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Mitochondria, sex and variation in routine metabolic rate

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Abstract

Variation in the metabolic costs associated with organismal maintenance may play a key role in determining fitness, and thus these differences among individuals are likely to be subject to natural selection. Although the evolvability of maintenance metabolism depends on its underlying genetic architecture, relatively little is known about the nature of genetic variation that underlies this trait. To address this, we measured variation in routine metabolic rate ($\dot{M}O_{2routine}$), an index of maintenance metabolism, within and among three populations of Atlantic killifish, *Fundulus heteroclitus*, including a population from a region of genetic admixture between two subspecies. Polygenic association tests among individuals from the admixed population identified 54 single nucleotide polymorphisms (SNPs) that were associated with $\dot{M}O_{2routine}$, and these SNPs accounted for 43% of interindividual variation in this trait. However, genetic associations with $\dot{M}O_{2routine}$ involved different SNPs if females and males were analysed separately, and there was a sex-dependent effect of mitochondrial genotype on variation in routine metabolism. These results imply that there are sex-specific genetic mechanisms, and potential mitonuclear interactions, that underlie variation in $\dot{M}O_{2routine}$. Additionally, there was evidence for epistatic interactions between 17% of the possible pairs of trait-associated SNPs, suggesting that epistatic effects on $\dot{M}O_{2routine}$ are common. These data demonstrate not only that phenotypic variation in this ecologically important trait has a polygenic basis with considerable epistasis among loci, but also that these underlying genetic mechanisms, and particularly the role of mitochondrial genotype, may be sex-specific.

KEYWORDS
epistatic interactions, *Fundulus heteroclitus*, genome-wide association study, genotype–phenotype, interindividual, oxygen consumption

1 | INTRODUCTION

Adaptive responses of organisms depend on the genetic basis of traits that underlie variation in performance and fitness (e.g., Barrett & Schluter, 2008; Bay et al., 2017; Radwan & Babik, 2012; Shaw & Etterson, 2012; Visser, 2008). One key trait that is likely to be involved in these responses is the metabolic rate required to meet the minimum energetic costs of maintaining cellular structures and functions (i.e., "basal" in endotherms or "standard" in ectotherms; Dillon, Wang, & Huey, 2010; Hoegh-Guldberg & Bruno, 2010; Montoya & Raffaelli, 2010; O'Connor et al., 2007). Variation in basal or standard metabolic rate has been demonstrated to have substantial consequences for fitness in a variety of taxa (Álvarez & Nicieza, 2005; Blackmer et al., 2005; Boratyński, Koskela, Mappes, Mills, & Mokkonen, 2018; Boratyński, Koskela, Mappes, & Oksanen, 2010; Boratyński, Koskela, Mappes, &

Schroderus, 2013; Boratyński & Koteja, 2009, 2010; Burton, Killen, Armstrong, & Metcalfe, 2011; Metcalfe, Taylor, & Thorpe, 1995; Rønning et al., 2016). However, standard conditions (i.e., postabsorptive, nonreproductive and inactive) are rarely experienced in the field (e.g., Murchie, Cooke, Danylchuk, & Suski, 2011), and as a result, routine metabolic rate ($\dot{M}O_{2routine}$), which includes metabolic costs due to minimal activity levels and growth, is often utilized as an alternative measure of maintenance metabolic rate in ectothermic organisms (McBryan, Anttila, Healy, & Schulte, 2013; Metcalfe, Leeuwen, & Killen, 2016; Treberg, Killen, MacCormack, Lamarre, & Enders, 2016). Variation in $\dot{M}O_{2routine}$ has also been associated with differences in fitness (e.g., Killen, Marras, & McKenzie, 2011; Pettersen, White, & Marshall, 2016), and thus, it is likely that both standard and routine metabolism will play important roles in adaptive responses.

A growing number of studies have demonstrated variations in basal, standard or routine metabolic rate that are either heritable or can respond to artificial selection (e.g., Boratyński et al., 2013; Książek, Konarzewski, & Łapo, 2004; Nespolo, Bustamante, Bacigalupe, & Bozinovic, 2005; Nilsson, Åkesson, & Nilsson, 2009; Pettersen, Marshall, & White, 2018; Rønning, Jensen, Moe, & Bech, 2007; Sadowska et al., 2005), which indicates that these traits are at least partially genetically determined. In addition, several studies have demonstrated that both the genetic basis for, and fitness consequences of, basal metabolic rate are sex-dependent (Boratyński, Ketola, Koskela, & Mappes, 2016; Boratyński et al., 2013, 2018; Šíchová, Koskela, Mappes, Lantová, & Boratyński, 2014), implying that the mechanisms underlying variation in the energetic costs of maintenance differ between females and males in at least some species. Yet, despite these observations, the genetic loci and physiological mechanisms that underlie this variation remain largely unknown (but see Brennan et al., 2018; Do et al., 2008).

Given the central role of the mitochondrion in metabolism and oxygen consumption, one obvious candidate that could be involved in determining $\dot{M}O_{2routine}$ is the mitochondrial genome. Indeed, variation in mitochondrial genotype has been associated with differences in $\dot{M}O_{2routine}$ or basal metabolic rate in several taxa (Arnqvist et al., 2010; Boratyński et al., 2016; Hoekstra, Siddiq, & Montooth, 2013; Kurbalija Novičić et al., 2015; Šíchová et al., 2014), and in some cases these effects are sex-specific (Boratyński et al., 2016; Kurbalija Novičić et al., 2015; Šíchová et al., 2014). Sex-specific effects of mitochondrial genotype on variation in $\dot{M}O_{2routine}$ are particularly interesting due to the potential for interactive effects between the mitochondrial and nuclear genomes (i.e., mitonuclear interactions). In independently evolving taxa, the mitochondrial genome is expected to coevolve with the nuclear genome, which may result in intergenomic incompatibilities when hybridization introduces foreign mitochondrial DNA onto a novel nuclear background (e.g., Burton, Pereira, & Barreto, 2013; Hill, 2015; Rand, Haney, & Fry, 2004). As mitochondria are typically maternally inherited in animals, theory predicts that these incompatibilities may manifest differentially between the sexes (e.g., Hill, 2015; Hill et al., 2018), and this has been observed for several traits (Aw, Garvin, Melvin, & Ballard, 2017; Camus & Dowling, 2018; Camus, Wolf, Morrow, & Dowling, 2015; Vaught & Dowling, 2018), including basal metabolic rate in bank voles, *Myodes glareolus* (Boratyński et al., 2016, 2018, 2013; Šíchová et al., 2014). However, establishing the generality of these effects across taxa and identifying the nuclear loci involved remain open tasks.

In the current study, we investigate genetic associations with variation in $\dot{M}O_{2routine}$ in the Atlantic killifish, *Fundulus heteroclitus*. There are two genetically and physiologically distinct subspecies of this topminnow found in intertidal saltmarshes along the east coast of North America (e.g., Morin & Able, 1983): *F. h. heteroclitus* from Florida to New Jersey, USA ("southern") and *F. h. macrolepidotus* from New Jersey to Canada ("northern"). The northern subspecies has a higher $\dot{M}O_{2routine}$ than the southern subspecies (Fangue, Richards, & Schulte, 2009; Healy & Schulte, 2012a, 2012b; McBryan, Healy, Haakons, & Schulte, 2016) consistent with intraspecific countergradient variation associated with latitude and temperature (Conover & Schultz, 1995). Along the coastline of New Jersey, the subspecies meet and interbreed, resulting in a stable contact zone of genetic admixture (e.g., McKenzie, Dhillon, & Schulte, 2015; Strand, Williams, Oleksiak, & Sotka, 2012). Across this region there is a steep transition from a fixed southern to a fixed northern mitochondrial genotype (e.g., González-Vilaseñor & Powers, 1990), and there is some evidence of disequilibrium between the mitochondrial genotypes and nuclear genetic variants (Baris, Blier, Pichaud, Crawford, & Oleksiak, 2016; Baris et al., 2017; McKenzie et al., 2019). Thus, contact zone populations of killifish offer an ideal opportunity to use admixture mapping to associate genetic variation with $\dot{M}O_{2routine}$ variation in natural populations. Admixture mapping not only facilitates dissociation of neutral variation between the subspecies from variation that contributes to phenotypic differences, but also minimizes environmental effects (e.g., irreversible developmental plasticity) that could affect phenotypic variation between distant populations. Consequently, in the current study we use this approach with genotyped individuals from a population in New Jersey (Healy, Brennan, Whitehead, & Schulte, 2018a) to address the following questions: (a) Which genetic loci are associated with interindividual variation in $\dot{M}O_{2routine}$ and what proportion of phenotypic variation can be attributed to these loci? (b) What is the extent of epistatic interactions among these loci? (c) Are the loci associated with interindividual variation also consistent with genetic and phenotypic variation among populations? (d) Do the loci associated with variation in $\dot{M}O_{2routine}$ depend on sex? (e) Are there effects of mitochondrial genotype or mitonuclear interactions on $\dot{M}O_{2routine}$ variation in this species?

2 | MATERIALS AND METHODS

2.1 | Fish collection

The Atlantic killifish used in the current study were a subset of those used in a previous study examining variation in upper thermal tolerance and hypoxia tolerance (Healy et al., 2018a). These fish were collected with minnow traps from Hudson Creek, Georgia,

USA (GA), Beaverdam Creek, New Jersey, USA (NJ) and Taylor River, New Hampshire, USA (NH) in autumn 2012, then shipped overnight to the University of British Columbia (Vancouver, Canada). Individuals were uniquely marked with fluorescent tags (Northwest Marine Technology), and held in 200-L tanks that were connected to a recirculating water system for laboratory acclimation. Acclimation conditions were 15°C, 20 ppt salinity, 12:12-hr light/dark, and were maintained for at least 1 month prior to the start of experiments. Fish were fed once daily to satiation with Nutrafin Max Tropical Fish Flakes (Hagen) on all days except those immediately preceding days of experimental trials or tissue sampling. Husbandry was in accordance with an approved UBC animal care permit (A11-0372). For geographical coordinates, sample sizes and previously determined mitochondrial genotype frequencies for each population see Table S1.

2.2 | Measurement of routine metabolic rate

Routine metabolic rate ($\dot{M}O_{2routine}$) was measured using similar methods to those previously published for killifish (e.g., Brennan et al., 2018; Fangue et al., 2009; Healy & Schulte, 2012b). $\dot{M}O_{2routine}$ trials occurred prior to other tolerance trials reported elsewhere (Healy et al., 2018a), and thus any potential effects of other tolerance trials on variation in $\dot{M}O_{2routine}$ did not affect the data collected for the current study. For $\dot{M}O_{2routine}$ trials, fish were held in 250-ml glass respirometers under acclimation conditions overnight. In the morning, trials were started by sealing the respirometers, and oxygen levels inside the respirometers were monitored with a sampling rate of once every 10 s using NeoFox oxygen probes (Ocean Optics). After 1 hr of measurements, respirometers were opened, tags were inspected, mass and length were determined, and fish were returned to their acclimation tanks. Oxygen levels at the end of the measurement periods were 75%–85% of air-saturated levels. The 5-min measurement interval with the lowest constant rate of oxygen decrease (i.e., linear decrease in oxygen level with $R^2 \geq 0.95$) was used to calculate $\dot{M}O_{2routine}$. Fish were generally quiescent throughout the measurement period; however, from visual assessment, a small number of fish (26 or ~7% of all assessed) demonstrated excessive and consistent activity relative to other fish in the study at both the start and the end of their measurement period. These fish were excluded from analyses to minimize the effects of activity levels on variation in $\dot{M}O_{2routine}$ (the sample sizes presented in Table S1 reflect these exclusions). Between trials, respirometers were rinsed with 75% ethanol to maintain bacterial respiration at negligible levels relative to fish respiration (<2% of total estimated $\dot{M}O_{2routine}$).

2.3 | Genotype data acquisition

Data for mitochondrial genotypes and 73,398 single nucleotide polymorphisms (SNPs) throughout the nuclear genome were obtained for the NJ individuals in the current study from the supplemental materials and data repositories associated with Healy et al. (2018a; National Center for Biotechnology Information BioProject PRJNA477712 [Healy, Brennan, Whitehead, & Schulte, 2018c]; European Bioinformatics Institute Biostudy S-BSST170 [Healy, Brennan, Whitehead, & Schulte, 2018b]). Mitochondrial and nuclear genotypes were available for 296 of the 366 NJ fish that were used for measurements of $\dot{M}O_{2routine}$. Genotyping methods are detailed in Healy et al. (2018a); mitochondrial genotypes were determined by genotype-specific quantitative real-time polymerase chain reactions, and nuclear SNPs were obtained using a restriction-site-associated DNA sequencing (RADseq) approach. Briefly, RADseq libraries were prepared with pools of indexed genomic DNA isolated from 96 individuals as in Ali et al. (2016) with *Sbf*I biotinylated RAD adapters and NEBNext Ultra DNA Library Prep Kits (New England Biolabs). After preliminary sequencing to determine appropriate normalization among libraries, libraries were sequenced on two lanes of an Illumina HiSeq 4000 with 150-bp paired-end sequencing at the University of California Davis Genome Center. BWA-MEM version 0.7.12 (Li & Durbin, 2009) was used to map the resulting sequencing reads to the *Fundulus heteroclitus* reference genome 3.0.2 (Reid et al., 2017; National Center for Biotechnology Information [NCBI] GenBank accession GCA_000826765.1), and FREEBAYES version 0.9.21-19-gc003cl (Garrison & Marth, 2012) was used to call genetic variants among individuals. Biallelic SNPs were retained and quality filtered using VCFTOOLS version 0.1.15 (Danecek et al., 2011) and HDPLOT (McKinney, Waples, Seeb, & Seeb, 2017). For the current study, we repeated this filtering process using only the NJ fish for which both SNP and $\dot{M}O_{2routine}$ data were available. After filtering, high-quality genotypic calls at 73,218 SNPs in 286 individuals remained, and these data were used for the genotype–phenotype association analyses reported here.

2.4 | Data analysis and statistics

Statistical analyses were performed in R version 3.4.0 (R Core Team, 2017) with $\alpha = .05$. Effects of population, sex and body mass on $\dot{M}O_{2routine}$ were assessed with a general linear model followed by analysis of covariance (ANCOVA) using data for the GA, NJ and NH populations. To account for the allometric relationship between body mass and $\dot{M}O_{2routine}$, data were log transformed prior to analysis, and after transformation assumptions of normality and homogeneity of variances were met. The linear model was simplified by the removal of nonsignificant factors prior to the final ANCOVA (see Table S2 for the full and reduced ANCOVA tables). Post-hoc pairwise comparisons among groups were performed with Tukey tests. Mass-corrected $\dot{M}O_{2routine}$ was calculated using the residuals of the ANCOVA model as if each individual weighed 4 g to examine mass-independent variation in $\dot{M}O_{2routine}$. Variation in mass-corrected $\dot{M}O_{2routine}$ was assessed statistically as described above for uncorrected $\dot{M}O_{2routine}$, but body mass was removed as a potential explanatory factor. A second linear model with mitochondrial genotype, sex and their interaction as explanatory factors was used to assess the effects of mitochondrial genotype on mass-corrected $\dot{M}O_{2routine}$ in the NJ killifish. Again, $\dot{M}O_{2routine}$ data were log transformed, and

all assumptions were met. Note that this separate model was necessary as the GA and NH populations are fixed for the southern and northern mitochondrial genotype, respectively, making it impossible to test effects of mitochondrial genotype within these populations.

Associations between SNP variation and $\dot{M}O_{2routine}$ variation among the admixed NJ individuals were assessed in a polygenic context using random forest (RF) models (Goldstein, Hubbard, Cutler, & Barcellos, 2010; Liaw & Wiener, 2002). For these models, missing genotypes (<5% per SNP) were imputed with BEAGLE version 4.1 (Browning & Browning, 2016), effects of body mass were accounted for by the use of mass-corrected $\dot{M}O_{2routine}$ (calculated as described above), and effects of population structure were accounted for as described by Zhao et al. (2012). We generated regression models with 10,000 trees, and the best explanatory model was identified using an iterative purging approach as described elsewhere (Brieuc, Ono, Drinan, & Naish, 2015; Healy et al., 2018a; Holliday, Wang, & Aitken, 2012; summarized in Figure S1). Once this approach identified the SNPs contributing to the best explanatory model for variation in $\dot{M}O_{2routine}$, this model was rerun 100 independent times. The percentage of phenotypic variation explained was averaged across these runs, and the $\dot{M}O_{2routine}$-associated SNPs were ranked based on their average importance scores. Mitochondrial genotype and sex were included as potential explanatory factors in the initial model essentially in an equivalent manner to each nuclear SNP. However, this may somewhat underestimate their overall effects, and may lead to removal of these factors at early iterations, which precludes their function as "covariates" later in the analysis. Consequently, we repeated our RF procedure in females and males separately as an alternative to including sex as a factor in the main RF model. In addition, we used Fisher's exact tests to examine potential interactions between mitochondrial genotype and nuclear SNPs associated with variation in $\dot{M}O_{2routine}$ in these sex-specific analyses. Differences in nuclear genotypic proportions (i.e., homozygous allele 1, heterozygous and homozygous allele 2) between fish of each mitochondrial genotype were assessed in females or males for SNPs identified in the corresponding sex-specific RF analysis. Candidate genes potentially contributing to variation in $\dot{M}O_{2routine}$ were identified as those either containing or within 27 kb (an approximation of the size of linkage blocks in killifish [Brennan et al., 2018]) of the $\dot{M}O_{2routine}$-associated SNPs. Functional enrichment analysis of the candidate genes was performed with gene ontology (GO) annotations obtained from Healy, Bryant, and Schulte (2017) and GOSEQ version 1.28.0 (Young, Wakefield, Smyth, & Oshlack, 2010), and the Benjamini–Hochberg method was used to correct for multiple comparisons (Benjamini & Hochberg, 1995).

Epistatic interactions among $\dot{M}O_{2routine}$-associated SNPs identified by the models with all individuals were assessed as in Holliday et al. (2012). Models were run five times, and this was iterated such that between successive iterations the SNP with the lowest average importance score was removed. Interactions between the removed SNP and the remaining SNPs were determined by significant changes in the importance of a SNP between subsequent steps as assessed by Student's *t* tests followed by false-discovery rate correction (Benjamini & Hochberg, 1995). This method assesses all but one of these possible interactions, as RF models cannot be run with a single marker, and therefore an interaction between the two most important SNPs cannot be tested. A significant increase in the importance value of an SNP following removal of another is consistent with disruptive epistasis between the SNPs, whereas a significant decrease in importance value is consistent with a synergistic epistatic interaction (Holliday et al., 2012).

3 | RESULTS

3.1 | Variation in routine metabolic rate

Across the three populations in our study, the interactive effects among sex, body mass and population, and the main effect of sex did not significantly affect variation in $\dot{M}O_{2routine}$ ($F \leq 1.70$, $p \geq .19$; Table S2), and thus, these terms were removed hierarchically from our statistical model (Table S2). $\dot{M}O_{2routine}$ was significantly affected by both population ($F_{2,443} = 7.75$, $p = 4.9 \times 10^{-4}$; Figure 1a) and body mass ($F_{1,443} = 3{,}246.76$, $p < 2.2 \times 10^{-16}$; Figure S2), and the allometric effects of body mass on $\dot{M}O_{2routine}$ demonstrated a typical scaling exponent ($b = 0.73$; e.g., Gillooly, Brown, West, Savage, & Charnov, 2001). We

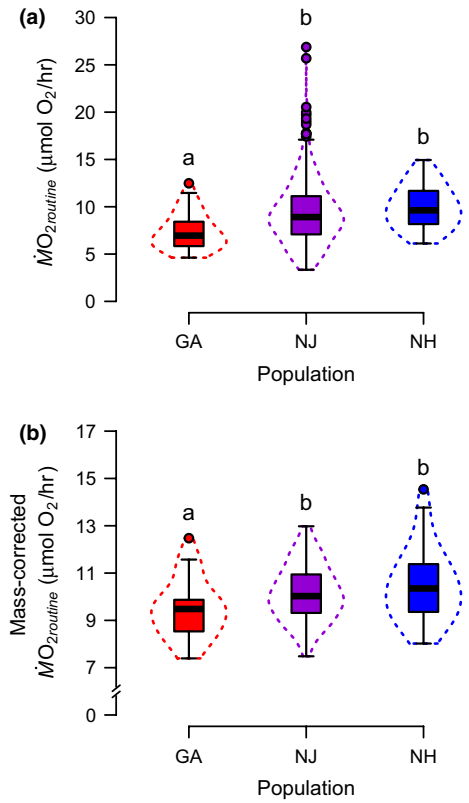

FIGURE 1 Variation in routine metabolic rate ($\dot{M}O_{2routine}$; a) and mass-corrected $\dot{M}O_{2routine}$ (b) among populations of killifish: southern—GA, red; northern—NH, blue; admixed—NJ, purple. Data are displayed as box plots surrounded by density distributions (dashed lines); lower case letters indicate results of post-hoc comparisons among populations [Colour figure can be viewed at wileyonlinelibrary.com]

used this model to calculate mass-corrected $\dot{M}O_{2routine}$, and variation in corrected $\dot{M}O_{2routine}$ displayed similar variation among populations to that observed for uncorrected $\dot{M}O_{2routine}$ ($F_{2,444} = 7.92$, $p = 4.2 \times 10^{-4}$; Figure 1b; Table S3). As a result, we consider the corrected data in the analyses below to focus on mass-independent differences in metabolism. In post-hoc tests, the GA population had lower $\dot{M}O_{2routine}$ than the NH population ($p < 1.0 \times 10^{-3}$), as expected based on previous comparisons of GA and NH killifish (e.g., Fangue et al., 2009; Healy & Schulte, 2012a, 2012b; McBryan et al., 2016). The $\dot{M}O_{2routine}$ of the NJ population was higher than that of the GA population ($p < 1.0 \times 10^{-3}$), and was statistically indistinguishable from that of the NH population ($p = .41$; mean ± *SEM*: 9.5 ± 0.2, 10.2 ± 0.1 and 10.5 ± 0.2 μmol O_2/hr for GA, NJ and NH, respectively). Additionally, the range of $\dot{M}O_{2routine}$ among NJ killifish spanned most of the range encompassed by the $\dot{M}O_{2routine}$ values observed in both the GA and the NH populations (~77% of the total range, ~98% of the GA range and ~76% of the NH range; Figure 1b). Data for $\dot{M}O_{2routine}$, mass, length, sex and mitochondrial genotype for each fish are available in Table S4.

3.2 | Genetic associations with routine metabolic rate

To assess potential effects of mitochondrial genotype on variation in mass-corrected $\dot{M}O_{2routine}$, we ran a second linear model with the fish from the NJ population alone, as this population is segregating for the southern and northern mitochondrial types. Among NJ individuals, $\dot{M}O_{2routine}$ was significantly affected by an interaction between mitochondrial genotype and sex ($F_{1,291} = 5.80$, $p = 1.7 \times 10^{-2}$; see Table S5 for ANOVA table; $n = 65$ southern and 69 northern females, and 80 southern and 82 northern males; Figure 2). Comparisons of mean values suggested that, after correction for the effects of body mass, there were trends for females with southern mitochondrial genotypes to have higher $\dot{M}O_{2routine}$ than females with northern mitochondrial genotypes (mean ± *SEM*: 10.2 ± 0.2 and 9.9 ± 0.1 μmol O_2/hr, respectively), and for males with southern

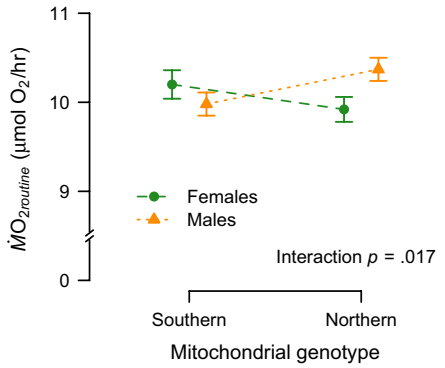

FIGURE 2 Routine metabolic rate ($\dot{M}O_{2routine}$) in the genetically admixed NJ population of killifish grouped by mitochondrial genotype and sex (♀—green circles, dashed line; ♂—orange triangles, dotted line). Interaction *p*-value between these factors is displayed above the *x*-axis [Colour figure can be viewed at wileyonlinelibrary.com]

mitochondrial genotypes to have lower $\dot{M}O_{2routine}$ than males with northern mitochondrial genotypes (mean ± *SEM*: 10.0 ± 0.1 and 10.4 ± 0.1 μmol O_2/hr, respectively). However, although there was a marginal *p*-value for a difference between males with northern or southern mitochondrial genotypes ($p = .09$), no differences among groups were resolved by post-hoc tests ($p \geq .14$ for all other comparisons). These results suggest that there are modest sex-specific effects of variation in mitochondrial genotype on $\dot{M}O_{2routine}$ in the NJ population of killifish.

Random forest models using the 286 NJ killifish with both phenotypic and genotypic data in the current study detected 54 nuclear SNPs as associated with variation in $\dot{M}O_{2routine}$ among individuals (Figure 3a; Table S6), and ~43% of the variation in $\dot{M}O_{2routine}$ (Figure S3a) could be attributed to genetic differences at these loci. Although these SNPs may only be linked to causal variants, 42 of the 54 $\dot{M}O_{2routine}$-associated SNPs were found within genes, and 13 were within coding sequences (seven nonsynonymous and six synonymous substitutions; Table S6). Examining the genes containing or nearby (≤27 kb) the 54 SNPs identified several candidates with plausible roles in metabolic processes (Table S6). It is possible that this simply reflects the large number of metabolic pathways within a cell, and after false-discovery rate correction, GO enrichment analyses did not detect any significantly enriched annotations within these candidate genes (Table S7). However, this lack of functional enrichments may partially be due to the low number of candidates identified (50 genes), as ~20% of the candidate genes encode proteins that function in signalling pathways associated with growth factors (*plcg1*, *hdac7* and *kif16b*) or major metabolic kinases (*inppl1*, *nlrx1*, *cndp2*, *myocd*, *rptor*, *znrf1*, *mtmr7* and *itpr3*). These genes do not group within a single GO annotation, but GO terms related to cellular kinases and growth factors (e.g., GO:00305011) met the threshold for statistical significance prior to corrections for multiple comparisons (Table S7), which provides some evidence for roles of these processes in the mechanistic basis of interindividual variation in $\dot{M}O_{2routine}$. Regardless of the specific pathways involved, our overall results are consistent with a polygenic basis for variation in $\dot{M}O_{2routine}$ that probably involves genes that function across many metabolic pathways.

Separate RF models for females and males ($n = 128$ and 158, respectively) detected 19 and 40 $\dot{M}O_{2routine}$-associated SNPs, respectively (Figure 3b; Table S6), and again high percentages of phenotypic variation among individuals could be attributed to this SNP variation (57% and 55%, respectively; Figure S3b,c). These analyses identified several of the same loci as those identified in the analysis using all individuals (16 of 54: four female only and 12 male only), but no SNP was shared between the final models for each sex (Figure 3b). This suggests that different loci are associated with variation in $\dot{M}O_{2routine}$ in female and male killifish. Taken together with the interactive effect of sex and mitochondrial genotype in the NJ population (described above), sex-specific associations with variation in $\dot{M}O_{2routine}$ for these SNPs may also indicate potential involvement in mitonuclear interactions that affect variation in $\dot{M}O_{2routine}$. However, following corrections for multiple comparisons, no SNP demonstrated significant

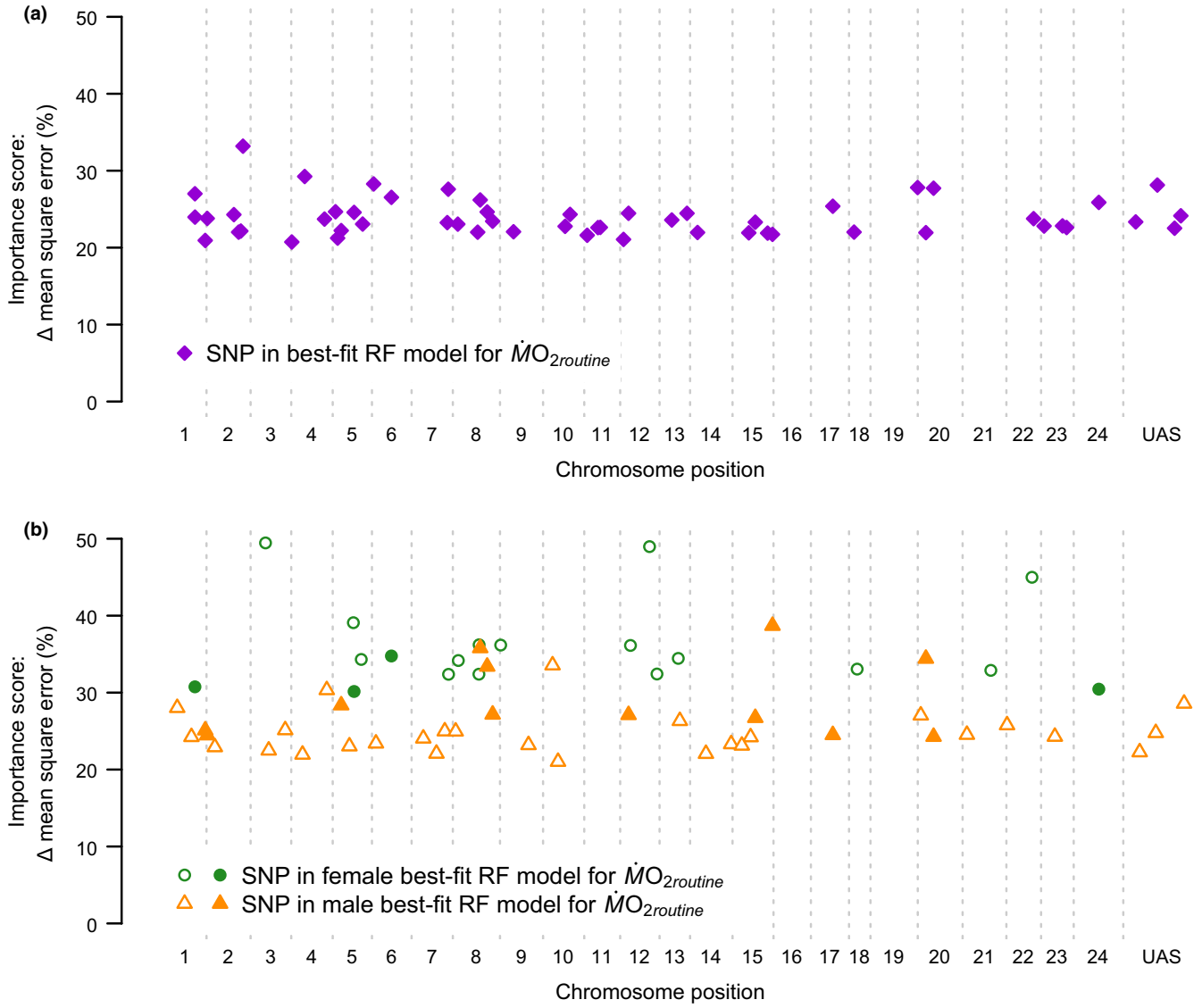

FIGURE 3 Importance scores for the $\dot{M}O_{2routine}$-associated nuclear SNPs from the best-fitting random forest (RF) models with all individuals (a; purple diamonds), only females (b; green circles) or only males (b; orange triangles). Solid symbols in (b) indicate SNPs also detected in the analysis with all individuals. Note that no SNPs were detected in both the male- and the female-specific analyses. Numbers along the *x*-axis are chromosome numbers and dashed grey lines display chromosomal boundaries (UAS = unassembled scaffolds) [Colour figure can be viewed at wileyonlinelibrary.com]

differences in genotypic frequencies between fish of each mitochondrial genotype (Fisher's exact tests $p \geq .02$ for all compared to Bonferroni-corrected $\alpha = .001$). Thus, these SNPs are unlikely to be tightly linked to genes involved in sex-specific mitonuclear interactions, or at a minimum the effects of these interactions are modest when analysed independently, which may be a consequence of the polygenic basis for variation in $\dot{M}O_{2routine}$.

3.3 | Epistatic interactions among nuclear loci associated with metabolic rate

The 54 SNPs associated with variation in $\dot{M}O_{2routine}$ (from RF models with both sexes of NJ killifish) create 1,431 possible pairwise interactions among trait-associated loci. For the majority (83%) of these pairs of trait-associated SNPs there was no statistical evidence for interactive effects between loci ($q \geq 5.1 \times 10^{-2}$), whereas 17% of SNP pairs demonstrated significant epistasis ($q \leq 4.8 \times 10^{-2}$) with a clear bias for disruptive rather than synergistic effects between SNPs (15% compared to 2%, respectively; Figure 4; Table S8). Despite this overall pattern for a lack of epistasis among $\dot{M}O_{2routine}$-associated SNPs, these data represent 240 epistatic interactions that influence variation in $\dot{M}O_{2routine}$. Therefore, although epistatic interactions among loci were found for a minority of SNP pairs, these interactions are common aspects of the genetic architecture that underlies variation in $\dot{M}O_{2routine}$ among killifish.

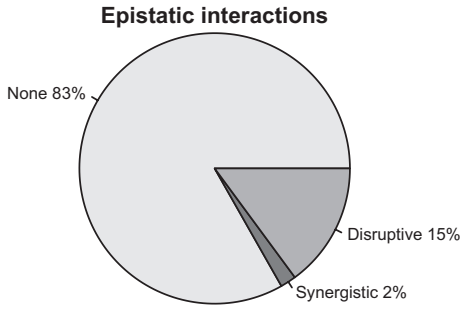

FIGURE 4 Pie chart summary of epistatic interactions among the 54 $\dot{M}O_{2routine}$-associated nuclear SNPs detected by RF models with all NJ killifish (synergistic epistasis—dark grey; disruptive epistasis—medium grey; no epistasis—light grey)

4 | DISCUSSION

Our results demonstrate that interindividual variation in metabolic rate has a complex polygenic basis, including epistatic interactions and sex-dependent effects of mitochondrial genotype. Furthermore, when analysed separately, $\dot{M}O_{2routine}$-associated SNPs were not shared between females and males, suggesting at least some of the genetic mechanisms that underlie variation in this trait are different between the sexes. Together, these data indicate that this ecologically important trait has the potential to respond to selection through polygenic adaptation (e.g., Sella & Barton, 2019), and highlight that these responses may involve different loci in females and males.

4.1 | Polygenic associations with variation in routine metabolic rate

In the current study, we examined interindividual variation in $\dot{M}O_{2routine}$ in a genetically admixed population of killifish. Our association results revealed a polygenic basis for this phenotypic variation with a large proportion of variation (~43%) attributable to 54 SNPs. This pattern is consistent with previous demonstrations of relatively high heritability for variation in the energetic costs of maintenance in some studies (reviewed by Pettersen et al., 2018), and suggests that these SNPs may play substantial roles in determining individual-level variation in this trait among killifish. However, interpreting the functional consequences of the $\dot{M}O_{2routine}$-associated SNPs in the current study is challenging, as these loci may only be linked to causal variants and the candidate genes identified by these SNPs were not enriched for any specific GO annotation. Yet, several of our candidates have regulatory functions within signalling pathways associated with growth factors and metabolic kinases. Thus, it is possible that differences in the regulation of metabolic demand may be an important component of the genetic mechanisms underlying interindividual variation in this trait, although this hypothesis requires direct testing through further experimental work.

Despite the common use of $\dot{M}O_{2routine}$ as a measure of the energetic costs of maintenance in ectothermic organisms, $\dot{M}O_{2routine}$ includes minimal costs associated with low levels of activity and growth (Metcalfe et al., 2016). Therefore, an important caveat of the current study is that some of the genetic associations observed here may involve genetically based differences in spontaneous activity rather than variation in energy use associated with maintenance. However, our methods follow the guidelines established by Chabot, Steffensen, and Farrell (2016) for measurement of $\dot{M}O_{2routine}$ in general, and our estimates represent the lowest observed to date for *Fundulus heteroclitus*, suggesting minimal contributions of activity to $\dot{M}O_{2routine}$ in the current study.

4.2 | Genetic interactions among loci associated with routine metabolic rate

We detected 240 instances of epistasis between pairs of our $\dot{M}O_{2routine}$-associated SNPs, demonstrating that interactive effects among associated loci are probably common for variation in this trait. The vast majority of these interactions were consistent with disruptive epistasis, which suggests that most of the effects of epistasis on $\dot{M}O_{2routine}$ are due to masking of the effects of one locus by the effects of another locus. That said, as discussed above, it is difficult to interpret the functional consequences of the $\dot{M}O_{2routine}$-associated SNPs identified in the current study, and few of our candidate genes were consistent with direct functional interactions. Thus, the mechanisms underlying epistatic effects among the SNPs associated with $\dot{M}O_{2routine}$ in the current study are likely to be complex.

Here we utilized the protocols of Holliday et al. (2012) to examine epistasis among trait-associated SNPs, and it is likely that these methods are subject to artificial changes in power due to the number of total loci examined and the number of RF models used at each iterative step of the analysis. Yet, clear networks of interacting genes have been identified by these approaches using fewer SNPs than those in the current study (Holliday et al., 2012), suggesting that it is unlikely we have grossly overestimated the number of epistatic interactions affecting $\dot{M}O_{2routine}$ in killifish.

4.3 | Interpopulation compared to interindividual variation in routine metabolic rate

Despite the relatively high proportion of interindividual variation in $\dot{M}O_{2routine}$ explained within the admixed NJ population in the current study, the effects of the associated SNPs on variation in $\dot{M}O_{2routine}$ among populations are less clear. Although the $\dot{M}O_{2routine}$ of NH killifish has repeatedly been demonstrated to be higher than that of GA killifish across several studies (e.g., Fangue et al., 2009; Healy & Schulte, 2012a, 2012b; McBryan et al., 2016; the current study), there are only relatively small allele frequency differences between these populations at the majority of our $\dot{M}O_{2routine}$-associated loci (Healy et al., 2018a; Figure S4; Table S6). Even with the expectation of relatively small shifts in allele frequencies at causal loci for polygenic traits (Bernatchez, 2016), these patterns for the GA and NH populations are perhaps surprising given that the NJ population contains similar proportions of northern and southern genotypes

in both the nuclear and mitochondrial genomes (e.g., McKenzie, Bucking, Moreira, & Schulte, 2017; McKenzie et al., 2015) and the phenotypic variation within this population spans much of the variation across the GA and NH populations (Figure 1). Regardless, these results indicate that there are probably differences in the genetic mechanisms underlying individual-level and population-level variation in $\dot{M}O_{2routine}$ in this species. However, for 12 of the SNPs associated with $\dot{M}O_{2routine}$ in NJ individuals, the variant associated with high $\dot{M}O_{2routine}$ also occurs at ≥20% higher frequencies in the NH population than in the GA population (Healy et al., 2018a; Figure S4; Table S6), which is consistent with the phenotypic difference in this trait between GA and NH. Therefore, our data also suggest that a subset of the 54 SNPs associated with $\dot{M}O_{2routine}$ in admixed killifish may contribute to the genetic basis for variation in this trait across populations.

Interestingly, none of the SNPs or genes associated with $\dot{M}O_{2routine}$ in our coastal population of killifish were also associated with $\dot{M}O_{2routine}$ in a Chesapeake admixed population in a previous study (Brennan et al., 2018). This finding is probably, in part, due to variation in the nuclear loci examined in each study as a result of using RADseq, and potentially the use of relatively small sample sizes for genome-wide analyses in these studies. Additionally, RADseq, RF models and iterative purging methods may fail to detect causative loci as a result of linkage and other masking effects, and may also inflate the phenotypic variance explained due to over-fitting (Brieuc, Waters, Drinan, & Naish, 2018; Forester, Lasky, Wagner, & Urban, 2018; Hoban et al., 2016). However, differences in the genetic basis for interindividual variation within separate populations are not atypical for polygenic traits (e.g., Bernatchez, 2016; Elmer & Meyer, 2011; Ralph & Coop, 2015; Yeaman, 2015), particularly for populations with high levels of polymorphism (Crawford, Schulte, Whitehead, & Oleksiak, in press).

4.4 | Sex-specific genetic effects on variation in routine metabolic rate

We observed no overlap in the $\dot{M}O_{2routine}$-associated SNPs detected by independent RF models for female and male killifish. These results suggest that the nuclear loci that underlie a large proportion of interindividual variation in $\dot{M}O_{2routine}$ are sex-specific. One possible explanation for these differences between the sexes is the use of $\dot{M}O_{2routine}$ in the current study, which does not require the organisms to be in a nonreproductive condition. Therefore, differences in reproductive costs between females and males may drive the lack of SNP overlap between these analyses, and it is possible that analyses using standard metabolic rate would be more similar between the sexes. However, several quantitative genetics studies in bank voles demonstrate not only that the genetic mechanisms underlying variation in basal metabolic rate vary between females and males (Boratyński et al., 2016, 2013; Šíchová et al., 2014), but also that selection for social dominance results in sex-specific phenotypic consequences within both dominant and subordinate breeding lines (Boratyński et al., 2018). Additionally, differences in resting metabolic rate and their relationships with locomotor activity are sex-specific in *Drosophila melanogaster* (Videlier, Rundle, & Careau, 2019), and there is some evidence of sex-dependent effects on variation in this trait in *D. subobscura* (Kurbalija Novičić et al., 2015). Taken together, these results suggest that the sex-specific patterns observed in the current study are not simply a consequence of examining associations with $\dot{M}O_{2routine}$, but rather that the loci involved in the major genetic effects underlying variation in maintenance metabolism may not be shared between the sexes in killifish.

Despite the similarities between our sex-specific results and those of previous studies in bank voles and fruit flies, it is possible that the lack of overlap in $\dot{M}O_{2routine}$-associated SNPs in the current study is, in part, a consequence of reduced statistical power due to splitting the NJ fish by sex, which inherently results in reduced sample sizes. Additionally, there is stochastic variation associated with fitting RF models, and it is possible that division of the NJ killifish into two groups results in identifying some amount of different loci due to examining different subsamples of individuals. However, given the complete lack of overlap between our sex-specific analyses, it is unlikely that these factors can completely explain the differences in our results between the sexes. Thus, our data imply that the loci that are most predictive of variation in $\dot{M}O_{2routine}$ are different between the female and male killifish in our study.

4.5 | Effects of mitochondrial genotype and mitonuclear interactions on routine metabolic rate

The phenotypic results obtained for the NJ population in the current study revealed an interactive effect of mitochondrial genotype and sex on interindividual variation in $\dot{M}O_{2routine}$, suggesting that there may be sex-specific mitonuclear interactions that have modest effects on this trait in killifish. Given this result, it is somewhat surprising that mitochondrial genotype was not an explanatory marker in any of the best-fitting RF models in our study, and that the sex-specific $\dot{M}O_{2routine}$-associated SNPs did not demonstrate genotypic frequencies that varied significantly between females and males. It is possible these results are a consequence of a modest effect associated with this interaction, or of technical noise in the early stages of model fitting due to the high number of SNPs examined or other limitations of RF models (discussed above). Alternatively, this may suggest that nuclear loci that were undetected by our RADseq approach are involved in these interactions. Regardless, and consistent with the phenotypic results presented here, effects of mitochondrial genotype on $\dot{M}O_{2routine}$ or basal metabolic rate have been observed previously in insects (Arnqvist et al., 2010; Hoekstra et al., 2013; Kurbalija Novičić et al., 2015) and birds (Tieleman et al., 2009), and there is some evidence that these effects are sex-specific in fruit flies (Kurbalija Novičić et al., 2015) and bank voles (Boratyński et al., 2016; Šíchová et al., 2014). Furthermore, complex I-fuelled oxidative phosphorylation rates in *D. melanogaster* demonstrate variation in the effects of mitochondrial genotype between females and males (Aw et al., 2017).

Potential effects of mitonuclear incompatibilities on $\dot{M}O_{2routine}$ in killifish are particularly relevant in the context of an interaction between sex and mitochondrial genotype, as mitonuclear interactions may vary between the sexes in some cases (e.g., Hill et al., 2018). For example, the "Mother's curse" hypotheses posits that because mitochondria are maternally inherited, mutations deleterious to males can escape purifying selection if they are beneficial in females (Frank & Hurst, 1996). Our data may be consistent with this hypothesis as metabolic rate represents a physiological cost, and in fish with northern mitochondrial genotypes, males tended to have higher $\dot{M}O_{2routine}$ than females. Higher metabolic rates have been associated with hybrid dysfunction in insects (Hoekstra et al., 2013; McFarlane, Sirkiä, Ålund, & Qvarnström, 2016), and higher basal metabolism is associated with reduced performance in fitness-related traits in *M. glareolus* (Boratyński et al., 2013, 2018). However, in the absence of resource limitations or other stresses, a higher cost of living (i.e., higher $\dot{M}O_{2routine}$) would not necessarily be expected to have negative fitness consequences (e.g., Burton et al., 2011). Yet, in killifish, intersubspecies crosses between females with southern mitochondrial genotypes and males with northern mitochondrial genotypes result in offspring with developmental rates that are faster than those of offspring from within subspecies crosses, and eggs from these intersubspecies crosses also have low hatching success (McKenzie et al., 2017). Furthermore, females with southern mitochondrial genotypes demonstrate a preference for within-subspecies matings (McKenzie et al., 2017), potentially consistent with variation in mitonuclear interactions between the sexes and mitonuclear effects on sexual selection, as has been suggested elsewhere (Hill, 2014a, 2014b, 2015). Therefore, further research is merited to understand both the potential for subtle mitonuclear incompatibilities in *F. heteroclitus* and the possibility that a northern mitochondrial genotype is beneficial for genetically admixed females at a cost to genetically admixed males.

5 | CONCLUSIONS

The results presented here provide insight into the genetic basis for variation in $\dot{M}O_{2routine}$, a trait that is probably consequential for fitness and is involved in the adaptive responses of organisms to changes in abiotic environmental conditions (e.g., temperature). Our study has demonstrated a highly polygenic basis for $\dot{M}O_{2routine}$ variation. Specifically, 43% of the interindividual variation in $\dot{M}O_{2routine}$ was attributable to 54 SNPs distributed throughout the genome in killifish. Epistatic interactions among these loci were common, indicating that interactions among trait-associated SNPs are an important component of the genetic architecture of $\dot{M}O_{2routine}$. In addition, consistent with previous observations in mammals and insects, our data suggest that the loci associated with variation in $\dot{M}O_{2routine}$ may at least partially depend on sex, and we observed a sex-specific effect of mitochondrial genotype on variation in $\dot{M}O_{2routine}$ in killifish from a genetically admixed population. Taken together, our findings highlight the immense complexity of the genetic basis for variation in $\dot{M}O_{2routine}$, show that adaptive responses involving this trait will probably proceed through polygenic adaptation, and suggest that sex-specific mitonuclear interactions may play a role in determining phenotypic variation, and possibly fitness, in admixed populations of Atlantic killifish.

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AUTHOR CONTRIBUTIONS

T.M.H., R.S.B., A.W. and P.M.S. designed the present study. T.M.H. collected the metabolic rate data and performed the analyses. R.S.B. performed the sequencing preparation and determined the SNP genotypes. A.W. and P.M.S. supervised the study. T.M.H. prepared the figures and tables, and all authors wrote and provided editorial feedback on the manuscript.

DATA AVAILABILITY STATEMENT

The sequencing reads used in the current study were obtained from the National Center for Biotechnology Information Sequence Read Archive available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA477712 (BioProject accession: PRJNA477712). Genetic variant call files and mitochondrial genotypes were obtained from the European Bioinformatics Institute BioStudy database available at https://www.ebi.ac.uk/biostudies/studies/S-BSST170 (BioStudy accession: S-BSST170). All other data are available as Supporting Information.

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