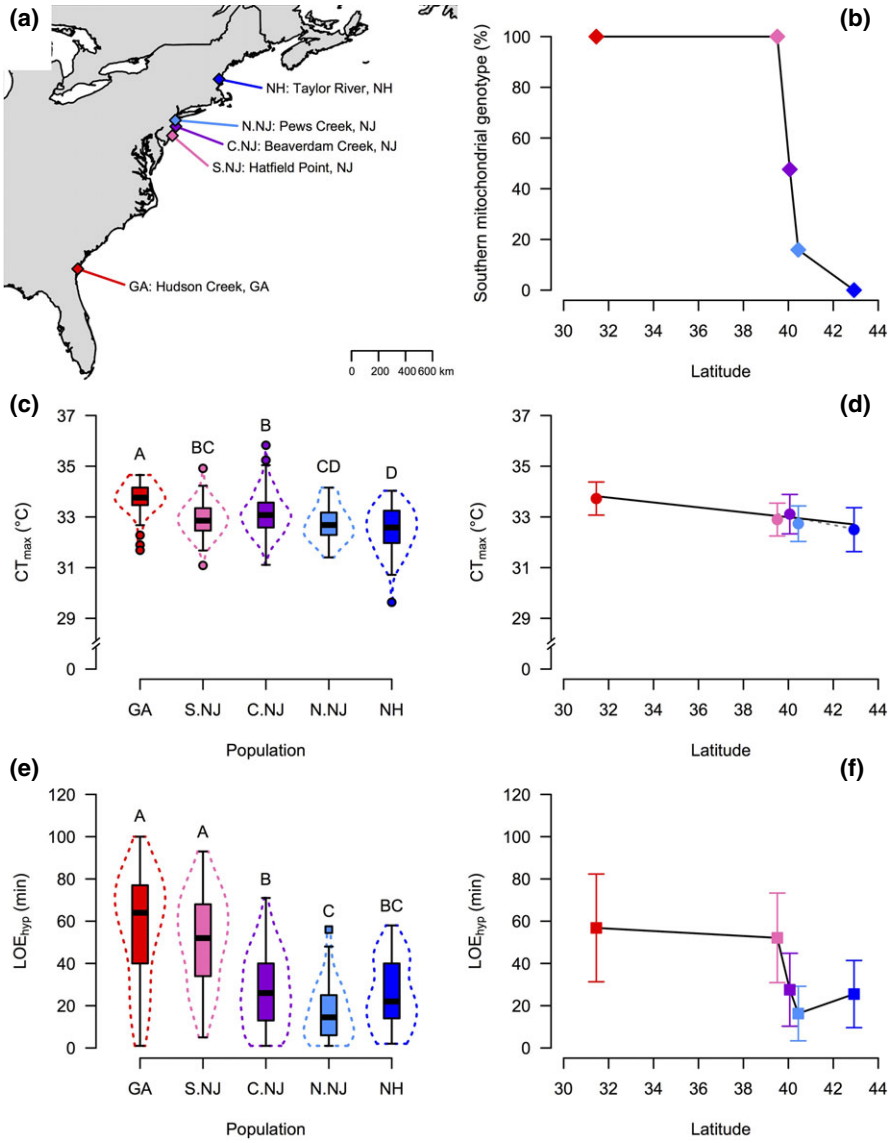

FIGURE 1 Variation in mitochondrial genotype, CT_{max} and LOE_{hyp} among populations of *F. heteroclitus*. (a) Locations of populations used along the Atlantic coast of North America (red – GA; pink – S.NJ; purple – C.NJ; light blue – N.NJ; dark blue – NH; population colors are consistent across all panels). (b) Southern mitochondrial genotype frequencies across latitudes. (c) Box plots for CT_{max} with violin plots showing data density distributions (dashed lines); shared uppercase letters indicate populations with values that are not significantly different. (d) Mean ± *SD* CT_{max} plotted against latitude; solid black line displays a significant linear fit ($CT_{max} = -0.09 \times lat. + 36.7$; $R^2 = 0.10$; $p = 4.9 \times 10^{-14}$); dashed gray line displays a similar fit if fish from the GA population are excluded ($CT_{max} = -0.16 \times lat. + 39.4$; $R^2 = 0.04$; $p = 1.2 \times 10^{-5}$). (e) Box plots for LOE_{hyp} with violin plots showing data density distributions (dashed lines); shared uppercase letters indicate populations with LOE_{hyp} values that are not significantly different. (f) Mean ± *SD* LOE_{hyp} plotted against latitude; solid black line segments connect mean values by latitude to display cline shape [Color figure can be viewed at wileyonlinelibrary.com]

(GA) had significantly higher CT_{max} ($p < 1.0 \times 10^{-4}$) and LOE_{hyp} ($p = 2.3 \times 10^{-9}$) than fish from the most northern population (NH), which was expected based on previously published results (Fangue et al., 2006; McBryan et al., 2016). However, variation in upper thermal tolerance and hypoxia tolerance exhibited different latitudinal patterns among populations. CT_{max} had an approximately linear relationship with latitude (Figure 1d), whereas there was a steep phenotypic transition from southern to northern LOE_{hyp} values over ~ 65 km along NJ (Figure 1f). This pattern for LOE_{hyp} is similar to that observed for mitochondrial genotype frequencies among these populations (Figure 1b), although there is a nonsignificant trend of lower LOE_{hyp} in the N.NJ population than the NH population, whereas the transition from southern to northern mitochondrial genotype is incomplete in N.NJ and complete in NH. These results indicate that variation in CT_{max} is not correlated with variation in LOE_{hyp} among populations. Furthermore, variation in these traits was not correlated among individuals collected from a population in a region of genetic admixture between the northern and southern

subspecies along the central coast of NJ (C.NJ; $p = 0.18$; Supporting Information Figure S3). CT_{max}, LOE_{hyp}, mitochondrial genotype and mass data for individual fish are available in Supporting Information Table S6.

3.2 | Genotype–phenotype associations in killifish from a genetically admixed population

Despite the similarities between the latitudinal patterns for LOE_{hyp} and mitochondrial genotype frequencies among populations (Figure 1b,f), LOE_{hyp} was not significantly different between killifish with northern or southern mitochondrial genotypes from the admixed C.NJ population (southern: 27.5 ± 1.4 min; northern: 27.6 ± 1.4 min; $p = 0.93$). This was also the case for CT_{max} (southern: 33.0 ± 0.1°C; northern: 33.2 ± 0.1°C; $p = 0.23$), suggesting that mitochondrial genotype does not play a major role in determining variation in either trait in killifish. Additionally, site-by-site association approaches failed to detect any significant associations between variation in thermal tolerance and the 77,084 nuclear single nucleotide polymorphisms (SNPs) assessed in this study (Figure 2a, Supporting Information Table S7), and only four SNPs were significantly associated with variation in hypoxia tolerance (Figure 2b, Supporting Information Table S8).

For complex physiological traits such as thermal tolerance and hypoxia tolerance any genetic bases for trait variation are likely to be polygenic (Bernatchez, 2016; Rose, Bay, Morikawa, & Palumbi, 2018). Therefore, site-by-site approaches may have limited power for detecting genetic associations with variation in CT_{max} or LOE_{hyp}. Consistent with this possibility, polygenic mapping analyses utilizing random forest models revealed that on average 43.4% of the variation in thermal tolerance (Figure 2c; range = 43.05%–43.65%), and 51.9% of the variation in hypoxia tolerance (Figure 2d; range = 51.66%–52.26%) among killifish individuals from the central NJ population (C.NJ) could be accounted for by the genetic variation observed at the 47 and 35 most explanatory (i.e., "important") sites for CT_{max} and LOE_{hyp}, respectively (Figure 2a,b, Supporting Information Tables S4 and S5). Note that for LOE_{hyp} the four SNPs detected with site-by-site association mapping were also detected by polygenic mapping. Consistent with the phenotypic results described above, none of the SNPs in the final models were shared between the traits, and mitochondrial genotype was not detected as an important predictor for either trait in the random forest models.

Genetic variants that are associated with interindividual phenotypic differences within a population are not necessarily also associated with phenotypic variation among populations. Thus, we compared allele frequencies among populations for the 47 and 35 SNPs from the polygenic association models for CT_{max} and LOE_{hyp}, respectively (Supporting Information Tables S4 and S5). For both traits, a subset of the markers associated with interindividual trait variation in C.NJ (8/47 for CT_{max}; 4/35 for LOE_{hyp}) demonstrated latitudinal clines in allele frequencies that were similar to the latitudinal variation in upper thermal tolerance or hypoxia tolerance among populations (Figure 3, Supporting Information Tables S4 and S5). This suggests that at least some of the phenotypic variation observed among populations may be underpinned by variation at SNPs associated with interindividual variation in phenotype within the C.NJ population. Furthermore, there was significant evidence for selection between the S.NJ and N.NJ populations at 561 of the SNPs in our study (Table S9), and one of the SNPs associated with interindividual variation in each tolerance trait also displayed evidence for selection (Figure 4, Supporting Information Tables S4 and S5). In addition, chromosomes 1, 2, 9, 13, 19, 23 and 24 had SNPs demonstrating strong evidence of selection ($q < 1 \times 10^{-9}$). Although these SNPs were not associated with variation in upper thermal tolerance or hypoxia tolerance, they are excellent candidates for involvement in local adaptation across the *F. heteroclitus* contact zone generally. Genes nearest the SNPs consistent with evidence of selection are listed in Supporting Information Table S9.

4 | DISCUSSION

Here, we demonstrate that two tolerance traits thought to be key indicators of climate change resilience in aquatic organisms (upper thermal tolerance and hypoxia tolerance) are independent in Atlantic killifish. That is, CT_{max} and LOE_{hyp} did not exhibit the same patterns of variation across latitudes, were not correlated among individuals, and did not share any SNPs that were associated with trait variation. Together these observations suggest that organismal responses to anthropogenic climate change that are influenced by variation in these traits will neither be facilitated nor hindered by an association between them. Our results reveal independent polygenic bases for variation in CT_{max} and LOE_{hyp} in killifish that can explain up to 43.4% and 51.9% of interindividual variation in these traits, respectively. This suggests not only that a large amount of variation in CT_{max} and LOE_{hyp} is likely genetically based, but also that these traits have the potential to independently respond to natural selection as a result of climate change or other environmental perturbations. This "modularity" is considered an important architectural feature of biological systems that contributes to the evolvability of organisms in complex environments (Wagner & Altenberg, 1996).

Correlations between upper thermal tolerance and hypoxia tolerance have been observed in other species of fish (Anttila et al., 2013; Smale & Rabeni, 1995), and similar physiological mechanisms have been suggested to influence both traits (Iftikar & Hickey, 2013; Lau et al., 2017; Mandic et al., 2009; Pörtner, 2001). In contrast, our results clearly indicate these traits do not share common underlying genetic mechanisms in killifish. This is perhaps somewhat surprising given the physical properties of water that result in decreases in oxygen solubility and content as temperature increases, which suggests that these two environmental variables are likely to be correlated. However, particularly in coastal ecosystems, inclusion of temperature in models that predict oxygen content provides little improvement in model power (Post et al., 2018). This is likely due to the impacts of the balance between respiration and photosynthesis in ecosystems combined with small-scale differences in water flow patterns, which play major roles in determining local oxygen

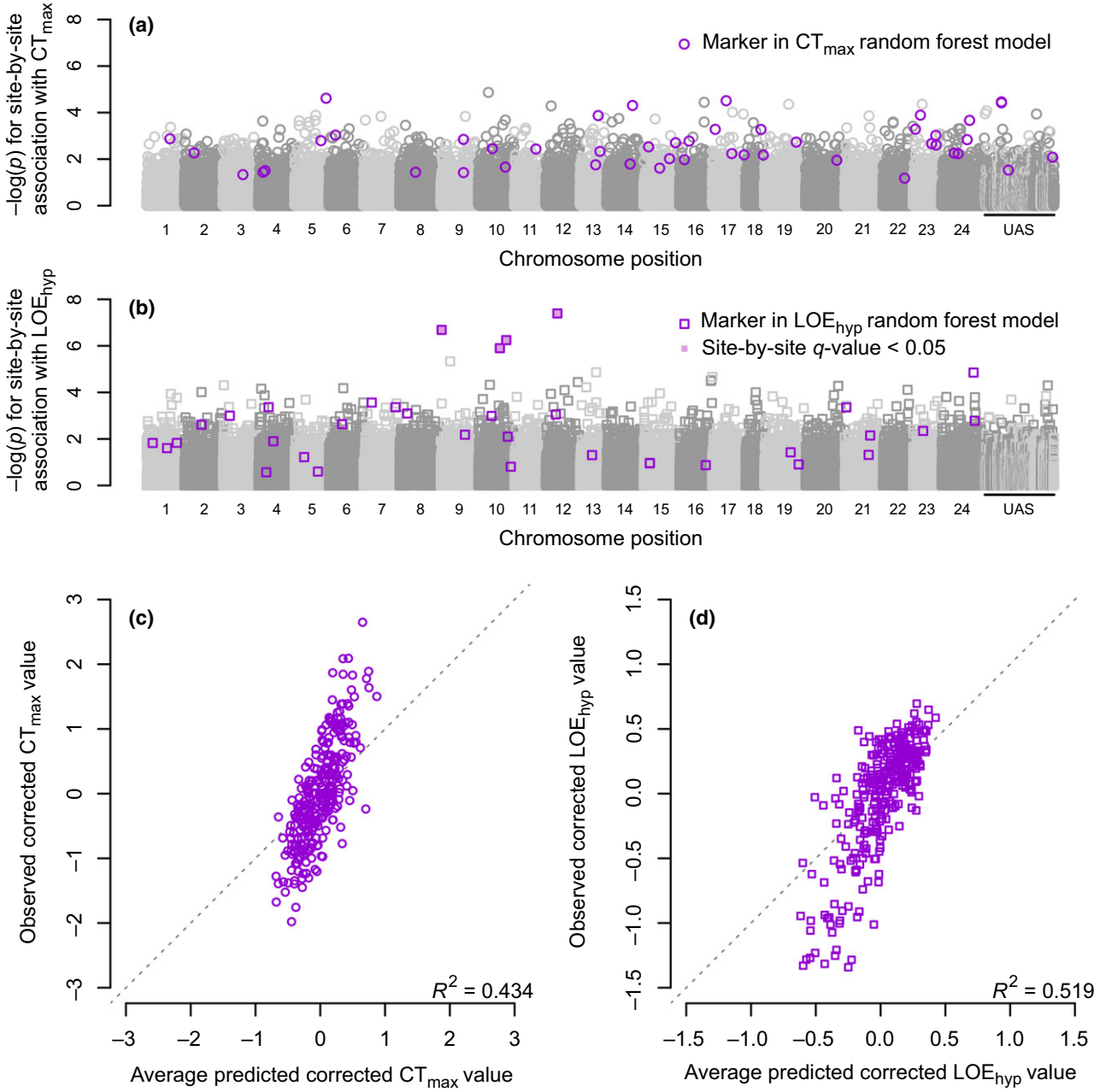

FIGURE 2 Genotype–phenotype associations with CT_{max} and LOE_{hyp}. For CT_{max} (a) and LOE_{hyp} (b) filled purple symbols indicate individual SNPs that were significantly associated with the trait; symbols outlined in purple indicate SNPs identified by the best fit random forest model; chromosomes are indicated by alternating light and dark gray symbols; x-axis numbers indicate chromosome numbers (UAS = unassembled scaffolds). (c) Observed versus average predicted values (empty purple circles) for the best fit random forest model for CT_{max}; the displayed R^2 value was calculated assuming a theoretical 1:1 fit (dashed line). (d) Observed versus average predicted values (empty purple squares) for the best fit random forest model for LOE_{hyp}; the displayed R^2 value was calculated assuming a theoretical 1:1 fit (dashed line) [Color figure can be viewed at wileyonlinelibrary.com]

conditions (e.g., Díaz & Rosenberg, 2008). Additionally, the capacity of organismal oxygen transport proteins, such as hemoglobin, to extract oxygen from the environment generally only becomes compromised at very low oxygen levels, which typically exceed those generated by environmental temperatures (Farrell, 2009) without additional factors such as metabolic activity in the ecosystem. Therefore, even given that oxygen content decreases with increasing temperature overall and that higher temperatures result in increased organismal metabolic activity (Altieri & Gedan, 2015), the relationships between extreme high temperatures and hypoxia, and their effects on organismal tolerance are complex.

Taken together, our results and those of previous studies strongly suggest that these tolerance traits are likely not determined by the same genetic mechanisms across species. However, it is

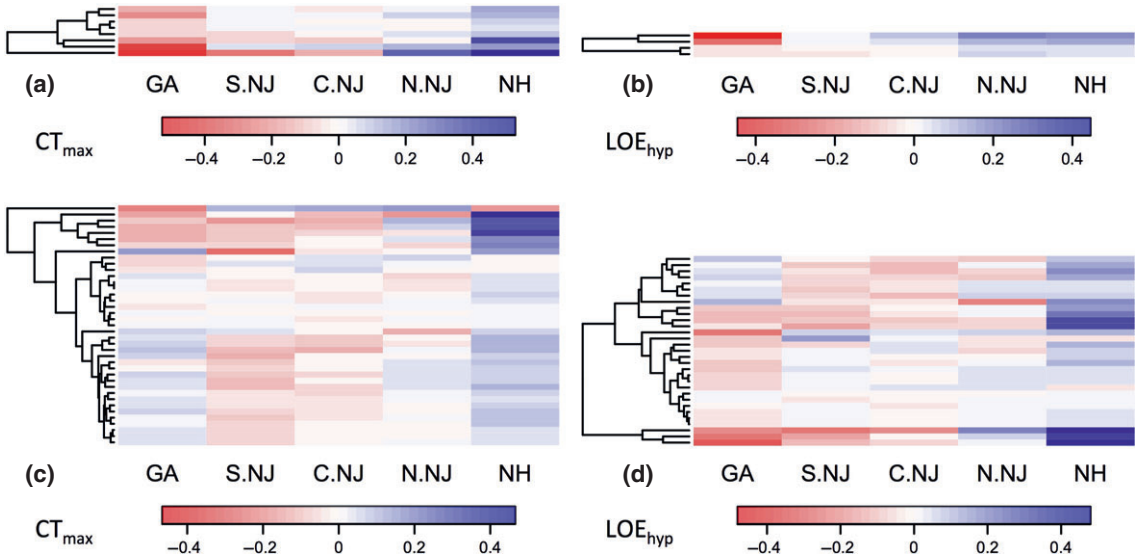

FIGURE 3 Heat maps of normalized allele frequency variation among populations of killifish for the SNPs identified from the best fit random forest models for CT_{max} and LOE_{hyp}. For CT_{max} (a) and LOE_{hyp} (b) the subset of SNPs with similar patterns of allele frequency variation and phenotypic variation among populations. For CT_{max} (c) and LOE_{hyp} (d) the remaining SNPs identified from the models. Each column corresponds to a population (labels at bottom), and each row corresponds to a SNP. Within panels, SNPs are clustered based on similarities of allele frequency changes across populations among SNPs, and clustering is indicated by the dendrogram shown beside each heat map. Red to white to blue colors indicate increasing occurrence of the "northern" allele in a population

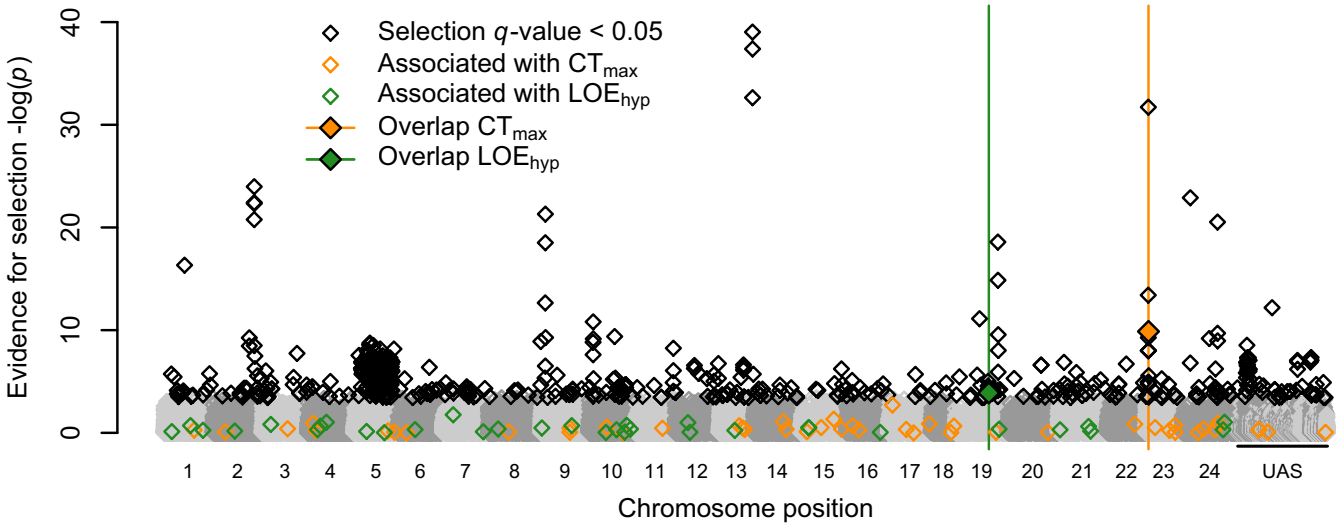

FIGURE 4 SNPs with evidence for selection from *pcadapt* analysis plotted across chromosomes. Chromosomes are indicated by alternating light and dark gray; x-axis numbers indicate chromosome numbers (UAS = unassembled scaffolds). Significant SNPs: empty black diamonds; SNPs associated with variation in CT_{max} from the best fit random forest model: empty orange diamonds; SNPs associated with variation in LOE_{hyp} from site-by-site mapping or the best fit random forest model: empty green diamonds; filled symbols and colored lines indicate SNPs with both evidence for selection and trait associations (CT_{max}: orange; LOE_{hyp}: green) [Color figure can be viewed at wileyonlinelibrary.com]

possible that genetic variants associated with each trait could have functional consequences that affect the same physiological process. For instance, CT_{max} and LOE_{hyp} are both likely determined in part by limits on the ability to maintain normal ion movements across cellular membranes (Miller & Stillman, 2011), and both CT_{max}-associated SNPs and LOE_{hyp}-associated SNPs in our study fall within genes encoding transmembrane ion channels (CT_{max}: potassium channel subfamily T member 1 [XM_012878483.1], transient receptor potential cation channel subfamily M member 1 [XM_012871459.1], voltage-dependent calcium channel subunit alpha-2/delta-4 [XM_012850819.1]; LOE_{hyp}: potassium voltage-gated channel subfamily H member 7 [XM_012874064.1]; Supporting Information

Tables S4 and S5). Thus, our results are not necessarily inconsistent with previous studies that have suggested that variation in a single physiological system, such as the cardiovascular system, is associated with variation in these two traits. If this is the case in killifish, it would suggest that although the same physiological systems may limit tolerance of high temperatures and low oxygen levels, different cellular and genetic mechanisms operating as a part of these systems are the weak links that lead to system failures in response to each stressor. In addition, this possibility could account for the often additive or synergistic effects of high temperature and low oxygen on tolerance in ectotherms (Rosa & Seibel, 2008; Schurmann & Steffensen, 1992) even given the lack of trait association observed in the current study.

Our data suggest that a large proportion of phenotypic variation in upper thermal tolerance and hypoxia tolerance is genetically determined. These results extend those of previous studies that have detected genetic bases for variation in environmental tolerance traits. For example, quantitative trait loci have been detected that can account for 7.5%–13% of the variation in upper thermal tolerance among families of rainbow trout, *Onchorhynchus mykiss* (Jackson et al., 1998; Perry, Danzmann, Ferguson, & Gibson, 2001) and channel catfish, *Ictalurus punctatus* (Jin et al., 2017), and 5%–32% of the variation in hypoxia tolerance among families of catfish (Wang et al., 2017; Zhong et al., 2017) and Nile tilapia, *Oreochromis niloticus* (Li et al., 2017). In comparison, our results explain >40% and >50% of the variation in upper thermal tolerance and hypoxia tolerance, respectively, in wild-caught killifish. It is possible that these are overestimates of the proportions of trait variation explained by the SNPs assessed in the current study, as genome-wide analytical approaches, including random forest association analyses, can result in false positives due to the number of loci and tests involved (Forester, Lasky, Wagner, & Urban, 2018; Lotterhos & Whitlock, 2014). In particular, random forest approaches utilizing high numbers of markers or iterative purging strategies may result in model overfitting (Brieuc, Waters, Drinan, & Naish, 2018; Forester et al., 2018). However, random forest models may also miss causal loci due to linkage among loci, overweighting of large effect loci and limits of statistical power (Forester et al., 2018). Furthermore, reduced representation sequencing strategies, such as RADseq, assess only a subset of the genome, meaning some causal loci are likely to be missed (Hoban et al., 2016). Despite these possible limitations, our results demonstrate multilocus associations with variation in both upper thermal tolerance and hypoxia tolerance, indicating that relatively high proportions of phenotypic variation in CT_{max} and LOE_{hyp} can be attributed to combinations of SNP variation across multiple loci (approximately 47 and 35 SNPs, respectively). Polygenic bases for phenotypic variation have been demonstrated for several ecologically relevant traits (Brieuc et al., 2015; Holliday et al., 2012; Laporte et al., 2015; Nemri et al., 2010), and recent studies in rainbow trout and corals also detected a polygenic basis for variation in thermal tolerance (Chen, Farrell, Matala, & Narum, 2018; Rose et al., 2018). Together these results highlight the importance of incorporating models of adaptive responses based on many loci of small effect into forecasts of the organismal responses to climate change (Bay et al., 2017; Pritchard & Di Rienzo, 2010).

For both tolerance traits, genotype–phenotype association models tended to predict the approximate order of tolerance values among individuals relatively well, but tended to underpredict high tolerance values and overpredict low tolerance values (Figure 2c,d). The reduced representation sequencing approach used in the current study may have influenced our ability to detect all the variant sites that contribute to variation in CT_{max} and LOE_{hyp} in killifish, which may constrain the predictive ability of our models. Alternatively, because the fish in our study were wild-caught, environmental contributions to phenotypic variation such as irreversible developmental plasticity (Schaefer & Ryan, 2006) could also explain the larger range of observed trait values compared to predicted trait values from our models. However, our results suggest that large proportions of the variations in these traits are genetically based, and that the genes located near (<5 kb) the associated SNPs include several promising candidate genes that may play direct functional roles in determining variation in upper thermal tolerance or hypoxia tolerance.

The eight CT_{max}-associated SNPs and four LOE_{hyp}-associated SNPs that demonstrated clinal allele frequencies similar to the patterns of latitudinal trait variation among populations are perhaps the best candidate loci to contribute to both interindividual and interpopulation variation in these traits. One of these CT_{max}-associated SNPs was located within the gene encoding sodium- and chloride-dependent creatine transporter 1 (*slc6a8*; XM_012855952.1), and another was located 499 bp downstream of the gene encoding G protein subunit alpha 11 (XM_012879775.1). These genes play roles in creatine transport and acetylcholine receptor signaling, respectively. Therefore, they are likely mechanistically involved in muscle contraction, and may represent weak physiological links that underlie loss of equilibrium at high temperatures. Consistent with this possibility, functional defects in *slc6a8* are involved in cerebral creatine deficiencies in humans, which can result in seizures (Nasrallah, Feki, & Kaabachi, 2010; Salomons et al., 2001). One LOE_{hyp}-associated SNP was located in the gene encoding max-like protein X (XM_012878820.1), and one was located in the gene encoding mucolipin-1 (XM_012860631.1). Max-like protein X forms a protein complex involved in mito-nuclear communication and activation of glycolytic gene transcription (Sans, Satterwhite, Stoltzman, Breen, & Ayer, 2006), a key component of tissue-level hypoxia responses (Richards, 2009). Mucolipin-1 (*mcoln1*) is a lysosomal calcium channel that is known to respond to cellular nutrient levels (Wang et al., 2015), and may be involved in two aspects of cellular hypoxia responses: (a) *mcoln1* is involved in regulation of the mechanistic target of rapamycin complex 1 (Li et al., 2016), a key regulator of cellular transcriptional responses in hypoxia (Arsham, Howell, & Simon, 2003), and (b) *mcoln1* is activated by mitochondrial reactive oxygen species (ROS) leading to autophagy and removal of damaged macromolecules (Zhang et al., 2016). Excess ROS are typically produced in hypoxia, and hypoxia-tolerant organisms often mount anticipatory antioxidant defenses (Lushchak & Bagnyukova, 2006). Furthermore,

inactivation of *mcoln1* is known to prevent removal of damaged mitochondria and clearance of excess ROS (Li et al., 2016).

One associated SNP for each trait overlapped with the SNPs that demonstrated evidence for selection between the S.NJ and N.NJ populations. For upper thermal tolerance, this SNP fell within the gene models of three overlapping genes; one of which was an E3 ubiquitin ligase (XM_012861443.1), and ubiquitination and degradation of improperly folded proteins is thought to be an important aspect of maintaining cellular function at high temperatures (Hochachka & Somero, 2002). For hypoxia tolerance, the overlapping SNP fell within the gene models of two genes encoded on opposite strands: a nuclear hormone receptor (XM_012881081.1) and calphoglin (XM_012881115.1) which is a coactivator of the aryl hydrocarbon receptor that plays a role in regulating many cellular responses to hypoxia via its interaction with hypoxia-inducible factor 1 alpha (Richards, 2009).

Taken together, we detected polygenic SNP variation in killifish that could explain 43.4% of the variation in upper thermal tolerance, and 51.9% of the variation in hypoxia tolerance among individuals. However, the genetic bases of these traits were not shared, and the absence of correlation between CT_{max} and LOE_{hyp} among or within populations suggests that there are distinct physiological and genetic mechanisms underlying variation in these traits in this species. Although trait associations may hinder or enable organismal adaptation to changing environments, depending on whether the directions of change in the traits are negatively or positively correlated, lack of trait associations potentially provides crucial evolutionary flexibility to fine-tune adaptive responses to specific local conditions.

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AUTHOR CONTRIBUTIONS

TMH, RSB, AW, and PMS designed the experiments and wrote the manuscript. TMH collected the phenotypic data, and RSB collected the genotypic data. TMH and RSB analyzed the data. TMH prepared the figures and tables.

DATA AVAILABILITY

Variant call format files and sequencing reads are available online (European Bioinformatics Institute (EBI) Biostudies database: S-BSST170; NCBI Sequence Read Archive (SRA): BioProject PRJNA477712). All other data used in the analyses presented in this study are provided in the Supporting Information Data S1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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