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Phenotypic and molecular consequences of stepwise temperature increase across generations in a coral reef fish

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Abstract

Global warming will have far-reaching consequences for marine species over coming decades, yet the magnitude of these effects may depend on the rate of warming across generations. Recent experiments show coral reef fishes can compensate the metabolic challenges of elevated temperature when warm conditions are maintained across generations. However, the effects of a gradual temperature increase across generations remain unknown. In the present study, we analysed metabolic and molecular traits in the damselfish *Acanthochromis polyacanthus* that were exposed to +1.5°C in the first generation and +3.0°C in the second (Step +3.0°C). This treatment of stepwise warming was compared to fish reared at current-day temperatures (Control), second-generation fish of control parents reared at +3.0°C (Developmental +3.0°C) and fish exposed to elevated temperatures for two generations (Transgenerational +1.5°C and Transgenerational +3.0°C). Hepatosomatic index, oxygen consumption and liver gene expression were compared in second-generation fish of the multiple treatments. Hepatosomatic index increased in fish that developed at +3.0°C, regardless of the parental temperature. Routine oxygen consumption of Step +3.0°C fish was significantly higher than Control; however, their aerobic scope recovered to the same level as Control fish. Step +3.0°C fish exhibited significant upregulation of genes related to mitochondrial activity and energy production, which could be associated with their increased metabolic rates. These results indicate that restoration of aerobic scope is possible when fish experience gradual thermal increase across multiple generations, but the metabolic and molecular responses are different from fish reared at the same elevated thermal conditions in successive generations.

KEYWORDS

acclimation, aerobic scope, climate change, gene expression, hepatosomatic index, transgenerational plasticity

1 | INTRODUCTION

The accumulation of greenhouse gases in the atmosphere is causing rapid warming of the oceans, and if the current emission trajectory is maintained, average ocean temperatures are predicted to increase between 2°C and 4°C by 2100 (RCP 8.5; IPCC, 2014). This substantial increase in water temperature could have far-reaching consequences for marine organisms, since most of them are ectotherms and their physiology is closely associated with thermal conditions of the surrounding environment (Doney et al., 2012; Hoegh-Guldberg

& Bruno, 2010; Laffoley & Baxter, 2016). This tight relationship between marine organisms and temperature leads to the expectation that future warming will result in population declines and shifts in the geographic distribution of many species (Poloczanska et al., 2013; Pörtner & Farrell, 2008; Sunday et al., 2015). However, some species might acclimate to environmental change through phenotypic plasticity (Munday, Warner, Monro, Pandolfi, & Marshall, 2013; Reusch, 2014; Schulte, Healy, & Fangue, 2011; Somero, 2010), which could help to offset the effects of climate change. Indeed, recent studies have revealed that metabolic compensation to warmer temperatures can occur in some marine fishes (Donelson, Munday, McCormick, & Pitcher, 2012; Salinas & Munch, 2012). Yet, these beneficial responses to higher temperatures depend on the length of exposure (Warren, Donelson, McCormick, Ferrari, & Munday, 2016), the life stage at which warming is experienced (Donelson, 2015) and the thermal conditions experienced by parents (Donelson et al., 2012; Salinas & Munch, 2012; Shama, Strobel, Mark, & Wegner, 2014; Shama et al., 2016). Further, most experimental studies conducted to date have exposed fish to projected future temperatures without considering the rate of warming within and between generations. Considering that changes in sea surface temperature will occur over years and decades, and consequently over multiple generations for most marine species, understanding the effects of warming across generations is crucial for making reliable predictions about the future impacts of climate change on marine ecosystems.

In the particular case of coral reef fishes, increases in average ocean temperature by a few degrees Celsius can result in adverse effects on the ecology, physiology and reproduction of multiple species (Munday, Jones, Pratchett, & Williams, 2008). Ocean warming can affect a range of traits including: developmental rate (McLeod, Jones, Jones, Takahashi, & McCormick, 2015), aerobic scope (Nilsson, Crawley, Lunde, & Munday, 2009), swimming capacity (Johansen & Jones, 2011), behavioural interactions (Allan, Domenici, Munday, & McCormick, 2015), reproductive output (Donelson, Munday, McCormick, Pankhurst, & Pankhurst, 2010) and sex determination (Rodgers, Donelson, & Munday, 2017). However, improvements in performance can occur when individuals are exposed to warmer water early in life (developmental plasticity: Donelson, Munday, McCormick, & Nilsson, 2011; Grenchik, Donelson, & Munday, 2013; Donelson, McCormick, Booth, & Munday, 2014), and when parents also experience the same thermal conditions as the offspring (transgenerational plasticity: Donelson et al., 2012; Donelson & Munday, 2015). Parents can potentially influence the performance of their offspring in stressful environmental conditions, through mechanisms such as transmission of beneficial epigenetic marks, nutrients, proteins, hormones or even through differences in parental care (Bonduriansky, Crean, & Day, 2012; Hofmann, 2017; Torda et al., 2017). Indeed, improvements in performance as a result of adaptive parental effects or transgenerational plasticity have been observed in a number of fish species, including pipefishes (Roth & Landis, 2017), sheepshead minnows (Salinas & Munch, 2012), skates (Lighten et al., 2016), three-spine sticklebacks (Shama et al., 2014, 2016) and tropical damselfish (Donelson et al., 2012). However, in the majority of multigenerational studies that evaluate the effects of climate change, end-of-the-century temperature projections are immediately applied in one generation and kept stable across generations, even though most species will experience ocean warming across multiple generations. It is important to understand the differences in phenotypic and molecular responses that might occur with stepwise temperature increase across generations, as a mismatch in temperatures between parents and progeny could hinder transgenerational plasticity (Shama et al., 2016). Alternatively, a stepwise temperature increase across generations may provide the opportunity for developmental plasticity to occur on top of transgenerational effects from a previous generation (Donelson, Wong, Booth, & Munday, 2016).

The present study focuses on the spiny chromis, *Acanthochromis polyacanthus*, a species of coral reef fish for which responses to temperature increase are well known. In the spiny chromis, elevated summer water temperature raises resting metabolic rate (Donelson et al., 2011; Nilsson et al., 2009), lowers aerobic scope (Donelson & Munday, 2012; Nilsson et al., 2009), reduces growth and condition (Donelson et al., 2011; Munday, Kingsford, O'Callaghan, & Donelson, 2008), decreases reproductive output (Donelson et al., 2010, 2016) and causes masculation of developing juveniles (Donelson & Munday, 2015). Yet, many of these traits, including aerobic scope, are restored at higher temperature if the individual's parents also experienced the same elevated temperature (i.e., +3.0°C in first and second generation; Donelson et al., 2012; Donelson & Munday, 2015). Analyses of gene expression have revealed that this aerobic compensation via transgenerational plasticity is accompanied by upregulation of genes related to metabolism, immune response and transcriptional regulation (Veilleux et al., 2015). Further, potential epigenetic mechanisms for transgenerational acclimation in *A. polyacanthus* have been detected, specifically differential methylation of genes involved in energy homeostasis, mitochondria activity, oxygen consumption and angiogenesis (Ryu, Veilleux, Donelson, Munday, & Ravasi, 2018). However, it remains to be determined if metabolic compensation occurs when there is a stepwise increase in temperature across generations (i.e., from +1.5°C in the first generation, to +3.0°C in the second).

In this study, we compared standard length (SL), weight, hepatosomatic index (HSI), oxygen consumption and patterns of liver gene expression of fish that experience a stepwise increase in temperature across generations (+1.5°C in the first generation, and +3.0°C in the second generation; Step +3.0°C) with: (a) fish maintained at present-day water temperature conditions across two generations (Control); (b) fish exposed to projected future ocean temperatures from hatching (Developmental +3.0°C); (c) and second-generation fish exposed to the same elevated temperature conditions as their parents (Transgenerational +1.5°C and Transgenerational +3.0°C; Figure 1). Liver was chosen for the analyses of gene expression as this tissue is closely associated with metabolic processes, and previous studies have successfully used it to understand response to elevated temperatures (Smith, Bernatchez, & Beheregaray, 2013; Kim, Costa, Esteve-Codina, & Velando, 2017). The samples analysed here correspond to a

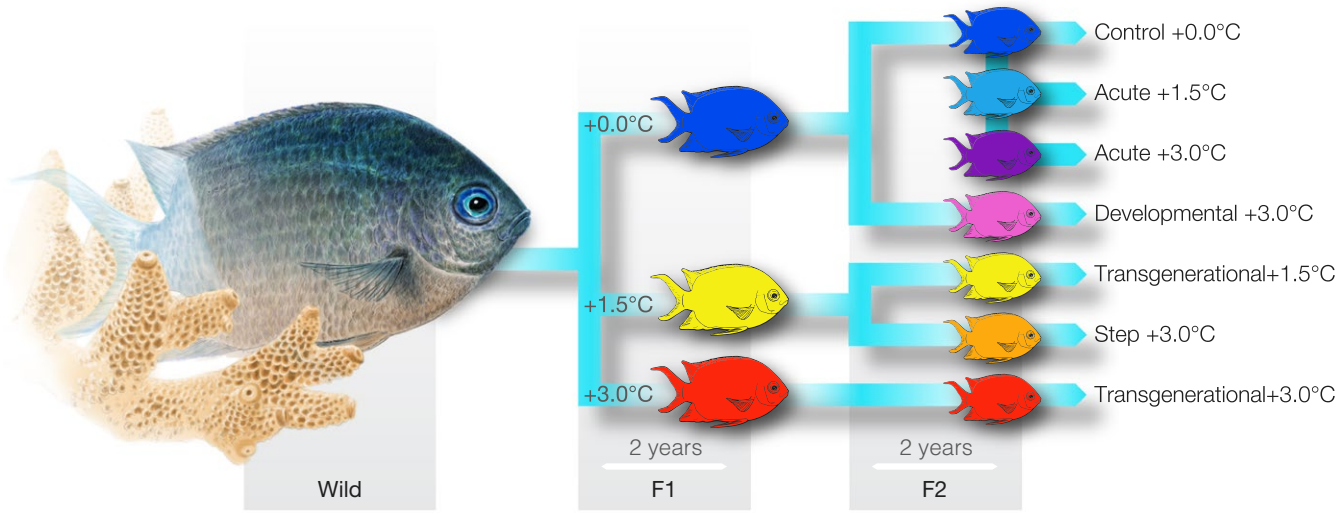

FIGURE 1 Experimental design depicting the cross generational thermal conditions for fish in each of the five F2 treatments: Control, Developmental +3.0°C, Transgenerational +1.5°C, Step +3.0°C, and Transgenerational +3.0°C. A portion of the F2 Control individuals were acutely exposed to elevated temperatures for seven days (Acute +1.5°C and +3.0°C), as reference of acute oxygen consumption [Colour figure can be viewed at wileyonlinelibrary.com]

multi-generational experiment first presented by Donelson et al. (2012), to understand thermal acclimation via transgenerational plasticity in a coral reef fish. This long running experiment is the basis for other projects aimed at understanding molecular mechanisms associated with metabolic compensation (Ryu et al., 2018; Veilleux et al., 2015), as well reproductive compensation at elevated temperatures (Donelson et al., 2016; Veilleux, Donelson, & Munday, 2018) in *A. polyacanthus*. The present study expands on the results of the previous research by exploring in detail the molecular and phenotypic response of a damselfish to stepwise warming, and by presenting new phenotypic data on previously examined treatments (HSI, SL and weight). Previous analyses including the Step +3.0°C treatment have been limited to investigating the effects of incremental warming on reproductive capacity (Donelson et al., 2016) or understanding the relationship between patterns of gene methylation and phenotypic traits (Ryu et al., 2018). By delving into the phenotypic and molecular changes that occur with temperature increases across generations, this study represents a more ecologically relevant approach for measuring the response of coral reef fishes to ocean warming over longer timescales.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The spiny chromis, *A. polyacanthus*, is common on reefs throughout Indonesia, Philippines, northern Australia and Melanesia (Randall, Allen, & Steene, 1997). It forms monogamous breeding pairs and is one of the few species of coral reef fish that possesses direct development, where individuals hatch as juveniles that remain close to the spawning site for several months (Robertson, 1973).

Nine established breeding pairs of spiny chromis were collected in August of 2007 (aged 2–8 years), from the Palm Island region (18°37′S, 146°30′E) in the central Great Barrier Reef, Queensland, Australia. The wild adult fish were maintained in aquaria at James Cook University, producing F1 offspring that were divided into three seasonally cycling temperature treatments shortly after hatching (Figure 1). The current-day Control treatment (+0.0°C) followed the average seasonal temperature cycle experienced by this damselfish at the collection site, where the average winter and summer temperatures are 23.2°C and 28.5°C, respectively (temperature records available at http://data.aims.gov.au/). The two elevated temperature treatments matched projections for ocean warming by the end of the century under moderate (+1.5°C) and high (+3.0°C) emissions scenarios (Hobday & Lough, 2011; IPCC, 2014). Thus, the summer temperatures in the +1.5°C and +3.0°C temperature treatments were 30.0°C and 31.5°C, respectively. The F1 individuals developed in these three treatments until they reached maturity (prior to the summer breeding season) at 2 years of age. All F1 fish were paired with another fish from an unrelated family. Between December of 2009 and February of 2010, the F1 pairs produced F2 juveniles which hatched at the same temperature as the parents. Newly hatched F2 juveniles from F1 Control parents either continued to be reared in control conditions, or were transferred to +3.0°C treatment conditions on the day of hatching to produce the Developmental +3.0°C treatment (Figure 1). Meanwhile, juveniles produced by F1 + 1.5°C parents either continued to be reared in +1.5°C conditions (Transgenerational +1.5°C) or were transferred to +3.0°C conditions on the day of hatching (Step +3.0°C). Finally, juveniles produced by F1 + 3.0°C parents were only reared at the same +3.0°C conditions (Transgenerational +3.0°C; Figure 1). F2 fish of all treatments were reared in the described condition for 2 years until testing the phenotypic and metabolic traits described below. At 2 years, a portion of the second-generation Control

fish were exposed to +1.5°C and +3.0°C (30.0°C and 31.5°C) for 7 days, to test the effect of elevated water temperature on metabolic rate without developmental or transgenerational exposure (Acute +1.5°C and +3.0°C treatments; only oxygen consumption was evaluated on them). In all cases where fish were moved between temperature treatments (either at hatching or for acute testing), the change of water temperature occurred over a period of 6 hr (i.e., 1.5°C or 3.0°C per 6 hr). The proportion of F2 fish analysed in this study that correspond to each F0 breeding pair (grandparents) are available on Supporting information Table S1 and Table S2.

2.2 | Phenotypic traits

Standard length (SL) in mm (Control $n = 20$, Developmental +3.0°C $n = 10$, Step +3.0°C $n = 8$, Transgenerational +1.5°C $n = 16$, and Transgenerational +3.0°C $n = 16$), total weight to nearest 0.01 g (Control $n = 21$, Developmental +3.0°C $n = 14$, Step +3.0°C $n = 9$, Transgenerational +1.5°C $n = 17$, and Transgenerational +3.0°C $n = 17$), and liver weight to nearest 0.01 g (Control $n = 14$, Developmental +3.0°C $n = 6$, Step +3.0°C $n = 5$, Transgenerational +1.5°C $n = 9$, and Transgenerational +3.0°C $n = 10$) were measured in randomly selected 2-year-old F2 individuals. In most of the comparisons, there was a similar number of males and females evaluated, with the exception of liver weight for Transgenerational +1.5°C, where the number of females ($n = 6$) was twice the number of males ($n = 3$). Hepatosomatic index (HSI), a measure of relative liver size across treatments, was calculated by dividing liver weight by the total weight. The Kruskal–Wallis test on ranks was used to determine differences across temperature treatments for length, weight and HSI, as the data were not normally distributed and the sample sizes were relatively small. When significant differences between treatments were found, Mann–Whitney U tests were used to assess pairwise differences in phenotypic measurements between two treatments, employing the Benjamini–Hochberg Correction for multiple comparisons.

Routine oxygen consumption ($MO_{2Routine}$) and maximum oxygen consumption (MO_{2Max}) were measured as a proxy for metabolic rate. Fishes were starved for 12–24 hr prior to testing to eliminate any effects caused by digestion. For $MO_{2Routine}$, fish were placed in 3.25L transparent plastic respirometers and allowed to acclimate for 1 hr with constant water flow prior to testing (Donelson & Munday, 2012; Donelson et al., 2011; Seebacher, Ward, & Wilson, 2013). Placement of fish into chambers involved gently corralling the fish allowing them to swim into the chamber, thus, there was no stress caused by handling or netting. This short habituation time has been found to be sufficient to achieve routine measurements in this species with this handling method (Supplementary Methods; Supporting information Figure S1). Following habituation, the chamber was sealed and oxygen concentrations were monitored for 30–40 min to determine $MO_{2Routine}$ (measured with Presens Fibox 3 non-invasive optical set-up). Static measures produce consistent and reliable measures of $MO_{2Routine}$ for this species, likely due to natural constant pectoral fin movement (Supplemental Methods; Supporting information Figure S1). All chambers were washed between uses to remove build-up of biological material. Background respiration was found to be negligible at all testing temperatures, and this was consistent with previous work showing it takes close to six hours for background respiration to have a significant effect (Rodgers et al., 2017). Measurements of MO_{2Max} were obtained for the same fish, at least 3 hours after $MO_{2Routine}$ measurements, by moving each individual to a circular swim chamber (2.75 L) set to the maximum aerobic swimming speed of the fish (Donelson & Munday, 2012; Seebacher et al., 2013). Each fish was swum for 10 min, and the steepest five minutes of the trial were used for analysis. The net aerobic scope (NAS) of each individual was obtained by subtracting the $MO_{2Routine}$ from the MO_{2Max}. For both trials, the respirometer was submerged in a temperature-controlled aquarium to maintain a stable temperature.

All fish were tested at the average summer temperature of their treatment (i.e., Control = 28.5°C, $n = 15$; Acute +1.5°C = 30.0°C, $n = 6$; Acute +3.0°C = 31.5°C, $n = 6$; Developmental +3.0°C = 31.5°C, $n = 7$; Transgenerational +1.5°C = 30.0°C, $n = 6$; Step +3.0°C = 31.5°C, $n = 6$; Transgenerational +3.0°C = 31.5°C, $n = 10$). Acute testing of Control fish at elevated temperatures allowed us to determine the presence or absence of metabolic compensation in developmental and transgenerational treatments. All oxygen consumption measures were corrected for temperature-dependent solubility of oxygen in seawater. The values of NAS for Control, Developmental and Transgenerational treatments have been previously presented in Veilleux et al. (2015) and are reported here for the purpose of comparing them with Step +3.0°C fish, shown here for the first time. The gender proportions (M:F) for all of fish tested were as follows: Control= 43:57, Acute +1.5°C = 50:50, Acute +3.0°C = 50:50, Developmental +3.0°C = 58:42, Transgenerational +1.5°C = 55:45, Step +3.0°C = 60:40 and Transgenerational +3.0°C = 53:47.

Due to the allometric relationship between metabolic attributes and fish mass, differences in $MO_{2Routine}$, MO_{2Max} and NAS between treatments were assessed using a Generalized Linear Model (GLM), with body weight as the covariate, adhering to the model's assumptions. Specifically, testing for homogeneity of variance was completed with Levene's test for which both $MO_{2Routine}$ and MO_{2Max} were not significant ($p > 0.05$). In the case of NAS, variation was examined further with both Cochran C and Hartley F-max tests, which were both found to be not significant ($p > 0.05$). Distribution adherence to model assumptions for all traits was checked with quantile–quantile plots. Considering the unbalanced nature of thermal treatments and testing temperatures, these two variables were combined into a single fixed factor (Control, Acute +1.5°C, Acute +3.0°C, Developmental +3.0°C, Transgenerational +1.5°C, Step +3.0°C and Transgenerational +3.0°C). The testing temperature used for the five aforementioned treatment factors was as follows: Control= 28.5°C, +1.5°C = 30°C or +3.0°C = 31.5°C. Weight (g) and metabolic attributes (mgO_2/hr) were log-transformed, in order to fit the linear relationship between the dependent traits and the covariate. Least square means for all traits and individuals were extracted and back transformed from log values for graphical presentation. When significant

differences between treatments existed, pairwise comparisons were done with Fisher's LSD. Statistical analyses were performed using SPSS statistics v23.0.0.2. The mean weight of fish in the analyses was 16.9 g (±3.61 g *SD*).

2.3 | Analysis of gene expression

Most of the sequences used for this study were reported in Veilleux et al. (2015) and are presented here only to compare them to the patterns of gene expression of Step +3.0°C fish (all sequences available at GenBank under BioProject ID PRJNA255544). For the library preparation, total RNA was extracted from F2 transgenerational Control (n = 5), Developmental +3.0°C (n = 5), Transgenerational +1.5°C (n = 4), Step +3.0°C (n = 4) and Transgenerational +3.0°C (n = 5, Figure 1) treatments. For most of the treatments, we analysed three males and two females, with the exception of Transgenerational +1.5°C and Step +3.0°C (one male and three females). Whole livers were homogenized (average weight for all treatments 0.27 mg) and samples were purified with PerfectPure kit (following the manufacturer's instructions) which includes treatment with DNAase. Final RNA extractions yielded between 0.5 and 1.5 μg of total RNA, with a minimum RNA integrity number (RIN) of 7.5, as recommended by Macrogen Inc, Korea. Libraries for paired-end fragments of 101 bp were prepared using the Illumina TruSeq protocol and sequenced at Macrogen Inc with an Illumina HiSeq2000. Raw sequences were processed for quality and adaptor removal using TRIMMOMATIC (Bolger, Lohse, & Usadel, 2014), retaining only paired-end fragments with a Phred score of 33 or higher, reads larger than 30 bp and sliding window trimming of 5:20. The analyses of liver gene expression of the present study use the genome of *A. polyacanthus* as reference, whereas Veilleux et al. (2015) used a de novo transcriptome. The genome was assembled using ABYSS v1.5.2 (Simpson et al., 2009), resulting in 993 Mb with an N50 of 334,400 bp, and the annotation of the 25,301 gene models was done against the NCBI nr, Eukaryotic nt and UNIPROT databases using BLAST2GO 3.1.2 (Schunter et al., 2016; Ryu et al., 2018; Conesa et al., 2005; NCBI Accession Number GCF_002109545.1). The BLAST2GO platform was also used to annotate each gene with their corresponding Gene Ontology (Gene Ontology Consortium; Ashburner et al., 2000). The resulting RNA-seq reads were mapped to the genome of *A. polyacanthus* using HISAT2 (Kim, Langmead, & Salzberg, 2015). Counts for each of the genes were obtained from the SAM files generated with HISAT2, using the function "FEATURECOUNTS" of the Subreads package (Liao, Smyth, & Shi, 2014). The minimum Mapping Quality Score, a measure of mapping confidence for paired-end reads, was set to 20, and read length was limited from 50 to 101 bp. Only fragments with both pairs successfully aligned to reference transcripts were considered for the analysis. All samples analysed in this study correspond to the same experiment and sampling scheme. Further, preparation of libraries and Illumina sequencing took place at the same time. Thus, we are confident there is no bias in measures of gene expression between Step +3.0°C and the rest of the temperature treatments.

Statistical analyses of the RNA-seq counts were performed in R 3.3.2 (R Development Core Team, 2016), using the program DESEQ2 version 1.18 (Love, Huber, & Anders, 2014). A likelihood ratio test (LRT) was used to identify differentially expressed genes (DEG) between the thermal treatments of the experiment (i.e., Control, Developmental +3.0°C, Transgenerational +1.5°C, Step +3.0°C and Transgenerational +3.0°C). The LRT evaluates the goodness of fit of a full model (differences between thermal treatments and within thermal treatments) versus a reduced one (differences within thermal treatments). DEG were those that passed a multiple test corrected significance cut-off of $p < 0.05$ (adjusted p-value) for at least one of the thermal treatments. A Principal Coordinate Analysis (PCoA) of all individuals was done with the DEG from the LRT, employing the variance stabilized data of the raw counts using the R Package VEGAN (Oksanen et al., 2017). To determine whether a particular gene had differential expression significantly distinct from zero between two conditions (adjusted $p < 0.05$), pairwise comparisons between thermal treatments were done with the "Contrast" function of DESEQ2. Cook's distances were estimated to detect any potential outlier read counts for each gene. The minimum number for outlier replacements was set to three, which means that an outlier could be replaced by the average number of counts when three or more replicates were available for that particular treatment (Love et al., 2014). If multiple samples contained Cook's distances above the estimated cut-off for a particular gene, the adjusted p-value would not be reported, and the specific gene would not be considered for downstream analyses. Heatmaps of the variance stabilized counts of loci with differential gene expression in pairwise comparisons (adjusted $p < 0.05$) were produced using the R package pHeatmap (Kolde, 2015).

To assess enrichment of Gene Ontology (GO) categories, we performed a Mann–Whitney U (GO-MWU) test, with a Benjamini–Hochberg correction (Wright, Aglyamova, Meyer, & Matz, 2015; scripts available at: https://github.com/z0on/GO_MWU). This analysis is based on the logarithm of the uncorrected p-values of the pairwise contrast of all genes, as the test performs better with raw measures (Wright et al., 2015). The uncorrected p-values of upregulated genes were forced to have a positive value (multiplying by −1), and downregulated genes were maintained negative. This particular test measures the distribution of GO categories at the bottom or top of the arrangement of log uncorrected p-values, while collapsing any redundant GO terms (those with more than >75% of similarity; Wright et al., 2015). Only categories that are represented by more than five GO terms were taken into consideration for the analyses. For each of the pairwise comparisons, the enrichment test was done separately for the three GO domains: Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). The output of the scripts is a table with p-values adjusted with the Benjamini–Hochberg correction for the enrichment assessment, and clustering distance graphs of the up- and downregulated GO categories, showing the categories with adjusted p-values of 0.01, 0.05 and 0.1 with different font sizes.

3 | RESULTS

3.1 | Phenotypic traits

Fish used in this study were between 60.82 and 83.18 mm (SL) and between 10.81 g and 26.84 g. There were no significant differences in fish length or weight between treatments (Kruskal–Wallis test, $df = 4$, $p > 0.05$; Supporting information Figure S2a,b). HSI differed significantly between temperature treatments (KW = 18.87, df = 4, $p < 0.05$; Figure 2). Specifically, all treatments in which fish were reared at +3.0°C had significantly higher HSI compared with current-day control fish (Developmental +3.0°C: $p = 0.019$, Transgenerational +3.0°C: $p = 0.004$, Step +3.0°C: $p = 0.004$), regardless of the parental condition. Furthermore, HSI in the Step +3.0°C ($p = 0.005$) and Transgenerational +3.0°C ($p = 0.019$) treatments was also significantly higher than Transgenerational +1.5°C fish (Figure 2).

The combination of rearing treatment and water temperature had a significant effect on $MO_{2Routine}$ ($F_{6,68} = 3.254$, $p = 0.007$; Figure 3a) and NAS ($F_{6,46}$=2.774, $p = 0.022$; Figure 3b), but not MO_{2Max} ($F_{6,46} = 1.185$, $p = 0.33$; Supporting information Table S3; Figure S3). Significant differences in relation to Control were observed with Acute +3.0°C ($MO_{2Routine}$ $p = 0.001$; NAS $p = 0.016$) and Developmental +3.0°C ($MO_{2Routine}$ $p = 0.006$; NAS $p = 0.037$). In contrast, no significant differences were observed when Control was compared to Acute +1.5°C ($MO_{2Routine}$ $p = 0.163$; NAS $p = 0.075$), Transgenerational +1.5°C ($MO_{2Routine}$ $p = 0.511$; NAS $p = 0.702$) or Transgenerational +3.0°C ($MO_{2Routine}$ $p = 0.287$; NAS $p = 0.504$; Figure 3). Step +3.0°C fish showed significantly higher $MO_{2Routine}$ than Control ($p = 0.037$), yet the stepwise treatment was not distinct from Transgenerational +3.0°C ($p = 0.205$; Figure 3a). Restoration of NAS was observed in Step +3.0°C fish as values were not significantly different to Control fish ($p = 0.504$). Step +3.0°C NAS was also similar to Transgenerational +3.0°C fish ($p = 0.862$; Figure 3b), which could be due to a slightly higher, but not significant, MO_{2Max} in Step +3.0°C with respect to Control ($p = 0.722$; Supporting information Figure S3).

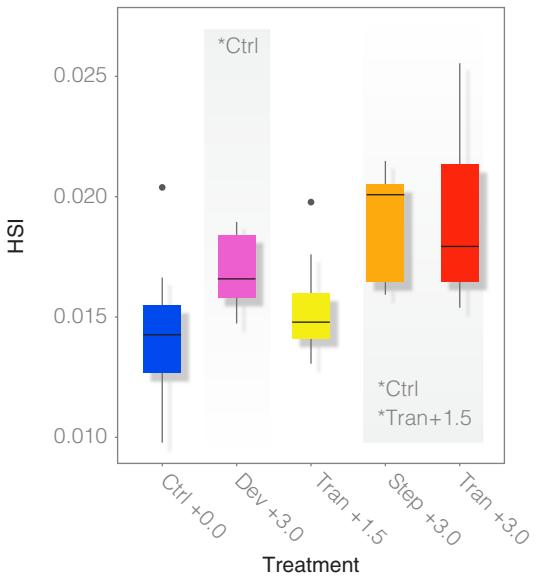

FIGURE 2 Boxplots of the hepatosomatic index of fish at different thermal treatment. Significant differences ($p < 0.05$) estimated with a Mann–Whitney U tests are indicated on the figure with an asterisk [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Analysis of gene expression

After quality filtering of reads and removal of adaptors, the average percent of mapping to the reference genome was 89%, which represents a slight increase in the average efficiency of mapping with respect to the denovo transcriptome (82%). In total, 26,183 transcripts had counts for expression, 91% of them were successfully annotated to gene level, while 56% had annotations to GO categories.

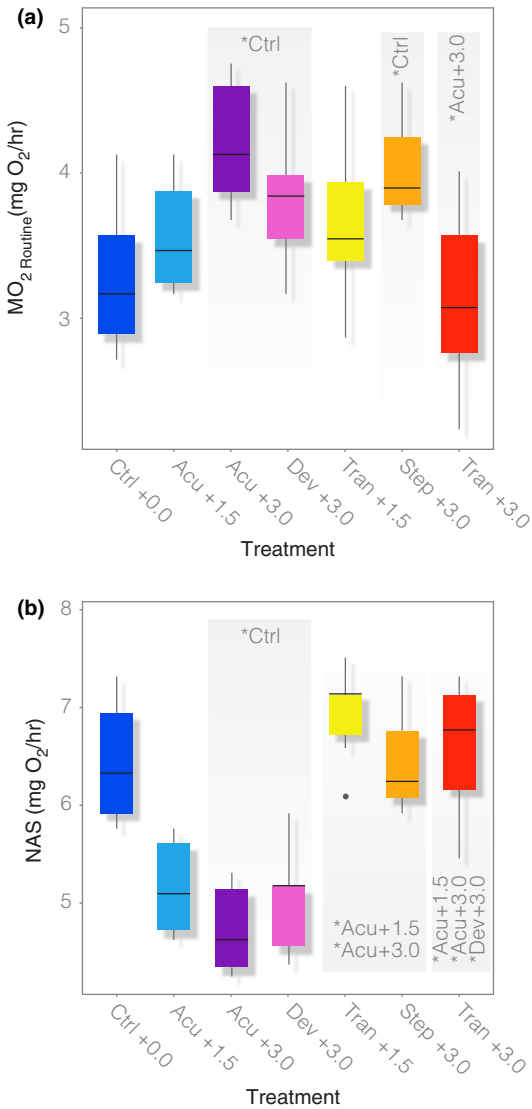

FIGURE 3 Boxplots of routine oxygen consumption (MO2Routine; a) and net aerobic scope (NAS; b) for the different thermal treatments. Values are mass corrected least squared means of oxygen consumption (mg O_2/hr) for each treatment adjusted for the covariate individual weight (g). Significant differences from Fishers LSD post hoc are indicated with an asterisk ($p < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

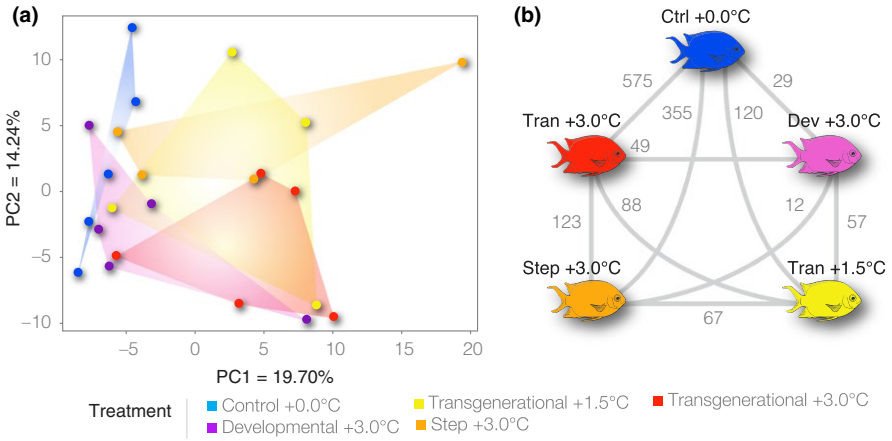

FIGURE 4 Principal coordinate analysis of normalized read counts from significant genes with the LRT (adjusted $p < 0.05$; a) and number of differentially expressed genes resulting from the pairwise comparisons between thermal treatments (b) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Gene Ontology categories that were most up- and downregulated for the comparisons of Step +3.0°C with Control and Transgenerational treatments, according to the GO-MWU ranks test (10% FDR)

	Biological process	Cellular component	Molecular function
Control			
Up	Lipoprotein Metabolic Process (4/10)—*10*	Mitochondrion (7/15)—*3*	Glutathione peroxidase activity (2/7)—*9*
Down	DNA Replication (8/49)—*6*	*None*	Phosphatase Regulator Activity (2/11)—*10*
Transgenerational +1.5°C			
Up	Electron Transport Chain (2/6)—*12*	Proton-transporting ATP synthase complex, catalytic core F (2/5)—*6*	NADH dehydrogenase (quinone) activity (3/5)—*9*
Down	rRNA processing (2/12)—*7*	Na/K exchanging ATPase Complex (1/7)—*4*	protein phosphatase type 2A regulator activity (1/6) —*10*
Transgenerational +3.0°C			
Up	Electron Transport Chain (3/6)—*38*	Prefoldin Complex (4/9) —*17*	Oxidoreductase activity, acting on NAD(P)H, quinone or similar (7/13) —*9*
Down	DNA replication (11/49)—*3*	Chromosome, centromeric region (2/5)—*8*	Galactosyltransferase activity (1/18)—*1*

Notes. Numbers in parenthesis indicate the number of genes found in this analysis, over the total number of genes for that particular category. The total number of up- and down-regulated GO categories for each of the comparisons is italicized.

The average number of read counts per sample in the analysis of gene expression was 28,883,208 before normalization. The LRT revealed 889 genes were significantly differentially expressed (adjusted $p < 0.05$) in at least one of the comparisons between treatments. The greatest differences in gene expression were between individuals in the Transgenerational (both +1.5°C and +3.0°C) and Step +3.0°C treatment with respect to Control (Figure 4). The first principal component accounted for 19.70% of the variance, while the second principal component explained 14.24% of the variance (Figure 4a). This result is not unusual for analyses that include multiple treatments (five in this case) that have a sizeable effect on the patterns of gene expression of the studied samples.

Pairwise comparisons indicated significant differences in gene expression between Step, Developmental and Transgenerational treatments (Figure 4b), which also resulted in multiple enriched categories in the GO-MWU rank test (Table 1). The number of DEGs between Step and Control was 355 (Supporting information Figure S4), reflecting the differences in developmental temperature (+3.0°C vs. +0.0°C) and the parental conditions (+1.5°C vs. +0.0°C). There were 16 enriched categories for Biological Process (BP), 3 for Cellular Component (CC) and 19 for Molecular Function (MF) for the comparison of Step +3.0°C with Control (Supporting information Figure S5). Step +3.0°C fish showed upregulation for GO terms related to lipid transport, mitochondrial activity, signalling and ion transport. Meanwhile, downregulated GO categories were related to RNA processing, DNA replication, oxidation-reduction and catabolic processes. Furthermore, a key sensor of energy levels, X5 AMP Activated Protein Kinase Subunit Beta 2 (*PRKAB2*), was upregulated in the Step +3.0°C treatment compared with Control. There were multiple genes upregulated in Step +3.0°C which are related to inflammation, apoptosis and tumour suppression (Supporting information Table S4), potentially as a result of the increase in HSI with respect to Control. Interestingly, the gene Growth Arrest and DNA Damage-Inducible Protein (*GADD45*), which modulates cellular response to stress, was upregulated in Step +3.0°C fish with regard to all other treatments (Control, Developmental +3.0°C, Transgenerational +1.5°C and +3.0°C).

There were only 12 DEGs between Step +3.0°C and Developmental +3.0°C. The relatively low number of DEG is perhaps surprising, because these treatments differed in parental conditions (+0.0°C vs. +1.5°C) even though the fish developed at the same temperature (+3.0°C; Figure 1). Because of the small number of DEGs, we did not perform the additional analysis for enrichment of GO categories. Further, the heatmap did not separate fish from the two treatments consistently, as was the case for all other comparisons, so these differences were not further evaluated in our study. Two genes that are involved in tolerance of thermal shock (Warm Temperature Acclimation Related 65da protein, *WAP65*; Atrial Natriuretic Peptide Receptor, *NPR1*) were downregulated in Step +3.0°C with respect to Developmental +3.0°C, a result in-line with the parental conditions of the latter. Interestingly, the comparison between Developmental +3.0°C and Control resulted in 29 DEGs, also a relatively small number when compared to other treatments (Figure 4b; Supporting information Table S4). In this comparison, 10 of the upregulated genes in Developmental +3.0°C were also upregulated in Step +3.0°C with regard to Control (Supporting information Table S4), although none of them were associated with inflammation, apoptosis and tumour suppression. The relatively low number of DEG between Step +3.0°C and Developmental +3.0°C, as well as the overlap in upregulated genes with regard to Control, suggest similar overall patterns of liver gene expression between fish of these treatments. This is most likely caused by the fact that both treatments had differences in thermal conditions between parents and offspring (+0.0°C to +3.0°C for Developmental; +1.5°C to +3.0°C for Step), and that juveniles of both treatments developed at +3.0°C.

We observed 67 DEGs between the Step +3.0°C and Transgenerational +1.5°C (Figure 5a), leading to enrichment of 10 GO terms in CC, 19 for MF, and 22 on BP on the MWU ranks test. These results indicate differences between siblings that developed at different conditions, but with parents under the same temperature (+1.5°C). Gene categories such as oxidoreductase activity, ATPase activity, hydrogen ion transport and mitochondrion were upregulated in Step +3.0°C (Supporting information Figure S6). Additional genes upregulated in Step +3.0°C were related to response to thermal stress, apoptosis and inflammation, cholesterol and toxin metabolism, as well as mitochondrial activity. Similar to the comparison with Control fish, GO categories downregulated in Step +3.0°C included RNA processing, nucleic acid metabolic processing, cytoskeleton and helicase. Further, the gene *PRKAB2*, which was upregulated in Step +3.0°C compared to Control, was also upregulated in Step +3.0 in this comparison.

There were 123 DEGs between the Step +3.0°C and Transgenerational +3.0°C, showing the differences between a stepwise increase to +3.0°C over two generations versus fish subject to constant warm conditions (Figure 5b). The rank test showed the highest number of upregulated GO terms in this comparison: 41 for BP, 25 for CC and 10 for MF (Supporting information Figure S7). Step +3.0°C fish showed upregulation of multiple genes related to the mitochondrial activity, components of the respiratory chain, ion transport and biosynthesis of organic compounds (Supporting information Figure S7; Table S4). As seen in other comparisons, genes associated with DNA replication and transcription were downregulated in Step +3.0°C, as well as genes that regulate the formation of intracellular components. Both Step +3.0°C and Transgenerational +3.0°C showed upregulation of different genes related to inflammation and apoptosis, which is potentially associated with the increase in HSI with respect to other treatments. Finally, when Step +3.0°C and Transgenerational +3.0°C were compared to control, there was an overlap of 32 upregulated genes, including genes that regulated the metabolism of carbohydrates, repair after stress response, and respiration (Supporting information Table S4).

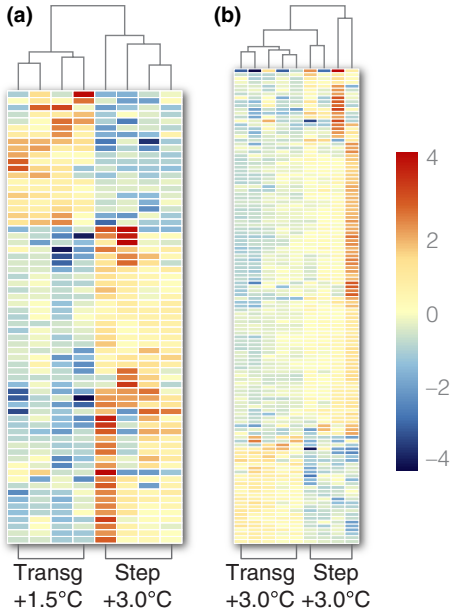

FIGURE 5 Heatmaps for comparisons of the Step +3.0°C treatment with Transgenerational +1.5°C (a) and Transgenerational +3.0°C (b). The color scale is the fold change relative to a gene's mean expression. Red and blue colors represent higher and lower expression, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

In this study, we explored the differences in physical condition, aerobic metabolism and gene expression of second-generation *A. polyacanthus* from parental lineages exposed to the average sea surface temperature of the central Great Barrier Reef, Australia, and projected temperatures for the end of the century under moderate (+1.5°C) and high (+3.0°C) warming scenarios. To better understand the phenotypic and molecular consequences of incremental warming across generations, we included a treatment with a stepwise temperature increase across generations, where parents were reared at +1.5°C and their progeny at +3.0°C. Our results indicate that Step +3.0°C fish exhibited partial compensation of $MO_{2Routine}$ and full restoration of NAS, whereas full restoration of both $MO_{2Routine}$ and NAS was observed in both transgenerational treatments (+1.5°C and

+3.0°C; Donelson et al., 2012; Veilleux et al., 2015). In conjunction with these metabolic changes, fish from the Step +3.0°C treatment showed upregulation of multiple genes related to mitochondrial activity, cellular respiration and stress response when compared to Control and Transgenerational fish, suggesting there are energetic cost and shifts in cellular processes as a result of gradual temperature increase across generations.

4.1 | Developing at warm temperatures led to liver enlargement

We observed an increase in the size of the liver relative to body size for all fish that developed in +3.0°C conditions: Developmental +3.0°C, Step +3.0°C and Transgenerational +3.0°C. These results are in-line with observations of the F1 generation, where breeding fish that developed in +3.0°C had higher HSI than sibling fish that developed in +0.0°C and +1.5°C (Donelson et al., 2012). Liver enlargement in fish has traditionally been associated with an increase in lipid storage, which is why the HSI is generally considered an indicator of energy reserves (Wootton, Evans, & Mills, 1978). However, observations in *A. polyacanthus* have shown that fat is mostly accumulated in the visceral region (Donelson et al., 2010); therefore, an enlarged liver may not be an indicator of lipid storage in this species. Considering that the liver is involved in a broad range of processes, such as digestion, detoxification, immune defence, nitrogen catabolism and reproduction (Brusle & Anadon, 1996), liver enlargement of the spiny chromis is most likely due to processes required for coping with elevated thermal conditions and metabolic stress.

The differences observed in HSI between Step +3.0°C and Control fish were accompanied by upregulation of multiple genes related with inflammation, apoptosis and tumour suppression. For example, we found upregulation of genes involved in attracting lymphocytes to the endothelium of hepatic cells (Chemokine *CCL4*; Menten, Wuyts, & Van Damme, 2002) and in mediating the expression of Toll-like Receptor and NF-Kb complex (*BCL10;* Mazzone et al., 2015). NF-Kb complex modulates the inflammatory genes *SPP2* and *G0S2* (upregulated in Step +3.0°C), which alter the permeability of the mitochondria, promoting apoptosis (Welch et al., 2009). Apoptosis is also controlled by the upregulated *TRIM39*, a gene which activates the Modulator of Apoptosis Protein (Huang et al., 2012). Changes in the membrane of the endoplasmic reticulum also lead to apoptosis, which is influenced by the upregulated gene *IKBIP* (Hofer-Warbinek et al., 2004). Further, 10 genes involved with the mediation of cell proliferation after inflammation and deactivation of reactive oxygen species after apoptosis were also upregulated in Step +3.0°C fish. However, this should be interpreted with caution, as many of these molecular pathways and their corresponding functions have been thoroughly described for mammals, and less so for fishes.

Interestingly, the gene *GADD45* was upregulated for Step +3.0°C in every pairwise comparison. This gene represents an essential checkpoint in the G2 phase of mitosis, being able to inhibit the genomic instabilities associated with tumours (Hollander & Fornace, 2002). Upregulation of this gene has been reported in individuals subject to chemical (turbot; Ruiz, Orbea, Rotchell, & Cajaraville, 2012) and osmotic stress (salmon; Maryoung et al., 2015) for extended periods of time. It is possible that this DNA repair gene is acting as a regulator of cell proliferation in Step +3.0°C after gradual temperature increase across generations.

Genes with similar functional categories, including inflammation, apoptosis and repair after stress response, were also differentially expressed in Transgenerational +3.0°C when compared to Control, supporting the idea that an enlarged liver is occurring in conjunction with a change in gene expression of said functional categories. However, the same trends in gene expression were not observed for the Developmental +3.0°C, even when this treatment still exhibited a significant increase of HSI. This may be due to the fact that Developmental +3.0°C fish possessed an intermediate increase in HSI between the Control and other +3.0°C treatments (Step and Transgenerational).

4.2 | Effects of temperature increase on metabolism

The metabolic rates of both Acute and Developmental treatments followed the established trend for several coral reef fishes that temperatures above the summer average cause an increase in $MO_{2Routine}$, and a decrease in the NAS (Donelson et al., 2012; Nilsson et al., 2009; Rummer et al., 2014). As observed previously, fish in the Transgenerational +3.0°C treatment restored their NAS to levels similar to Control fish at +0.0°C (Veilleux et al., 2015). Here, we show that a stepwise increase in temperature across generations (Step +3.0°C) also enables restoration of NAS. However, in contrast to the Transgenerational +3.0°C treatment, Step +3.0°C fish had a significantly higher $MO_{2Routine}$ when compared to Control. These results expand on our previous knowledge that stepwise warming across generations results in differing phenotypic responses in reproductive ability (Donelson et al., 2016), to show that there are also differing effects on aerobic metabolism compared with fish exposed to the same elevated temperature across generations. Thus, stepwise warming across generations allows restored aerobic scope and reproductive ability, but with the cost of higher basal metabolic demand. Further, it is possible that maintaining a larger liver when developing at elevated temperatures can also lead to higher metabolic costs (Hulbert & Else, 2000; Rolfe & Brown, 1997).

The higher metabolic rates of Step +3.0°C fish correlated with the activation of molecular pathways associated with energy metabolism. For example, we observed upregulation of GO terms related to mitochondria in Step +3.0°C, with regard to Control, Transgenerational +1.5°C and +3.0°C, including genes associated with the respiratory chain, and the formation and activation of cytochrome c oxidase. The high demand for energy was associated with changes in the catabolism of Step +3.0°C fish, as shown in the upregulation of *PRKAB2* with regard to Control and Transgenerational +1.5°C. This gene is a subunit of the AMPK sensor that responds to energy levels in the cell, being activated by binding to AMP when levels of ATP are low (Sanders, Grondin, Hegarty, Snowden, & Carling,

2007) and was also found to be upregulated in livers of goldfish subject to hypoxia (Jibb & Richards, 2008). In a similar way, *LAMTOR4*, a component of the Ragulator complex which senses energy levels and the presence of amino acids in the cell, was upregulated in Step +3.0°C compared to Transgenerational +3.0°C. This gene is part of a signalling pathway that controls cell growth, translation, transcription and lipid synthesis in vertebrates (Zhang et al., 2014). The activation of these two energy sensing genes in Step +3.0°C fish, along with the downregulation of GO categories related with RNA processing and DNA replication, indicates that Step +3.0°C individuals are shifting their energy investment during the process of compensating their metabolism to elevated temperature.

Taken together, these results indicate *A. polyacanthus* compensates for the effects of high temperature on aerobic metabolism differently when F2 fish reach elevated temperatures over two generations (Step +3.0°C treatment), compared to fish that experience higher temperatures consistently from the first generation (Transgenerational +1.5°C and +3.0°C treatments). Differences in the gene expression comparisons of Step +3.0°C with Control and Transgenerational +1.5°C are most likely due to the contrasting developmental and parental conditions of these treatments. However, differences between second-generation fish of Step +3.0°C and Transgenerational +3.0°C could also be influenced by selection that occurred in the F1 generation. A limited number of F1 pairs were capable of reproducing at +3.0°C, even when they developed in the elevated temperatures from hatching (Donelson et al., 2012, 2014). Ultimately, three out of four F1 breeding pairs were composed by an individual that came from the same F0 breeding pair (Donelson et al., 2012, 2014), suggesting that the F1 pairs that managed to reproduce at +3.0°C could represent genotypes that are better adapted to warmer conditions. In contrast, a larger number of F1 breeding pairs from a diversity of F0 genetic lineages reproduced in the +1.5°C condition (i.e., the F1 parents of Step +3.0°C fish). Moreover, some of the observed differences in this comparison could be related to an imbalance of the sexes analysed for Step +3.0°C fish (three females, one male) and Transgenerational +3.0°C (three males, two females). Previous studies have found differences in the metabolic and molecular response of fishes to temperature increase between males and females (McCairns, Smith, Sasaki, Bernatchez, & Beheregaray, 2016; Muñoz, Breckels, & Neff, 2012), yet these differences have not been directly evaluated in *A. polyacanthus*. Therefore, we cannot entirely rule out that the differences between Step +3.0°C and Transgenerational +3.0°C offspring used in our experiment could be influenced by a combination of developmental/transgenerational conditions, selection of genotypes able to reproduce at elevated temperatures and the gender imbalance of the analysed samples.

4.3 | Comparing stepwise and developmental treatments

The differences in the restoration of NAS between Step +3.0°C and Developmental +3.0°C fish observed here, as well as previous research on stepwise fish's reproductive performance (Donelson et al., 2016), indicate that fish have a greater capacity for coping with higher temperatures when their parents are exposed to elevated temperature, even if parental and offspring temperatures are not the same. Nevertheless, there was a relatively small number of DEGs between Step +3.0°C and Developmental +3.0°C fish. This was somewhat unexpected, but could be related to the similarities in the timing of exposure to warmer water: fish from both treatments were transferred from their parental treatments (+1.5°C or +0.0°C) to +3.0°C at exactly the same stage of development (within a few hours of hatching). Thus, it is possible that Step +3.0°C and Developmental +3.0°C fish are both reflecting the effects of developmental exposure during initial life stages as a result of a warmer environment. Further, it is important to recognize that this study was limited to an analysis of gene expression of the liver, and the expression of other tissues such as brain, gonads or heart could show more differences between developmental and stepwise treatments.

Despite the small number of DEGs between fish of these treatments, the upregulated genes in the Developmental +3.0°C provide information on the response of fish that developed at +3.0°C and had Control parents. Two notable examples are *NPR1* and *WAP65*. *NPR1* regulates sodium ions in the plasma, playing a large role in acid-base regulation (Tsukada, Rankin, & Takei, 2005), and has been associated with the capacity of cyprinids to acclimate to harsh environments (Xu et al., 2016). *WAP65* has been associated with seasonal acclimation to warm temperature in several fishes (goldfish, Kikuchi, Yamashita, Watabe, & Aida, 1995; mumichog killifish, Picard & Schulte, 2004; sweetfish, Li & Chen, 2013). This protein has high affinity to heme, and its main function is to transport this co-factor to the liver and sequester reactive oxygen species generated as a reaction to a particular stressor (Diaz-Rosales, Pereiro, Figueras, Novoa, & Dios, 2014). The *WAP65* gene was also upregulated in Transgenerational +3.0°C with regard to Step +3.0°C. It may be that this gene is upregulated when a relatively large temperature change occurs within a single generation, and the upregulation in Transgenerational +3.0°C fish is related to the fact that their parents were Developmental +3.0°C fish.

5 | CONCLUSIONS

In this study, we examined the challenges that fish will face with projected climate change over upcoming decades. The experimental design was such that it allowed us to identify the consequences of temperature increase across generations, compared with an increase in temperature within a generation and the maintenance of elevated temperatures across generations. The analysis of metabolic rates revealed that Step +3.0°C fully recovered aerobic scope, when compared to Control fish. However, offspring subjected to a stepwise treatment showed a significantly higher routine oxygen consumption, which was accompanied by upregulation of genes associated with energy production and cellular respiration, leading to changes in the catabolism and DNA replication machinery. This suggests that metabolic compensation is possible if fish experience warmer water than their parents, but

the physiological process is not the same as in fish that develop in the same elevated temperature conditions across generations. The results of this study confirm that for a tropical damselfish acclimation to warming oceans via plasticity will depend on the conditions at which the parents develop, as well as on the magnitude and rate of temperature increase across generations.

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DATA ACCESSIBILITY

The *Acanthochromis polyacanthus* draft genome used here as reference for the analysis of gene expression is available at: https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Acanthochromis_polyacanthus/100/. RNA-seq sequences of all individuals are available on GenBank under BioProject ID PRJNA255544: https://www.ncbi.nlm.nih.gov/bioproject/255544

AUTHORS' CONTRIBUTIONS

J.M.D., P.L.M. and H.D.V. conceived the project; J.M.D. managed and performed the experiments and gathered phenotypic measures; H.D.V. and T.R. managed the sample processing for library preparation as well as the transcriptomic sequences; M.A.B., J.M.D. and T.R. analysed the data; M.A.B., J.M.D., P.L.M. and T.R. wrote the paper. All authors provided intellectual input and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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