Molecular plasticity contributes to thermal resilience in two coastal fish species

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Abstract

Understanding species capacities to adjust to shifting thermal environments is crucial amidst current climate-mediated ocean warming. Fish populations displaying high thermal plasticity can undergo molecular, metabolic, and mitochondrial modifications in response to heat stress. Under the context of heat stress, such acclimation provides a means to maintain normal biological functions through alteration of thermal performance and provides a model to dictate which species will persist when this stress becomes prolonged. Here we combine measures of mitochondrial physiology (using a novel fluorescent technique) and gene expression analyses to investigate thermal resilience and acclimation capacity of two closely related endemic triplefin species, the intertidal common triplefin (*Forsterygion lapillum*) and the estuarine triplefin (*F. nigripenne*). Triplefins are an ideal evolutionary model to explore the molecular basis of thermal resilience. Both species evolved in thermally variable environments and are thus predicted to display resistance to heat stress. We observed enhanced mitochondrial function at higher temperatures, although only ATP production was significantly enhanced for both species.

Different gene expression profiles were detected between warm acclimated and control fish, with high interspecific variation in acclimatory responses across brain transcriptomes. Differential gene expression and gene ontology highlighted an induction of stress response pathways and oxidoreductase activity in warm acclimated tissues, alongside a rearrangement of metabolic functions facilitating increased carbohydrate metabolism. Our findings indicate thermal acclimation potential in both species, with plasticity in mitochondrial performance enhancing upper thermal tolerance and transcriptional evidence of thermal compensation and homeostatic adjustments under warming conditions. Overall, these results demonstrate robust mechanisms of resilience in coastal fish species that have evolved under climatic variable conditions and provide a new methodological approach for future thermal studies.

Introduction

Recent human activities have resulted in significant energy imbalances in the earth's climate system, and much of that energy imbalance manifests within the global oceans (Cheng et al., 2019; Schuckmann et al., 2023; Venegas et al., 2023). Marine environments are changing in multiple and dramatic ways, testing the capacity of marine species to withstand and acclimate to suboptimal conditions (Anderson et al., 2012; Morley et al., 2019). Temperature sits at the forefront of these anthropogenically driven effects, acting to modify physiological parameters within marine organisms (Miller & Stillman, 2012; Bates & Morley, 2020). For marine ectotherms to undergo thermal acclimation, they must exhibit plasticity in physiochemical and cellular traits, allowing biological function to be maintained in adverse environmental conditions (da Silva et al., 2019; Anderson et al., 2012; Seebacher et al., 2015). Central to the thermal performance of ectothermic organisms are mitochondria, providing the cellular energy (ATP) required for almost all biological processes to function (Little et al. 2020; Pichaud et al. 2017).

With the brain being the largest consumer of ATP in vertebrate bodies, brain mitochondria can produce ATP at a surplus, providing a physiological advantage when the homeostasis of biological systems is disrupted, and the energetic demands required to maintain these systems increase (Willis et al., 2021). As a result, it is believed that the brain is one of the principal organs dictating thermal limits in fish (Biederman et al., 2019; Ern et al., 2023). Yet, there have been very few studies investigating thermal plasticity of specific mitochondrial properties in brain mitochondria, such as those directly assessing the maintenance of mitochondrial membrane potential or ATP equilibrium dynamics (see Willis et al., 2021). Thermal limits in the brain and aerobic tissues are largely determined by the thermal plasticity of mitochondrial traits and functions (Iftikar et al., 2015; Iftikar & Hickey, 2013; Hilton et al., 2010). For instance, intertidal triplefin species that experience daily temperature fluctuations have more stable and efficient brain and heart mitochondrial function at higher temperatures relative to deeper-subtidal species where temperatures are more stable (Willis et al., 2021; Hilton et al., 2010). However, the molecular processes underlying thermal resilience are poorly understood.

A powerful technique for inferring thermal acclimation and physiology of fish is transcriptomics (Smith et al., 2013; Qian et al., 2014). Application of genomic and transcriptomic technology can highlight which specific genes and pathways provide thermal resilience in warm acclimated fish and how these change between species and populations (Bilyk & Cheng, 2014; Qian et al., 2014; Sandoval-Castillo et al., 2020). Transcriptomic research targeting thermal acclimation in liver, gill and muscle tissues has revealed significant alterations in genes involved in metabolic and cellular stress response pathways (Podrabsky & Somero, 2004; Kim et al., 2021; Harms et al., 2014; Newton et al., 2012; Momoda et al., 2007; Buckley et al., 2006). Despite the brain's importance in determining thermal resilience it has been the focus of fewer studies (but see Pan

et al., 2024; Miller & Stillman, 2012; Li et al., 2024; Bernal et al., 2022). Furthermore, comparative transcriptomics can highlight stress and acclimatory responses genetically conserved across different species (Ellison et al., 2020; Shin et al., 2012; Sandoval-Castillo et al., 2020). Although most current applications explore expression across fish from different habitat ecotypes or diets (Herrera et al., 2022; Narum & Campbell, 2015), by studying the transcriptional responses of closely related fish species to elevated temperatures, candidate genes and gene pathways representative of thermal acclimation can be identified, as demonstrated in transcriptional plasticity comparisons of two invasive goby species (Wellband & Heath, 2017) and of rainbowfish species from different bioregions (Sandoval-Castillo et al., 2020). Any observed differences in expression patterns broadens our understanding of the different mechanisms for and limitations of thermal acclimation across species and populations (Narum & Campbell, 2015; Wellband & Heath, 2017).

Here we combine mitochondrial fluororespirometry and RNA-seq analyses to investigate the underlying mechanisms of thermal resilience in the common triplefin (*Forsterygion lapillum*) and its sister species, the estuarine triplefin (*Forsterygion nigripenne*), by assessing their capacity to acclimate to elevated temperature. Triplefins present an ideal evolutionary model to explore the molecular basis of thermal resilience – 26 species have evolved through an adaptive radiation into distinct ecotypes that occupy different habitats (e.g., rockpool, estuarine, deep reef, pelagic) (Wellenreuther et al., 2007; Feary & Clements, 2006; Hilton et al., 2008; Hickey et al., 2009; Wellenreuther et al., 2008). Triplefin species from intertidal habitats have more stable and efficient brain and heart mitochondrial function at higher temperatures relative to deeper-subtidal species (Willis et al., 2021; Hilton et al., 2010), but it is not yet understood how these closely related species adjust or adapt to rising and variable water temperatures. Whilst previous

research indicates high thermal tolerance in the common triplefin based on metabolic scope and brain ATP dynamics (Khan et al., 2014; McArley et al., 2017; Willis et al., 2021), this study represents the first examination of the thermal physiology of the estuarine triplefin, and the first transcriptomic analyses conducted on either species. Both study species inhabit thermally fluctuating environments along New Zealand's coastlines (Feary & Clements, 2006; Wellenreuther et al., 2007). Given that phenotypic plasticity is typically higher in species from thermally variable compared to stable environments (Bhat et al., 2015; Sandoval-Castillo et al., 2020; Janzen, 1967), we hypothesised that both species would demonstrate high acclimation potential and tolerance. We specifically assessed the thermal compensation of mitochondrial respiration and transcriptional activity, the latter highlighting pathways of gene expression change under elevated temperatures. Few studies have investigated ATP equilibrium dynamics or the maintenance of mitochondrial membrane potential in terms of thermal acclimation and plasticity. We use a novel and low-cost fluorescent approach to assess the performance of these functions under acute heat shock. With this technique, we can directly measure the upper limits of individual mitochondrial properties without specialist physiological equipment. We predicted that both inner membrane potential and ATP equilibrium would be maintained at higher temperatures in the brain mitochondria of warm acclimated triplefins. Considering previously observed genetic differences associated with aerobic metabolism and the cellular stress response in thermally acclimated fish (Coughlin et al., 2020; Pandey et al., 2021), we further predicted that acclimation to elevated temperatures in these triplefin populations would be reflected through differential gene expression between temperature treatments in brain tissue, highlighting regulatory differences in genes involved in thermal stress and metabolic responses.

Materials and methods

Triplefin sampling and experimental design

Estuarine triplefin (*F. nigripenne*) and common triplefin (*F. lapillum*) adults of unknown sex were obtained using minnow traps in April 2022. The estuarine triplefin was collected from a single location in the Waikouaiti River Estuary (46.62138°S, 170.64495°E) and the intertidal common triplefin was collected from rockpools at Puketeraki (45.65266°S, 170.65383°E) and Mapoutahi (-45.73389°S, 170.61647°E) within the East Otago Tāiāpure (with permission from the East Otago Tāiāpure Management Committee) in the East Otago coastal region on the South Island of New Zealand (see Supplemental Material). Fish were transported to the University of Otago's Zoology Department and housed in 250 L recirculating, bio-filtering tanks under a controlled 12-hour light cycle and salinity of ~30 ppm. Tanks were monitored daily, and fish were fed every second day with Ridley aquaculture Nutragard Start pellets (3 mm in size). Sampling and husbandry of triplefin species were done under a University of Otago ethics protocol.

Fish were randomly allocated to one of four tanks and held at controlled pre-acclimation temperatures of 12°C for two weeks (n = 20 estuarine triplefin per tank, n = 20 common triplefin per tank). Temperatures in each tank were then raised or lowered by 2°C per day until the desired experimental temperature was reached, where they were maintained for four weeks. Temperature treatments were 10°C (control), 14°C, 18°C or 22°C, selected based on previous studies describing thermal tolerance range and limits of North Island common triplefin populations (Khan et al., 2014) and adjusted to South Island sea surface temperatures (SSTs) and local conditions (Portobello, Otago). mean monthly temperatures average 7°C in winter and mean

monthly temperatures average 16.1°C in summer but can go as high as 21.1°C (Shears & Bowen, 2017; Chiswell & Grant, 2018), but our own 2023/2024 temperature logger data from a nearby site shows that the fish experience temperatures ranging from 5 to 21 °C (see Supplemental Material). To achieve target temperatures, all tanks but the control were fitted with aquaria heaters. The tanks were thermostatically controlled using a glycol-based system that cooled the heated tanks to the desired temperature. Fish displayed no signs of disease or illness, and no mortalities occurred.

Mitochondrial respirometry and function assays

Following the four-week acclimation period, fish were sedated within an ice slurry before they were euthanised by severing the brain stem on the dorsal side of the head. The weight and length of each fish were measured postmortem before the opening of the skull and brain tissue removal. Fish were also sexed through dissection and examination of the gonads (though it was difficult to identify the gonads and sex of individuals, meaning that many fish appeared to be male to us – see Supplemental Material for estimated numbers of each sex). In all mitochondrial respirometry assays, preparation of brain tissue followed previously described tissue preparation workflows from Iftikar & Hickey (2013) up to the washing of small tissues pieces (1-2 mm^3) within the mitochondrial respiratory medium (MiR05). Following immersion in the respiratory medium, tissue pieces were randomly allocated to and shaken for 15 minutes within one of two wells. Both wells contained 200 µL of a modified respiration media (RM, 1 mL MiR05, 2 mM malate, 10 mM pyruvate, 10 mM glutamate, 10 mM succinate and 2.5 mM ADP) and a unique fluorescent dye used as a probe to measure either ATP equilibrium or mitochondrial membrane potential respectively. Mitochondrial ATP equilibrium was assayed by adding 1 µL of the yellow-orange fluorescent dye magnesium green (MgG), which binds free extra-mitochondrial

magnesium. As ATP also binds Mg^{2+}, MgG fluorescence decreases as ATP concentrations increase. Mitochondrial membrane potential was assayed using 0.5 µL of the red-pink fluorescent dye tetramethylrhodamine, methyl ester (TMRM), at a concentration of 1 µM, which detects shifts in membrane potential. Pyruvate, saponin and the respiration media were prepared as stock solutions every three days.

Permeabilised tissue sections were loaded with fluorescent probes and analysed using purpose built fluorescent microscopes designed to detect fluorescence intensity. The tissues were placed on small purpose-built holders, which held permeabilised brain tissue pieces in excess media under coverslips. Respiration assays began with a 5-minute run-in period before tissues were heated using Peltier Pads heater blocks from 12 to 30℃ and then followed by a 5-minute cool-down period. The fluorescent signal was followed using purpose-built USB microscopes. These consisted of 3D printed holders that held two opposing coloured (460 and 540 nm) LEDs aimed at 45° onto the focal point beneath the USB scope. The holder also held glass bandpass (540 nm) or long pass (600 nm) filter between the USB scope lens and the object. The image from the camera was visualised and recorded using OBS Studio (30.1.1) and changes in fluorescence recorded simply by used of a solar photovoltaic cell fixed to the computer monitor. The voltage from the photovoltaic cell was recorded using an ADInstruments 15T PowerLab. A T-type thermocouple was placed into a cavity within the sample slide. The thermocouple was connected to an ADInstrument T-Pod to measure and record the sample temperature directly (+/- 0.01 ℃) concomitantly with fluorescence signals. Therefore, both signals were recorded using T15 ADInstrument Powerlabs, and LabChart version 8 (ADInstrument, 2022) recorded at signals 1000 data points.s^{-1}. Mitochondrial membrane potential was recorded at a range of up to 2 V, whilst ATP equilibrium was recorded at a range of up to 5 V. Before running assays, controls

were conducted to test the accuracy of the TMRM and MgG probes (see Supplemental Material). Samples sizes for the intertidal common triplefins ranged from n = 5-11 fish per treatment and for estuarine triplefins ranged from n = 9-12 fish per treatment. We note that one of the Peltier Pads malfunctioned halfway through running assays. The resulting increased ramping speed possibly confounded accuracy in tracking mitochondrial membrane potential changes as the TMRM probe's ability to estimate potential can become compromised with fast changes (Zorova et al., 2018). Future studies may consider substitute options for controlling ramping rates. Data were smoothed within LabChart 8 using Bartlett (Triangular) windows at a width of 1-second samples and exported as time series measuring voltage changes in fluorescence. Data were normalised by the voltage at maximum temperature (30℃), then by the voltage recorded at the initial temperature (~12℃, after 5-minute warm-up periods) and finally as derivatives by creating bins and subtracting the averages of 10 data points. The software SegReg (Oosterbaan, 2017) was used to perform segmented linear regression analyses on each individual assay to detect thermal breakpoints in the derived data. The breakpoint temperature was detected with 95% confidence intervals and represented the temperature at which the assayed mitochondrial property begins to lose efficiency and become compromised.

All statistical analyses and models conducted on the mitochondrial data were run using R version 4.3.0 (R Core Team, 2023). General linear models compared differences in thermal breakpoints across acclimation temperatures for each mitochondrial property and species individually. Due to the length of time taken to run all assays (22 days), there were significant negative trends in mitochondrial membrane potential breakpoint with acclimation length for the estuarine triplefins (see Supplemental Material). Hence, the duration of acclimation period was included as a model covariate. ANOVA and Tukey's Post-Hoc tests were run on all models using the package *heplots*

in R v1.6.2 (Friendly, 2007), with significance set to $p < 0.05$. Models comparing condition factor (K) and interaction terms between temperature and duration of acclimation period were also run (see Supplemental Material) but as these found no significant effects, condition factor and interaction effects were not included in further analyses. Sex was not applied to any statistical models.

RNA-seq, gene annotation and differential expression analyses

Total RNA was extracted using TRI Reagent (Sigma) and 1-bromo-3-chloropropane (BCP, Sigma) for homogenisation, and a Norgen Total RNA Purification kit (Norgen Biotek) with an on-column DNA removal step from the brain tissue of ten triplefins acclimated to the control 10℃ temperature and ten acclimated to the warmest 22℃ temperature (5 per species per temperature treatment, 20 samples total). RNA quality and integrity assessments following the workflow in Ragsdale et al. (2022). RNA samples were prepared following procedures laid out in the Illumina TruSeq™ Stranded mRNA sample preparation kit at the Otago Genomics and Bioinformatics Facility at the University of Otago. Sequencing was performed using the Illumina HiSeq2000 (Illumina, USA) machine with the sample library. After RNA sequencing, the 2 x 51 bp single-reads underwent quality control checks using FastQC v0.11.9 (Babraham Bioinformatics, 2023) and MultiQC v1.13 (Ewels et al., 2016) and trimming using cutadapt v4.1. The two highest quality brain samples from each species and temperature treatment were re-sequenced using the same protocol to generate 2 x 151 bp pair-end reads on an Illumina NextSeq2000, generating ~29.04 Mio reads per sample to construct a *de novo* transcriptome. The detailed pipeline for the assembly of the de novo transcriptome, gene annotation and differential expression analyses can be found at https://github.com/breanariordan/triplefinRNA.To investigate sample clustering, we performed pairwise correlation analyses on gene count data for

samples using the Spearman correlation coefficient and *ggplot2* package in R v4.30.0. Count data was filtered and then normalised using the R package *edgeR* v4.0.16 functions 'filterbyexpr' followed by 'calcNormFactors' and 'logCPM' respectively (Smyth, 2005). Clustering based on correlation coefficients was visualised as heatmaps using the R package *pheatmap*. Multidimensional scaling plots (MDS) were also generated on normalised gene count data using *ggplot2* and found samples to form distinct clusters based on species and temperature treatment (see Supplementary Material). We then performed differential expression analyses between the two temperature treatments within each species independently. Linear models were fit to normalised gene count data using the 'voom' function of the *limma* package v3.58.1 (Law et al., 2014). The threshold for differentially expressed genes was set to those surpassing a false discovery rate (*p-adj*) of < 0.05 and a LogFC value of ≤ -1 (downregulated) or ≥ 1 (upregulated). Further analyses were conducted using the same analytical approach to compare gene expression profiles between species.

Gene ontology (GO) annotation was created as an SQLite database using SQLite v3.42.0 and Trinotate v3.3.2 (Bryant et al., 2017) and loaded with annotations from BLASTx and BLASTp hits against the Uniprot-Swissprot database (Altschul et al., 1990; The UniProt Consortium, 2015). Enriched GO terms were extracted from the database by extracting sample gene lengths and factor labelling using the *align_and_estimate.pl* in Trinity v214.0 (Grabherr et al., 2011) with Salmon v1.10.1. Following this, the packages *goseq* v1.54.0 (Young et al., 2010) and *qvalue* v2.34.0 (Storey, 2002) in R were employed to perform gene ontology analyses between temperature within each species, and between species, with the same significance and LogFC thresholds as used in the differential gene expression analyses.

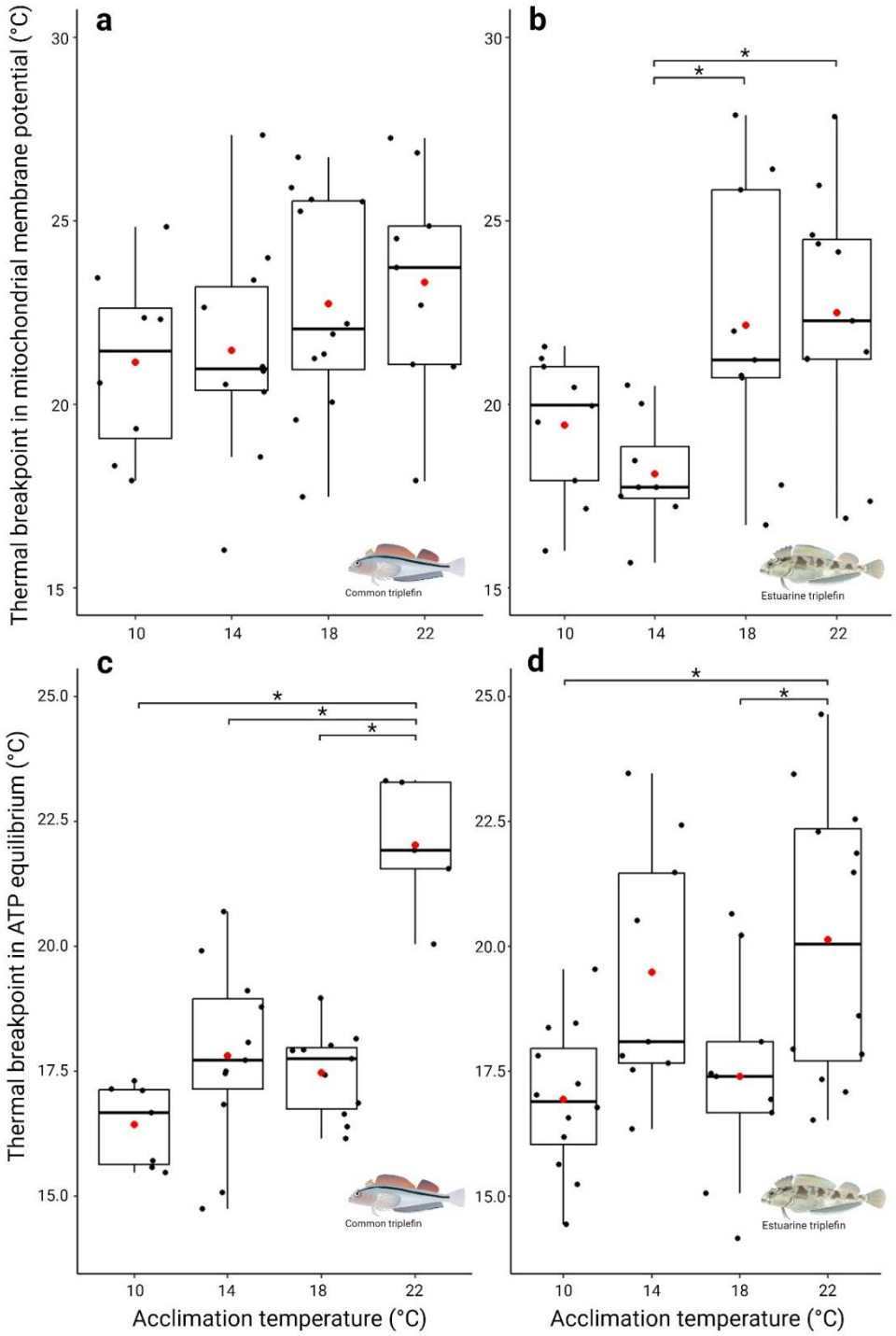
Results

Mitochondrial performance in thermally acclimated fish

With increasing temperature, brain mitochondrial membrane potential in both triplefin species became depolarised, indicating compromised integrity. The lowest thermal breakpoints of membrane potential were observed in the 14℃ treatments for the intertidal common triplefin and the estuarine triplefin. In the intertidal common triplefin, membrane potential thermal breakpoints were not significantly affected by temperature treatment ($F_{3, 34} = 1.19$, $p = 0.33$; Fig 1a), though there is a suggestive trend of increasing membrane potential with the lowest average (± SE) breakpoints in control fish at 21.14℃ ± 0.88 and highest in 22℃ acclimated fish at 23.32℃ ± 1.00. In the estuarine triplefin, membrane potential breakpoints significantly differed amongst temperature treatments ($F_{3, 32} = 4.66$, $p = < 0.008$; Fig 1b). The highest breakpoints were in the 18℃ and 22℃ treatments, averaging (± SE) 22.15℃ ± 1.28 and 22.49℃ ± 1.02, respectively, significantly higher than the 14℃ fish at 18.12℃ ± 0.55. Control fish at 10℃ had an average breakpoint temperature of 19.43℃ ± 0.66.

Thermal breakpoints of ATP equilibrium indicate a shift from ATP production to hydrolysis and a depletion of ATP availability in the brain. The lowest breakpoint temperatures in ATP equilibrium were observed in the intertidal common triplefin in the 14℃ treatment and in an estuarine triplefin acclimated to 18℃. Acclimation temperature significantly altered ATP equilibrium breakpoints in both the intertidal common ($F_{3,29} = 20.93$, $p < 0.001$; Fig 1c) and estuarine triplefins ($F_{3,37} = 4.71$, $p = < 0.007$; Fig 1d). Intertidal common triplefin brains at 22℃ recorded the highest average ATP equilibrium breakpoints (± SE) at 22.02℃ ± 0.61 (Fig 1d). These breakpoints were significantly higher than all cooler temperatures, with the 10℃, 14℃

and 18℃ displaying averages (± SE) of 16.43℃ ± 0.31, 17.81℃ ± 0.and 17.47℃ ± 0.26, respectively. Estuarine triplefin acclimated to 22℃ also displayed the highest breakpoints, averaging (± SE) 20.13℃ ± 0.82, significantly higher than control (10℃) and 18℃ acclimated fish at 16.94℃ ± 0.42 and 17.40℃ ± 0.70. Thermal breakpoints in 14℃ acclimated estuarine fish averaged 19.48℃ ± 0.84, marginally non-significantly higher than control fish.



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278 Figure 1. Thermal breakpoints of (a,b) mitochondrial membrane potential and (c,d) ATP
279 equilibrium in the brain tissue of the intertidal common triplefin, *Forsterygion lapillum* and the
280 estuarine triplefin, *F. nigripenne,* after an acclimation period of four to eight weeks in one of
281 four experimental temperature treatments: 10°C, 14°C, 18°C and 22°C. Samples sizes for

common triplefins ranged from *n* = 5-11 fish per treatment and for estuarine triplefins ranged from *n* = 9-12 fish per treatment. Total *n* = 76 fish. Black dots represent individual breakpoints, whilst boxes depict the distribution of the data and red dots the average breakpoint temperature for each treatment. Treatments with significant differences are notated with an asterisk. Triplefin images courtesy of Vivian Ward & Kendall Clements.

Differential Gene Expression Patterns and Ontology Pathways

After filtering, we obtained 232 million reads from 8 samples (14.5 mean; min: 11.3, max: 17) to generate a single *de novo* transcriptome for the estuarine and intertidal common triplefin. The transcriptome was used as a reference for 20 samples sequenced with 2 x 51 bp single-reads from which the expression of genes could be compared in the brain tissue of the triplefin species (5 samples per species and temperature). After trimming, 8.9 - 12.4 million reads were sequenced for each triplefin brain tissue experimental sample. Mapping assigned these reads to 195,401 gene transcripts, with 24.31-27.39% of reads uniquely assigned to a gene for each brain sample. No unassigned reads were in any samples, with the remaining reads being multi-mapped. Using the UniProt database, 36,289 sequences were recognised and aligned to protein sequences, generating subsequent gene ontology (GO) terms.

In triplefin brain transcriptomes, 8,383 genes were differentially expressed in the warm acclimation (22℃) compared to control (10℃) treatments (*p-adj* < 0.05; logFC ≤ -1 or ≥ 1). Of these genes, 4,680 were differentially expressed in the intertidal common triplefin and 5,471 in the estuarine triplefin. Highest correlations in gene expression were observed between individuals from the same species and treatment, whilst lowest correlations occurred between the different species (Fig 2a.) A total of 1,768 genes were differentially expressed to a logFC in expression of ≤ -1 or ≥ 1. All but five genes showed the same direction of differential expression in the 22℃ acclimated triplefins. The two that were annotated were *nap1l1* (nucleosome assembly protein 1 like 1) and *lctl* (lactase-like protein). Both were upregulated in warm

acclimated intertidal common triplefins but downregulated in warm acclimated estuarine triplefins. These, however, bear no association with thermal or stress response pathways to current knowledge.

In the intertidal common triplefins, 2,736 significantly differentially expressed genes were downregulated and 1,944 upregulated (Fig. 2b). The strongest upregulated gene was *ENOL*, encoding the glycolytic enzyme enolase (Li et al., 2015). The *Hk2* gene encoding for another glycolytic enzyme Hexokinase 2 was also one of the strongest upregulated in this species (Li et al., 2015). In the estuarine triplefins, 2,870 genes were downregulated (logFC $\leq$ -1) whilst 2,601 were upregulated (logFC $\geq$ 1; Fig. 2c). Of those strongly differentially expressed genes, many were of notable interest to this study due to their recognition as markers of mitochondrial performance or thermal stress (Shi et al., 2019; Huang et al., 2022; Akbarzadeh et al., 2018; Pandey et al., 2021), or due to their involvement in enriched gene ontology pathways within warm acclimated triplefins. Included in this subset for the estuarine triplefin was the upregulation of genes central to heat stress response systems cytochrome P450 monooxygenases (*cyp1b1*, *cyp3a56)*, neuronal signalling gene proteinase-activated receptor 1 (*F2r*) and mitochondrially-encoded cytochrome C oxidase II (*mt-co2*). Universal to both species was upregulation of heat shock proteins *hsp70*, *hsp90a*, and *SERPINH1*, the last of which was one of the strongest identifiable DEGs after warm acclimation.

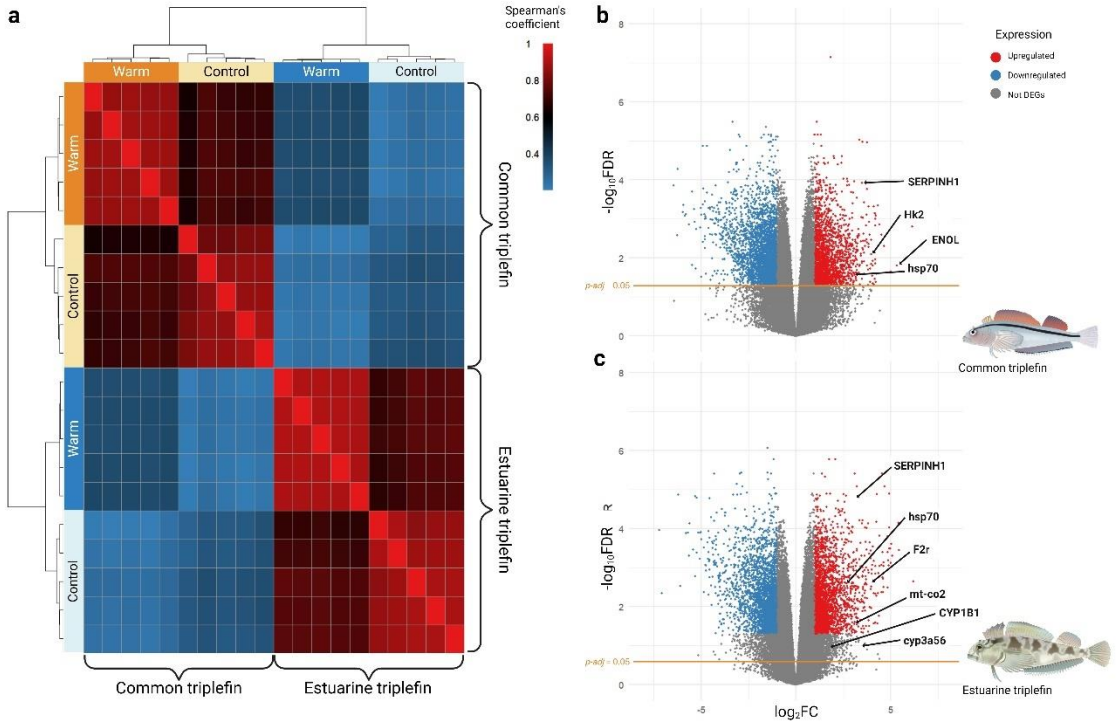

Figure 2. Differential expression analyses of gene transcripts identified in the brain tissue of the intertidal common triplefin, *Forsterygion lapillum* and the estuarine triplefin, *F. nigripenne*, experimentally exposed to control (10°C) or warm (22°C) temperature conditions (n = 5 per treatment) for an acclimation period of eight weeks. (a) Heatmap depicting the correlation in brain gene expression profiles between triplefin species and temperature treatments using only those differentially expressed genes found to pass the false discovery rate (*p-adj*) of < 0.05 and up- or down-regulated by at least a 2-fold change in expression (common triplefin n = 4,680, estuarine triplefin n = 5,471). Red colours indicate a higher correlation coefficient between samples whilst blue colours indicate low coefficients. Coloured bars underneath sample dendrograms represent the different species and temperature treatments. Volcano plots depicting the differential expression of all genes across temperature treatments for (b) the common triplefin, *Forsterygion lapillum*, and (c) the estuarine triplefin, *Forsterygion nigripenne*. Individual transcripts which show a 2-fold change or greater change in expression, and which have passed a false discovery rate (*p-adj*) of < 0.05 are shown in red (up-regulated) and blue (down-regulated). Genes of interest to this study have been labelled in the figures. Triplefin images courtesy of Vivian Ward & Kendall Clements.

Across the two triplefin species, 42 gene ontology terms passed a false discovery threshold (*p-adj*) of < 0.05 and were significantly enriched in warm acclimated fish (see Supplementary Material). In the intertidal common triplefin brains, warm acclimation saw 1,292 significantly

differentially expressed genes annotated into gene ontology pathways, with 17 terms significantly enriched (Fig. 3a). Estuarine triplefins had 1,550 differentially expressed genes (logFC $\leq$ -1/ $\geq$ 1) associated with at least one gene ontology term, with 25 gene terms significantly enriched (*p-adj* < 0.05; Fig. 3b). Interestingly, no significantly enriched pathways were shared between species. Intertidal common triplefin brains acclimated to 22℃ displayed an enrichment of defence and inflammatory response pathways (GO:0006952, GO:0006954), while estuarine triplefins were significantly enriched in metabolic processes including galactosidase activity (GO:0015925, GO:0004565) and vitamin binding (GO:0070279, GO:0019842), alongside oxidoreductase activity (GO:0016712). Pathways concerning neuronal cell migration and conductance channel activity were downregulated in both species but were not significant after false discovery rate correction (GO:1903977, $p < 0.05$, $q = 1$; GO:0060072, $p < 0.005$, $q = 1$). Enriched pathways that showed relevance to this study but were marginally non-significant after false discovery rate correction were thermal response pathways (GO:0050960, $p < 0.005$, $q = 0.34$; GO:0071502, $p < 0.01$, $q = 0.56$) and mitochondrial depolarisation (GO:0051901, GO:0051881, $p < 0.05$, $q = 0.86$) in intertidal common triplefins, and upregulation of other lipid metabolic processes (GO:0072330, $p < 0.0005$, $q = 0.07$, GO:0019752, $p < 0.0005$, $q = 0.09$, GO:0006629, $p < 0.0005$, $q = 0.13$) in estuarine triplefins.

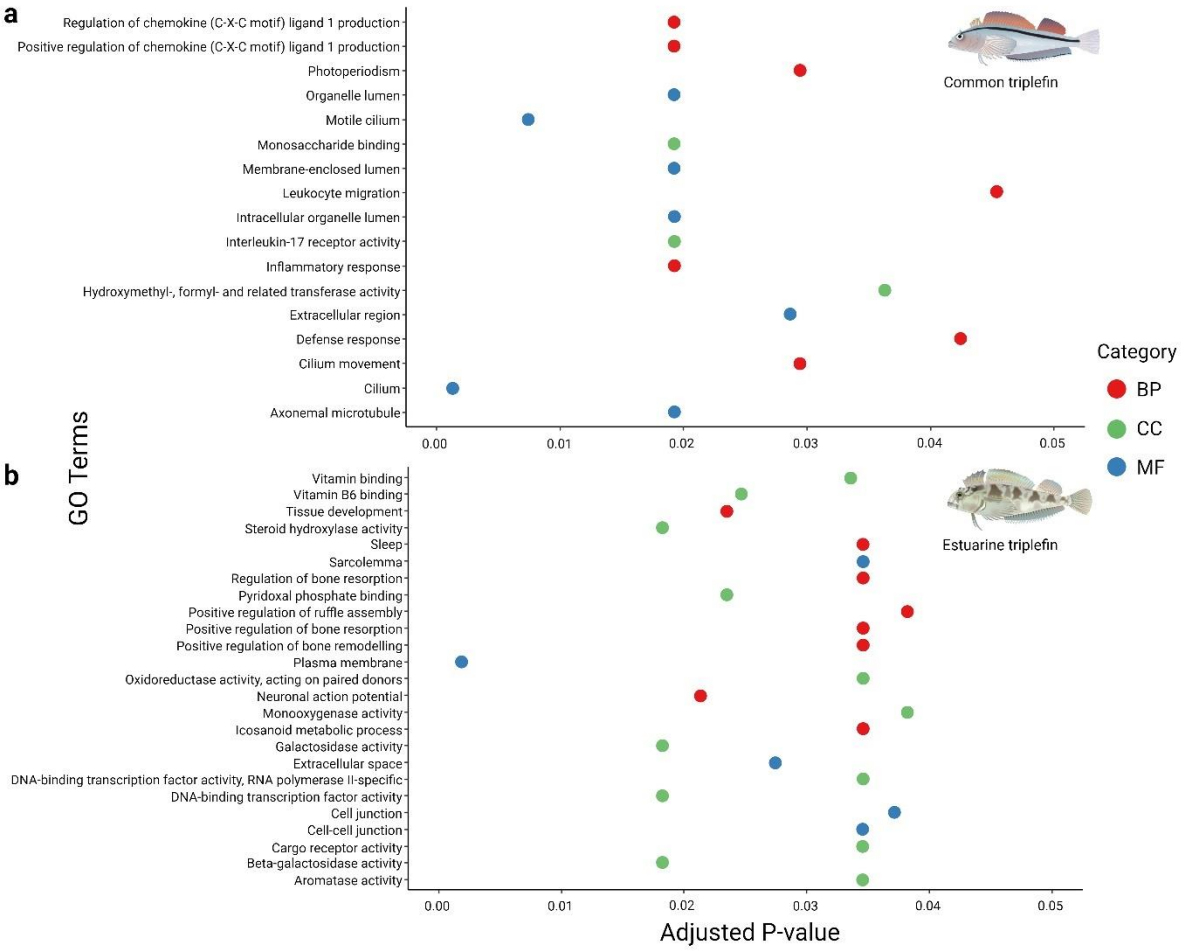

Figure 3. Significantly enriched gene ontology pathways associated with differentially expressed genes in the brain tissues of warm acclimated (a) intertidal common triplefin, *Forsterygion lapillum* fish (n = 17) and (b) estuarine triplefin, *F. nigripenne* fish (n = 25). Fish were experimentally exposed to control (10°C) or warm 22°C temperature conditions (n = 5 per treatment) for an acclimation period of eight weeks. Each circle represents a significantly enriched gene ontology pathway. Circle colour indicates gene ontology category of the pathway (BP: biological process, CC: cellular component, MF: molecular function). Differentially expressed genes pass a threshold log-fold change in expression of < -1 or > 1 and a false discovery rate (*p-adj*) of < 0.05. Triplefin images courtesy of Vivian Ward & Kendall Clements.

Discussion

This study assessed thermal resilience and acclimation potential in two coastal New Zealand triplefin species, the intertidal common intertidal (*F. lapillum*) and estuarine (*F. nigripenne*) triplefin. Prolonged elevated temperature exposure resulted in acclimation of mitochondrial function in both species, demonstrating how fluororespirometry techniques can determine mitochondrial function limits in teleost brain tissues. Warm acclimated estuarine triplefins enhanced mitochondrial membrane potential and ATP equilibrium maintenance, whilst the intertidal common triplefin only altered ATP equilibrium. Brain transcriptomes showed significant gene expression differences between warm acclimated fish (22℃) compared to control (10℃) fish, highlighting oxidative respiration, cellular stress response pathways and heat damage mitigation in brain tissues. Both findings provide insight into acclimatory processes in coastal marine teleosts and demonstrate that although these two species are sister taxa, they appear to utilise some different pathways to cope with increasing temperatures.

Thermal acclimation of mitochondrial properties

The thermal breakpoints of mitochondrial membrane potential represent upper performance limits before inner mitochondrial membrane impairment (Sokolova, 2023). Depolarisation beyond thermal breakpoints confirmed loss of membrane potential, similar to observations in spiny lobster (Palinuridae) and rainbow trout (*Oncorhynchus mykiss*) (Oellermann et al., 2020; Michaelsen et al., 2021). Warm acclimated triplefins from 18℃ and 22℃ treatments had the highest thermal breakpoints, significant only for the estuarine triplefin. The lack of acclimation in the intertidal common triplefins is unexpected given their generalist intertidal ecotype and previous evidence of thermal tolerance in their brain and cardiac mitochondria (McArley et al.,

2017; Willis et al., 2021; Hilton et al., 2010). Fish mitochondrial thermal limits often correlate with environmental temperatures, with liver mitochondrial breakpoint temperatures higher in tropical than Antarctic species (Biederman et al., 2021; Dahlhoff & Somero, 1993; Sokolova, 2023; Guderley, 2004). However, studies on Atlantic killifish (*Fundulus heteroclitus*) and Atlantic salmon (*Salmo salar*) did not find greater mitochondrial membrane potential maintenance in warm acclimated fish at high assay temperatures (Chung & Schulte, 2015; Gerber et al., 2020). The intertidal common triplefin may prioritise other physiological processes over mitochondrial plasticity to achieve whole-organism thermal tolerance.

Triplefin brain mitochondria observed higher ATP:ADP ratios until upper thermal limits, where ATP equilibrium shifted to favour hydrolysis, indicating ATP depletion and disrupted efficiency within tissue preparations. Both species showed thermal acclimation of ATP equilibrium, with breakpoints significantly higher in 22℃ acclimated triplefins. Enhanced plasticity in intertidal triplefin species likely results from exposure to variable thermal environments, as ATP capacities are believed to align with external temperatures, often used to measure upper critical thermal limits (CT_{MAX}) in fish species (Baris et al., 2016; Christen et al., 2018; Healy & Burton, 2023). Similar thermotolerance expansions were noted in killifish (*Fundulus* spp.), attributed to enhanced mitochondrial ATP production (Fangue et al., 2009; Baris et al., 2016). Willis et al. (2021) reported maintenance of ATP synthesis over hydrolysis across intertidal and rockpool inhabiting triplefins. Despite higher thermal breakpoints in 22℃ acclimated triplefins, 68% of fish held at 18℃ and 22℃ displayed ATP equilibrium breakpoints below their acclimation temperature. This contrasts with research suggesting mitochondria fail above an animal's CT_{MAX} (Ern et al., 2023; Chung & Schulte, 2020) and research on North Island intertidal common triplefins finding CT_{MAX} temperatures to sit above acclimation temperatures for all experimental

groups (McArley et al., 2017). In the McArley et al., (2017) study, individual CT_{MAX} values ranged between 29 to 32℃. Currently, the CT_{MAX} value is unknown for South Island intertidal common triplefin populations or for the estuarine triplefin species. Chronically elevated temperatures and stress deplete mitochondrial ATP reserves, reducing thermal breadth and potential for tolerance against additional or new stressors (Voituron et al., 2022; McArley et al., 2017; Willis et al., 2021). In Warrington, a nearby site within the East Otago Tāiāpure, temperature loggers rarely record temperatures as high as 22℃, even in rock pools (see Supplemental Material). It must be noted however, that fish kept in 18℃ and 22℃ conditions suffered no mortalities and were otherwise healthy, indicating CT_{MAX} must be higher than 22℃ for these South Island populations. Thus, whilst the mitochondria likely did not fail below acclimation temperatures in this study, chronic 18℃ and 22℃ exposure may have exhausted the brain mitochondrial ATP reservoir. Under an impaired thermal breadth and ATP synthesis ability, the additive stress of thermal ramping would cause a faster depletion of stored ATP and thereby lowering the temperature threshold at which ATP equilibrium is disturbed. In marine ectothermic species, such as eelpout from the family Zoarcidae, nuclear magnetic resonance (NMR) has been used to track changes in phosphocreatine (PCr) amounts and show how this energy reserve for ATP declines with adverse temperature shifts (Mark et al., 2002; Sommer et al., 1997; Bock et al., 2001). PCr acts as a buffer to the formation and protection of ATP stored within aerobic tissues (Watson et al., 2020). Any dramatic changes in PCr concentration can disrupt ATP concentration buffering, depleting energy resources (Watson et al., 2020). To confirm if this occurred within the warm exposed triplefin fish, tracking of PCr in brain tissues using spectroscopy techniques such as NMR would need to be applied (Pörtner et al., 2004).

Mitochondrial membrane potential thermal breakpoints were generally higher than those for ATP equilibrium. As brain mitochondria lose efficiency under warming, O_2 consumption increases to maintain membrane potential and proton motive force, despite decreases in ATP synthesis relative to O_2 consumption. Membrane potential breakpoints indicate polarity collapse and mitochondrial dysfunction, whilst ATP equilibrium breaks when ATP hydrolysis exceeds production (Abele et al., 2002). Acute warming increases ATP-consuming enzyme activity within the brain, including the sodium-potassium pump (Na+/K+-ATPase), calcium regulators (Ca^{2+}-ATPases), and other hydrolases, challenging ATP synthesis with almost exponential increases in hydrolysis rates. Thus, ATP equilibrium can shift to favour hydrolysis and ATP concentrations become insufficient. Conversely, loss of mitochondrial membrane potential typically always disrupts ATP production, due to the requirement of a proton motive force to drive ATP synthase and maintain ATP synthesis (Power et al., 2014; Chinopoulos et al., 2009). Conversely, Chung & Schulte (2020) have also suggested proton motive force to fail independently to CT_{MAX}, but this was associated with previous research by these authors finding no correlation between acclimation and loss of membrane potential (Chung & Schulte, 2015).

Methods of thermal compensation in mitochondrial membranes

Thermal acclimation of biological membranes involves altering membrane fluidity (Guderley, 2004; Dahlhoff & Somero, 1993). As temperatures increase, membranes become more fluid until integrity is lost, as demonstrated in abalone (genus *Haliotis*) and other ectotherms (Dalhoff & Somero, 1993; Biederman et al., 2019; Oellermann et al., 2020). This can be compensated through homeoviscous adaptation (Guderley, 2004; Kraffe et al., 2007; Biederman et al., 2021). In abalone, warm acclimation led to less fluid membranes and higher mitochondrial respiration breakpoints (Dahlhoff & Somero, 1993). Homeoviscous adaptation can involve altering

plasmalogen composition, notably phosphatidylethanolamine (PE) and phosphatidylcholine (PC), which influence cell membrane structure and mobility (Almsherqi, 2021; Bozelli & Epand, 2021). Warm acclimation in rainbow trout muscle mitochondria saw higher PE and PC proportions increase lipid packing and membrane thickness (Kraffe et al., 2007; Biederman et al., 2019; Bozelli & Epand, 2021; Price et al., 2017). Additionally, a higher PC/ PE ratio can reduce acyl chain flexibility and membrane fluidity, observed in warm acclimated carp (*Cyprinus carpio*), rainbow trout, and the brain mitochondria of American alligators (*Alligator mississippiensis*) (Wodtke, 1981; Hazel & Landrey, 1988; Price et al., 2017). Homeoviscous adaptation also involves changes in unsaturated fatty acids content (Guderley, 2004; Kraffe et al., 2007 Wodtke, 1981; Sokolova, 2023). Cold acclimated carp (*Cyprinus carpio*) muscle mitochondria increased membrane fluidity through raising unsaturated fatty acid proportions (Wodtke, 1981), whereas warm acclimated Antarctic fish (*Notothenia coriiceps*) increased saturated fatty acids, reducing fluidity (Biederman et al., 2021). Estuarine triplefins may display enhanced homeoviscous adaptation compared to the intertidal common triplefins, with evidence of overrepresentation of upregulated genes associated to fatty acid and lipid metabolism in this species, supporting the observed differences in membrane potential performance across the two species (Guderley, 2004).

Mitochondrial adjustments which decrease proton leak or promote ATP synthesis efficiently maintain coupling and ATP equilibrium under warming (Roussel & Voituron, 2020; Gerber et al., 2021). Warm acclimation demonstrated such adjustments in mosquitofish (*Gambusia affinis*) and zebrafish (*Danio rerio*) muscle mitochondria and Atlantic salmon (*Salmo salar*) cardiac mitochondria (LeRoy & Seebacher, 2020; LeRoy et al., 2021; Walesby & Johnston, 1980; Gerber et al., 2021). ATP synthesis is catalysed through the synergistic effect of the F0 and F1

subunits of ATP synthase (Complex V) (Whitehouse et al., 2019; Lane, 2010). Increased abundance of complex V subunits and proteins, as seen in the longjaw mudsucker (*Gillichthys mirabilis*), allows tighter regulation of complex activity, maintaining higher ATP:ADP ratios under decreased proton flow or facilitating higher ATP turnover rates (Jayasundara et al., 2015; O'Brien et al., 2018; LeRoy et al., 2021). However, as the specific activity and flux of respiratory complexes was not assessed in the current study, future studies may seek to utilise specific probes to isolate different complexes and determine their contributions to ATP equilibrium thermal performance.

Gene expression and warm acclimation

This study is among the first to examine the role of brain mitochondria in determining marine ectotherm thermal tolerance, as most research on triplefins and other fish species have focused on cardiac or liver mitochondria (Hilton et al., 2010; McArley et al., 2017; Iftikar & Hickey, 2013; Gerber et al., 2020; Michaelsen et al., 2021). Warm acclimated brain tissues in both intertidal common and estuarine triplefins displayed distinct transcriptomic profiles compared to control treatments, with 19,135 genes differentially expressed. This aligns with post-acclimation profiles from other teleosts, including marine sticklebacks (*Gasterosteus aculeatus*), tropical damselfish (*Acantochromis polyacanthus*) and zebrafish (Shama et al., 2016; Veilleux et al., 2015; Bernal et al., 2022; Vergauwen et al., 2010), indicating thermal plasticity through transcriptomic remodelling (Ragsdale et al., 2022). Acclimation to 22℃ led to more downregulated than upregulated genes in triplefin tissues, a common pattern of warm acclimation supported by previous research in the gill tissue of great spider crabs (*Hyas araneus*) after +5℃ acclimation and acclimated Antarctic killifish brain (Harms et al., 2014; Drown et al., 2022). In zebrafish liver transcriptomes, downregulation was thought to be a compensatory

mechanism for elevated temperatures, linked to suppression of biochemical pathways and transcripts (Vergauwen et al., 2010). In ectotherms, rising external temperatures accelerate biological reaction rates, meaning these processes like neuronal activity and cardiac contraction may not require upregulation (Miller & Stillman, 2012; Vornanen, 1996; Beltrán et al., 2021; Ito et al., 2015). In triplefins, warm acclimation downregulated neuronal excitability and cell migration in brain tissue, comparable with results found in tropical damselfish subjected to generational warming, where GO terms related to synaptic and neural activity were downregulated in +3.0℃ transgenerational offspring (Bernal et al., 2022).

Universal responses to heat stress

Warm acclimation induced cellular stress response pathways, significantly upregulating transcripts for molecular chaperones *Hsp70*, *Hsp90α* and *SERPINH1* in 22℃ triplefins. Molecular chaperones are biomarkers of physiological stress, induced with warm acclimation in green abalone (*Haliotis fulgens*), medaka (*Oryzias latipes*) and annual killifish (*Austrofundulus limnaeus*) (Tripp-Valdez et al., 2019; Ikeda et al., 2017; Podrabsky & Somero, 2004; Shama et al., 2016; Buckley et al., 2006). *SERPINH1*, also known as heat shock protein 47, activates under thermal stress to aid collagen maturation and synthesis, important for internal protection against heat damage, as seen in thermally stressed rainbow trout (Wang et al., 2015). Of the other chaperones, *Hsp90α* corrects misfolded protein configurations whilst *Hsp70* refolds damaged proteins, reducing denatured aggregates (Kassahn et al., 2009; Vergauwen et al., 2010; Goel et al., 2021). Warm acclimation facilitated stronger heat shock responses upon re-exposure to thermal stress in fish like spotted rose snapper (*Lutjanus guttatus*), longjaw mudsuckers and the striped snakehead (*Channa striata*) (Larios-Soriano et al., 2020; Purohit et al., 2014; Logan & Somero, 2011). The upregulation of heat shock protein genes in triplefin tissues indicates a heat

stress response has been mounted, protecting protein synthesis and activity. Supporting this was differential expression of cytochrome P450s in warm acclimated triplefins (Iwama et al., 1998; Yampolsky et al., 2014). This enzyme superfamily oxidises steroids, fatty acids, neurotransmitters and other compounds, aiding their biosynthesis (Uno et al., 2012; Niwa et al., 2015). Due to their role mitigating oxidative damage, P450s are often upregulated after environmental stress, seen after cadmium contamination in common carp (*Cyrpinus carpio*) and +4°C warming in marine sticklebacks (Harms et al., 2014; Rebl et al., 2013; Chen et al., 2019; Shama et al., 2016). In the estuarine triplefins, many P450s were involved in significantly enriched ontology pathways enhancing tissue development alongside oxidoreductase, monooxygenase and hydroxylase activity in warm acclimated brains. P450s use reactive oxygen species (ROS), such as hydrogen peroxide, to catalyse such oxidation and hydroxylation reactions, making them crucial for removing excess ROS (Harms et al., 2014; Chen et al., 2019; Pardhe et al., 2022). Enriched oxidoreductase activity suggests elevated mitochondrial respiration with warm acclimation, with this appearing stronger in the estuarine triplefin, requiring a stress response to counteract the effects of reactive oxygen species formation, a byproduct of respiration (Schulte, 2015).

Transcriptomic evidence for metabolic and mitochondrial compensation

Metabolic remodelling is key to thermal compensation under temperature stress (Shama et al., 2016; Madeira et al., 2017; Veilleux et al., 2015). Research on marine sticklebacks, rainbow trout, zebrafish and rainbowfishes found significant differential expression of metabolic genes with warm acclimation (Shama et al., 2016; de Nadal et al., 2011; Toni et al., 2019; Rebl et al., 2013; Smith et al. 2013; Sandoval-Castillo et al. 2020). Estuarine triplefin brains were enriched in metabolic processes, including galactosidases and carboxylic acids. Acid biosynthesis, aided

555 by the activity of enzymes such as the hydrolytic galactosidases, symbolise a shift towards
556 carbohydrate metabolism, converting proteins into important substrates for glycolysis and ATP
557 production (Hauf et al., 2000; Kitchener et al., 2024; Marzullo et al., 2022; Jayasundara et al.,
558 2015). Under elevated temperatures, increased glycolytic metabolism is apparent with enhanced
559 anaerobic potential and metabolic compensation in the gilthead sea bream (*Sparus aurata*) and
560 turbot (*Scophthalmus maximus*) (Madeira et al., 2017; Huang et al., 2022). Glycolytic encoded
561 genes were overexpressed in warm acclimated triplefins, including enolase (*ENOL*), hexokinase
562 (*Hk2*) and aldolase (*ALDOA*) in the intertidal common triplefin and lactate dehydrogenase (*ldhb*)
563 in the estuarine triplefin. These are showing similar expression patterns to heat-exposed longjaw
564 mudsuckers and hypoxic silver carp (*Hypophthalmichthys molitrix*) and goldfish (*Carassius*
565 *auratus*) (Buckley et al., 2006; Feng et al., 2022; West et al., f1999). The expression of these
566 genes, which included upregulation of mitochondrially-located aerobic enzymes alpha-
567 ketoglutarate dehydrogenase (*Ogdh*) and isocitrate dehydrogenase (*IDH2*), indicate attempts to
568 maintain aerobic metabolism in triplefin brain tissues and suggests triplefin brains can
569 aerobically compensate for the increased oxygen demands caused by elevated temperatures.
570 Despite upregulation of mitochondrial respiratory enzymes and complexes, no mitochondrial
571 ontology terms were significantly enriched in the triplefins, contrasting with previous
572 transcriptional evidence of enriched mitochondrial performance in warm acclimated fish (Shama
573 et al., 2016; Li et al., 2024; Bernal et al., 2022; Bernal et al., 2020).

574 Upregulation in warm acclimated intertidal common triplefin brains enriched inflammatory and
575 immune responses. The overexpression of immune-related transcripts suggests an energy shift
576 from cellular growth to repair due to oxidative tissue and DNA damage (Kassahn et al., 2009;
577 Komoroske et al., 2015). Although not reflected by an enrichment in GO terms, inflammatory

responses were observed in estuarine triplefins, involving genes like proteinase-activated receptor 1 (*F2r*) which was one of the strongest upregulated genes (logFC = 3.94), known to augment intracellular signalling and synaptic plasticity for tissue and neuron development (Han et al., 2011; Tucić et al., 2021; Midwood & Orend, 2009). Rather than directly upregulating inflammatory and immune response pathways, heat damage responses in the estuarine triplefin may invoke lysosomal pathways through neurotransmitter functions to remove and replace dying cells. Vitamin B6, significantly enriched in warm acclimated estuarine brains, controls neurotransmitter biosynthesis and plays a role in heat stress reduction in fish such as the cyprinid (*Gymnocypris chilianensis*) and in the olive flounder (*Paralichthys olivaceus*) (Parra et al., 2018; Zhao et al., 2022; Lee et al., 2023).

Conclusion

Intertidal common and estuarine triplefins demonstrated innate thermal plasticity and compensation, providing mechanisms for acclimation to ocean warming. Brain mitochondria maintained membrane potential and high apparent ATP concentrations after prolonged warming under thermal ramping. Brain tissues of warm acclimated individuals showed significant transcriptomic responses, indicating extensive metabolic and structural remodelling. All tissues upregulated heat shock proteins to mitigate oxidative damage and increased reliance on carbohydrate metabolism. Combined, these findings provide evidence for a coordinated acclimation response in the brains of both triplefin species. The estuarine triplefin showed a greater thermal acclimation response, adjusting mitochondrial membrane potential in functional measures and activating aerobic and glycolytic pathways, while the intertidal common triplefin activated cellular repair and inflammatory responses. Despite utilising different mechanisms, both species tolerated elevated temperatures and maintained biological and physiological

functions. While this study elucidates some of the plastic mechanisms that contribute to the resilience of coastal marine species that have evolved in climatically variable conditions, to check for an adaptive component it is necessary to compare how closely related species with divergent thermal niches (e.g., intertidal vs. subtidal species) adjust or adapt to rising and variable water temperatures.

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Author Contributions

Breana Riordan: Conceptualisation; data curation; formal analysis; investigation; methodology; project administration; validation; visualisation; writing – original draft; writing – review and editing. **Ludovic Dutoit:** Methodology; software; supervision; writing – review and editing. **Tania King:** Resources; data curation; methodology. **Luciano Beheregaray:** Writing – review and editing. **Neil Gemmell:** Resources; writing – review and editing. **Anthony Hickey:** Conceptualisation; methodology; software; resources; supervision; writing – review and editing. **Sheri Johnson:** Conceptualisation; data curation; investigation; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing.

Data Availability Statement

Raw sequence data generated to create the *de novo* transcriptome and perform subsequent RNA-seq and gene expression analyses within this manuscript are openly available and accessible through OSF at doi:10.17605/OSF.IO/BCDWU. All codes associated with this data and manuscript are available in the Github repository located at https://github.com/breanariordan/triplefinRNA.

References

Abele, D., Heise, K., Pörtner, H. O., & Puntarulo, S. (2002). Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *Journal of Experimental Biology*, *205*(13), 1831-1841. https://doi.org/10.1242/jeb.205.13.1831

Akbarzadeh, A., Günther, O., Houde, A. L., Shaorong, L., Ming, T. J., Jeffries, K. M., Hinch, S. G., & Miller, K. M. (2018). Developing specific molecular biomarkers for thermal stress in salmonids. *BMC Genomics*, *19*, 749. https://doi.org/10.1186/s12864-018-5108-9

637 Alix, M., Kjesbu, O. S., & Anderson, K. C. (2020). From gametogenesis to spawning: How
638 climate-driven warming affects teleost reproductive biology. *Journal of Fish Biology*, *97*(3),
639 607–632. https://doi.org/10.1111/jfb.14439

640 Almsherqi, Z.A. (2021). Potential role of plasmalogens in the modulation of biomembrane
641 morphology. *Frontiers in Cell and Developmental Biology*, 9.
642 https://doi.org/10.3389/fcell.2021.673917

643 Altschul, S.F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local
644 alignment search tool. *Journal of Molecular Biology*, *215*(3), 403-410.
645 https://doi.org/10.1016/S0022-2836(05)80360-2

646 Anderson, J. T., Panetta, A. M., & Mitchell-Olds, T. (2012). Evolutionary and ecological
647 responses to anthropogenic climate change: Update on anthropogenic climate change. *Plant*
648 *Physiology*, *160*(4), 1728–1740. https://doi.org/10.1104/pp.112.206219

649 Babraham Bioinformatics. (2023, March 1). *FastQC: A quality control tool for high throughput*
650 *sequence data.* https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

651 Baris, T. Z., Crawford, D. L., & Oleksiak, M. F. (2016). Acclimation and acute temperature
652 effects on population differences in oxidative phosphorylation. *American Journal of Physiology*
653 *– Regulatory, Integrative and Comparative Physiology*, *310*(2), R185–R196.
654 https://doi.org/10.1152/ajpregu.00421.2015

655 Bates, A. E., & Morley, S. A. (2020). Interpreting empirical estimates of experimentally derived
656 physiological and biological thermal limits in ectotherms. *Canadian Journal of Zoology*, *98*(4),
657 237-244. https://doi.org/10.1139/cjz-2018-0276

658 Beltrán, I., Herculano-Houzel, S., Sinervo, B., & Whiting, M. J. (2021). Are ectotherm brains
659 vulnerable to global warming? *Trends in Ecology & Evolution*, *36*(8), 691–699.
660 https://doi.org/10.1016/j.tree.2021.04.009

661 Bernal, M. A., Schmidt, E., Donelson, J. M., Munday, P. L., & Ravasi, T. (2022). Molecular
662 response of the brain to cross-generational warming in a coral reef fish. *Frontiers in Marine*
663 *Science*, *9*. https://doi.org/10.3389/fmars.2022.784418

Bernal, M.A., Schunter, C., Lehmann, R., Lightfoot, D. J., Allan, B. J. M., Veilleux, H. D., Rummer, J. L., Munday, P. L., & Ravasi, T. (2020). Species-specific molecular responses of wild coral reef fishes during a marine heatwave. *Science Advances, 6*(12), eaay3423. https://doi.org/10.1126/sciadv.aay3423

Bhat, A., Greulich, M. M., & Martins, E. P. (2015). Behavioral plasticity in response to environmental manipulation among zebrafish (*Danio rerio*) populations. *PLoS One, 10*(4), e0125097. https://doi.org/10.1371/journal.pone.0125097

Biederman, A. M., Kuhn, D. E., O'Brien, K. M., & Crockett, E. L. (2019). Mitochondrial membranes in cardiac muscle from Antarctic notothenioid fishes vary in phospholipid composition and membrane fluidity. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 235*, 46-53. https://doi.org/10.1016/j.cbpb.2019.05.011

Biederman, A. M., O'Brien, K. M., & Crockett, E. L. (2021). Homeoviscous adaptation occurs with thermal acclimation in biological membranes from heart and gill, but not the brain, in the Antarctic fish *Notothenia coriiceps. Journal of Comparative Physiology B, 191*(2), 289–300. https://doi.org/10.1007/s00360-020-01339-5

Bilyk, K. T., & Cheng, C-H. C. (2014). RNA-seq analyses of cellular responses to elevated body temperature in the high Antarctic cryopelagic nototheniid fish *Pagothenia borchgrevinki*. *Marine Genomics, 18*(2), 163–171. https://doi.org/10.1016/j.margen.2014.06.006

Bock, C., Sartoris, F. J., Wittig, R., & Pörtner, H. O. (2001). Temperature-dependent pH regulation in stenothermal Antarctic and eurythermal temperate eelpout (Zoarcidae): an in-vivo NMR study. *Polar Biology, 24*, 869-874. https://doi.org/10.1007/s003000100298

Bozelli, J. C., & Epand, R. M. (2021). Plasmalogen replacement therapy. *Membranes (Basel), 11*(11), 838. https://doi.org/10.3390/membranes11110838

Bryant, D. M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M. B., Payzin-Dogru, D., Lee, T. J., Leigh, N. D., Kuo, T. H., & David, F. G. (2017). A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. *Cell Reports, 18*(3), 762-776. https://doi.org/10.1016/j.celrep.2016.12.063

691 Buckley, B. A., Gracey, A. Y., & Somero, G. N. (2006). The cellular response to heat stress in
692 the goby *Gillichthys mirabilis*: a cDNA microarray and protein-level analysis. *Journal of*
693 *Experimental Biology, 209*(14), 2660–2677. https://doi.org/10.1242/jeb.02292

694 Chen, L., Pan, Y., Cheng, J., Zhu, X., Chu, W., Meng, Y. Y., Bin, S., & Zhang, J. (2019).
695 Characterization of myosin heavy chain (MYH) genes and their differential expression in white
696 and red muscles of Chinese perch, *Siniperca chuatsi*. *International Journal of Biological*
697 *Macromolecules*, *250*, 125907. https://doi.org/10.1016/j.ijbiomac.2023.125907

698 Cheng, L., Abraham, J., Hausfather, Z., & Trenberth, K. E. (2019). How fast are the oceans
699 warming? *Science, 363(6423),* 128-129. https://doi.org/10.1126/science.aav7619

700 Chinopoulos, C., Vajda, S., Csanády, L., Mándi, M., Mathe, K., & Adam-Vizi, V. (2009). A
701 novel kinetic assay of mitochondrial ATP-ADP exchange rate mediated by the ANT. *Biophysical*
702 *Journal, 96*(6), 2490–2504. https://doi.org/10.1016/j.bpj.2008.12.3915

703 Chiswell, S., & Grant, B. (2018). *New Zealand coastal sea surface temperature* (Report No.:
704 2018295WN). National Institute of Water and Atmospheric Research Ltd.
705 https://environment.govt.nz/assets/Publications/nz-coastal-sea-surface-temperature/

706 Christen, F., Desrosiers, V., Dupont-Cyr, B. A., Vandenberg, G. W., Le François, N. R., Tardif,
707 J. C., Dufresne, F., Lamarre, S. G., & Blier, P. U. (2018). Thermal tolerance and thermal
708 sensitivity of heart mitochondria: Mitochondrial integrity and ROS production. *Free Radical*
709 *Biology and Medicine*, *116*, 11–18. https://doi.org/10.1016/j.freeradbiomed.2017.12.037

710 Chung, D. J., & Schulte, P. M. (2015). Mechanisms and costs of mitochondrial thermal
711 acclimation in a eurythermal killifish (*Fundulus heteroclitus*). *Journal of Experimental Biology*,
712 *218*(11), 1621-1631. https://doi.org/10.1242/jeb.120444

713 Chung, D. J., & Schulte, P. M. (2020). Mitochondria and the thermal limits of ectotherms.
714 *Journal of Experimental Biology, 223*(20), 227801. https://doi.org/10.1242/jeb.227801

715 Coughlin, D. J., Wilson, L. T., Kwon, E. S., & Travitz, L. S. (2020). Thermal acclimation of
716 rainbow trout myotomal muscle, can trout acclimate to a warming environment?. *Comparative*

Biochemistry and Physiology Part A: Molecular & Integrative Physiology, *245*, 110702. https://doi.org/10.1016/j.cbpa.2020.110702

da Silva, C. R. B., Riginos, C., & Wilson, R. S. (2019). An intertidal fish shows thermal acclimation despite living in a rapidly fluctuating environment. *Journal of Comparative Physiology B*, *189*(3), 385–398. https://doi.org/10.1007/s00360-019-01212-0

Dahlhoff, E., & Somero, G. N. (1993). Effects of temperature on mitochondria from abalone (Genus *Haliotis*): *Adaptive Plasticity and its Limits. Journal of Experimental Biology*, *185*(1), 151–168. https://doi.org/10.1242/jeb.185.1.151

de Nadal, E., Ammerer, G., & Posas, F. (2011). Controlling gene expression in response to stress. *Nature Review Genetics*, *12*(12), 833–845. https://doi.org/10.1038/nrg3055

Drown, M. K., Douglas, L. C., & Marjorie, F. O. (2022). Transcriptomic analysis provides insights into molecular mechanisms of thermal physiology. *BMC Genomics*, *23*, 421. https://doi.org/10.1186/s12864-022-08653-y

Ellison, A. R., Webster, T. M. U., Rodriguez-Barreto, D., de Leaniz, C. G., Consuegra, S., Orozco-terWengel, P., & Cable, J. (2020). Comparative transcriptomics reveal conserved impacts of rearing density on immune response of two important aquaculture species. *Fish & Shellfish Immunology, 104*, 192-201. https://doi.org/10.1016/j.fsi.2020.05.043

Ern, R., Andreassen, A. H., & Jutfelt, F. (2023). Physiological mechanisms of acute upper thermal tolerance in fish. *Physiology*, *38*(3), 141–158. https://doi.org/10.1152/physiol.00027.2022

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047-3048. https://doi.org/10.1093/bioinformatics/btw354

Fangue, N. A., Richards, J. G., & Schulte, P. M. (2009). Do mitochondrial properties explain intraspecific variation in thermal tolerance?. *Journal of Experimental Biology*, *212*(4), 514-522. https://doi.org/10.1242/jeb.024034

743 Feary, D. A., & Clements, K. D. (2006). Habitat use by triplefin species (Tripterygiidae) on
744 rocky reefs in New Zealand. *Journal of Fish Biology, 69,* 1031-1046.
745 https://doi.org/10/1111/j.1095-8649.2006.01178.x.

746 Feng, C., Li, X., Sha, H., Luo, X., Zou, G., & Liang, H. (2022). Comparative transcriptome
747 analysis provides novel insights into the molecular mechanism of the silver carp
748 (*Hypophthalmichthys molitrix*) brain in response to hypoxia stress. *Comparative Biochemistry*
749 *and Physiology Part D: Genomics and Proteomics, 41,* 100951.
750 https://doi.org/10.1016/j.cdb.2021.100951

751 Friendly, M. (2007). HE plots for Multivariate General Linear Models. *Journal of Computational*
752 *and Graphical Statistics*, *16*(2), 421-444. https://doi.org/10.1198/106186007X208407

753 Gerber, L., Clow, K.A., & Gamperl, A. K. (2021). Acclimation to warm temperatures has
754 important implications for mitochondrial function in Atlantic salmon (*Salmo salar*). *Journal of*
755 *Experimental Biology*, *224*(2), jeb236257. https://doi.org/10.1242/jeb.236257

756 Gerber, L., Clow, K. A., Mark, F. C., & Gamperl, A. K. (2020). Improved mitochondrial
757 function in salmon (*Salmo salar*) following high temperature acclimation suggests that there are
758 cracks in the proverbial 'ceiling'. *Scientific Reports, 10*(1), 21636.
759 https://doi.org/10.1038/s41598-020-78519-4

760 Goel, A., Ncho, C. M., & Choi, Y. H. (2021). Regulation of gene expression in chickens by heat
761 stress. *Journal of Animal Science and Biotechnology*, *12*(1), 11. https://doi.org/10.1186/s40104-
762 020-00523-5

763 Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X.,
764 Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N.,
765 Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., …Regev, A. (2011). Trinity:
766 reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nature*
767 *Biotechnology*, *29*(7), 644-652. https://doi.org/10.1038/nbt.1883

768 Guderley, H. (2004). Metabolic responses to low temperature in fish muscle. *Biological Reviews,*
769 *79*(2), 409–427. https://doi.org/10.1017/S1464793103006328

Han, K. S., Mannaioni, G., Hamill, C. E., Lee, J., Junge, C. E., Lee, C. J., & Traynelis, S. F. (2011). Activation of protease activated receptor 1 increases the excitability of the dentate granule neurons of hippocampus. *Molecular Brain, 4*(1), 32. https://doi.org/10.1186/1756-6606-4-32

Harms, L., Frickenhaus, S., Schiffer, M., Mark, F. C., Storch, D., Held, C., Pörtner, H.-O., & Lucassen, M. (2014). Gene expression profiling in gills of the great spider crab *Hyas araneus* in response to ocean acidification and warming. *BMC Genomics, 15*(1), 789. https://doi.org/10.1186/1471-2164-15-789

Hauf, J., Zimmermann, F. K., & Müller, S. (2000). Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology*, *26*(9):688–698. https://doi.org/10.1016/S0141-0229(00)00160-5

Hazel, J. R., & Landrey, S. R. (1988). Time course of thermal adaptation in plasma membranes of trout kidney. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *255*(4), R622–R627. https://doi.org/10.1152/ajpregu.1988.255.4.R622

Healy, T. M., & Burton, R. S. (2023). Loss of mitochondrial performance at high temperatures is correlated with upper thermal tolerance among populations of an intertidal copepod. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 266*, 110836. https://doi.org/10.1016/j.cbpb.2023.110836

Herrera, M. J., Heras, J., & German, D. P. (2022). Comparative transcriptomics reveal tissue level specialization towards diet in prickleback fishes. *Journal of Comparative Physiology B, 192*, 275-295. https://doi.org/10.1007/s00360-021-01426-1

Hickey, A. J., Lavery, S. D., Hannan, D. A., Baker, C. S., & Clements, K. D. (2009). New Zealand triplefin fishes (family Tripterygiidae): contrasting population structure and mtDNA diversity within a marine species flock. *Molecular Ecology, 18*, 680-696. https://doi.org/10.1111/j.1365-294X.2008.04052

795 Hilton, Z., Clements, K. D., & Hickey, A. J. R. (2010). Temperature sensitivity of cardiac
796 mitochondria in intertidal and subtidal triplefin fishes. *Journal of Comparative Physiology B,*
797 *180*(7), 979–990. https://doi.org/10.1007/s00360-010-0477-7

798 Hilton, Z., Wellenreuther, M., & Clements, K. D. (2008). Physiology underpins habitat
799 partitioning in a sympatric sister-species pair of intertidal fishes. *Functional Ecology, 22,* 1108-
800 1117. https://doi.org/10.1111/j.1365-2435.2008.01465

801 Huang, Z., Guo, X., Wang, Q., Ma, A., Zhao, T., Qiao, X., & Li, M. (2022). Digital RNA-seq
802 analysis of the cardiac transcriptome response to thermal stress in turbot *Scophthalmus maximus.*
803 *Journal of Thermal Biology, 104*, 103141. https://doi.org/10.1016/j.jtherbio.2021.103141

804 Iftikar, F. I., & Hickey, A. J. R. (2013). Do mitochondria limit hot fish hearts? Understanding the
805 role of mitochondrial function with heat stress in *Notolabrus celidotus. PLOS ONE*, *8*(5),
806 e64120. https://doi.org/10.1371/journal.pone.0064120

807 Iftikar, F. I., Morash, A. J., Cook, D. G., Herbert, N. A., & Hickey, A. J. R. (2015). Temperature
808 acclimation of mitochondria function from the hearts of a temperate wrasse (*Notolabrus*
809 *celidotus). Comparative Biochemistry and Physiology Part A: Molecular & Integrative*
810 *Physiology*, *184*:46–55. https://doi.org/10.1016/j.cbpa.2015.01.017

811 Ikeda, D., Koyama, H., Mizusawa, N., Kan-no, N., Tan, E., Asakawa, S., & Watabe, S. (2017).
812 Global gene expression analysis of the muscle tissues of medaka acclimated to low and high
813 environmental temperatures. *Comparative Biochemistry and Physiology Part D: Genomics and*
814 *Proteomics, 24,* 19–28. https://doi.org/10.1016/j.cbd.2017.07.002

815 Ito, E., Ikemoto, Y., & Yoshioka, T. (2015). Thermodynamic implications of high Q_{10} of
816 thermoTRP channels in living cells. *11,* 33-38. https://doi.org/10.2142/biophysics.11.33

817 Iwama, G. K., Thomas, P. T., Forsyth, R. B., & Vijayan, M. M. (1998). Heat shock protein
818 expression in fish. *Reviews in Fish Biology and Fisheries, 8*(1), 35–56.
819 https://doi.org/10.1023/A:1008812500650

820 Janzen, D. H. (1967). Why mountain passes are higher in the tropics. The American Naturalist,
821 101, 233–249.

Jayasundara, N., Tomanek, L., Dowd, W. W., & Somero, G. N. (2015). Proteomic analysis of cardiac response to thermal acclimation in the eurythermal goby fish *Gillichthys mirabilis.* *Journal of Experimental Biology, 218*(9), 1359–1372. https://doi.org/10.1242/jeb.118760

Kassahn, K. S., Crozier, R. H., Pörtner, H. O., & Caley, M. J. (2009). Animal performance and stress: responses and tolerance limits at different levels of biological organisation. *Biological Reviews, 84*(2), 277–292. https://doi.org/10.1111/j.1469-185X.2008.00073.x

Khan, J. R., Iftikar, F. I., Herbert, N. A., Gnaiger, E., & Hickey, A. J. R. (2014). Thermal plasticity of skeletal muscle mitochondrial activity and whole animal respiration in a common intertidal triplefin fish, *Forsterygion lapillum* (Family: Tripterygiidae). *Journal of Comparative Physiology B, 184*(8), 991-1001. https://doi.org/10.1007/s00360-014-0861-9

Kim, W.-J., Lee, K., Lee, D., Kim, H.-C., Nam, B.-H., Jung, H., Yi, S.-J., & Kim, K. (2021). Transcriptome profiling of olive flounder responses under acute and chronic heat stress. *Genes & Genomics, 43*(2), 151–159. https://doi.org/10.1007/s13258-021-01053-8

Kitchener, E. J. A., Dundee, J. M., & Brown, G. C. (2024). Activated microglia release β−galactosidase that promotes inflammatory neurodegeneration. *Frontiers in Aging Neuroscience, 15.* https://doi.org/10.3389/fnagi.2023.1327756

Komoroske, L. M., Connon, R. E., Jeffries, K. M., & Fangue, N. A. (2015). Linking transcriptional responses to organismal tolerance reveals mechanisms of thermal sensitivity in a mesothermal endangered fish. *Molecular Ecology, 24*(19), 4960–4981. https://doi.org/10.1111/mec.13373

Kraffe, E., Marty, Y., & Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions. *Journal of Experimental Biology, 210*(1): 149-165. https://doi/org/10.1242/jeb.02628

Lane, N. (2010). Why are cells powered by proton gradients?. *Nature Education, 3*(9), 18. https://www.nature.com/scitable/topicpage/why-are-cells-powered-by-proton-gradients-14373960/

Larios-Soriano, E., Re-Araujo, A. D., Díaz, F., Sánchez, C. G., López-Galindo, L., Castro, L. I., & Ramírez, D. T. (2020). Effect of acclimation temperature on thermoregulatory behaviour, thermal tolerance and respiratory metabolism *of Lutjanus guttatus* and the response of heat shock protein 70 (*Hsp70*) and lactate dehydrogenase (*Ldh-a*) genes. *Aquaculture Research, 51*(3), 1089–1100. https://doi/org/10.1111/are.14455

Law, C. W., Chen, Y., Shi, W., & Smyth, G. K. (2014). Voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biology*, *15*: R29. https://doi/org/10.1186/gb-2014-15-2-r29

Lee, S., Moniruzzaman, M., Farris, N., Min, T., & Bai, S. C. (2023). Interactive effect of dietary gamma-aminobutyric acid (GABA) and water temperature of growth performance, blood plasma indices, heat shock proteins and GABAergic gene expression in juvenile olive flounder *Paralichthys olivaceus. Metabolites*, *13*(5), 619. https://doi/org/10.3390/metabo13050619

LeRoy, A., Mazué, G. P. F., Metcalfe, N. B., & Seebacher, F. (2021). Diet and temperature modify the relationship between energy use and ATP production to influence behavior in zebrafish (*Danio rerio*). *Ecology and Evolution*, *11*(14), 9791–9803. https://doi/org/10.1002/ece3.7806

LeRoy, A., & Seebacher, F. (2020). Mismatched light and temperature cues disrupt locomotion and energetics via thyroid-dependent mechanisms. *Conservation Physiology*, *8*(1), coaa051. https://doi/org/10.1093/conphys/coaa051

Li, J., Duan, Y., Kong, W., Gao, H., Fu, S., Li, H., Zhou, Y., Liu, H., Yuan, D., & Zhou, C. (2024). Heat stress affects swimming performance and induces biochemical, structural, and transcriptional changes in the heart of *Gymnocypris eckloni. Aquaculture Reports, 35,* 101998. https://doi/org/10.1016/j.aqrep.2024.101998

Li, X., Gu, J., & Zhou, Q. (2015). Review of aerobic glycolysis and its key enzymes – new targets for lung cancer therapy. *Thoracic Cancer, 6(1),* 17-24. https://doi.org/10.1111/1759-7714.12148

Little, A. G., Hardison, E., Kraskura, K., Dressler, T., Prystay, T. S., Hendriks, B., Pruitt, J. N., Farrell, A. P., Cooke, S. J., Patterson, D. A., Hinch, S. G., & Eliason, E. J. (2020). Reduced lactate dehydrogenase activity in the heart and suppressed sex hormone levels are associated with female-biased mortality during thermal stress in Pacific salmon. *Journal of Experimental Biology*, *223*(14), jeb214841. https://doi.org/10.1242/jeb.214841

Logan, C. A., & Somero, G. N. (2011). Effects of thermal acclimation on transcriptional responses to acute heat stress in the eurythermal fish *Gillichthys mirabilis* (Cooper). *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 300*(6), 1373-1383. https://doi.org/10.1152/ajpregu.00689.2010

Madeira, D., Araújo, J. E., Vitorino, R., Costa, P. M., Capelo, J. L., Vinagre, C., & Diniz, M. S. (2017). Molecular plasticity under ocean warming: Proteomics and fitness data provides clues for a better understanding of the thermal tolerance in fish. *Frontiers in Physiology*, *8*. https://doi.org/10.3389/fphys.2017.00825

Mark, F. C., Bock, C., & Pörtner, H. O. (2002). Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and 31P-MRS. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 283(*5), 1254-1262. https://doi.org/10.1152/ajpregu.00167.2002

Marzullo, M., Maï, M. E., & Ferreira, M. G. (2022). Whole-mount senescence-associated beta-galactosidase (SA-β-GAL) activity detection protocol for adult zebrafish. *Bio-protocol, 12*(13), e4457. https://doi.org/10.21769/BioProtoc.4457

McArley, T. J., Hickey, A. J. R., & Herbert, N. A. (2017). Chronic warm exposure impairs growth performance and reduces thermal safety margins in the common triplefin fish (*Forsterygion lapillum*). *Journal of Experimental Biology*, *220*(19), 3527–3535. https://doi.org/10.1242/jeb.162099

Michaelsen, J., Fago, A., & Bundgaard, A. (2021). High temperature impairs mitochondrial function in rainbow trout cardiac mitochondria. *Journal of Experimental Biology*, *224*(9), jeb242382. https://doi.org/10.1242/jeb.242382

Midwood, K. S., & Orend, G. (2009). The role of tenascin-C in tissue injury and tumorigenesis. *Journal Cellular Communication and Signaling*, *3*(3), 287–310. https://doi.org/10.1007/s12079-009-0075-1

Miller, N. A., & Stillman, J. H. (2012). Neural thermal performance in porcelain crabs, genus *Petrolisthes. Physiological and Biochemical Zoology*, *85*(1), 29–39. https://doi.org/10.1086/663633

Momoda, T. S., Schwindt, A. R., Feist, G. W., Gerwick, L., Bayne, C. J., & Schreck, C. B. (2007). Gene expression in the liver of rainbow trout, *Oncorhynchus mykiss*, during the stress response. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *2*(4), 303–315. https://doi.org/10.1016/j.cbd.2007.06.002

Morley, S. A., Peck, L. S., Sunday, J. M., Heiser, S., & Bates, A. E. (2019). Physiological acclimation and persistence of ectothermic species under extreme heat events. *Global Ecology and Biogeography*, *28*(7), 1018–1037. doi:10.1111/geb.12911

Narum, S. R., & Campbell, N. R. (2015). Transcriptomic response to heat stress among ecologically divergent populations of redband trout. *BMC Genomics, 16*, 103. https://doi.org/10.1186/s12864-015-1246-5

Newton, J. R., De Santis, C., & Jerry, D. R. (2012). The gene expression response of the catadromous perciform barramundi *Lates calcarifer* to an acute heat stress. *Journal of Fish Biology*, *81*(1), 81–93. https://doi.org/10.1111/j.1095-8649.2012.03310.x

Niwa, T., Murayama, N., Imagawa, Y., & Yamazaki, H. (2015). Regioselective hydroxylation of steroid hormones by human cytochromes P450. *Drug Metabolism Reviews*, *47*(2), 89–110. https://doi.org/10.3109/03602532.2015.1011658

O'Brien, K. M., Rix, A. S., Egginton, S., Farrell, A. P., Crockett, E. L., Schlauch, K., Woolsey, R., Hoffman, M., & Merriman, S. (2018). Cardiac mitochondrial metabolism may contribute to differences in thermal tolerance of red- and white-blooded Antarctic notothenioid fishes. *Journal of Experimental Biology*, *221*(15), jeb177816. https://doi.org/10.1242/jeb.177816

928 Oellermann, M., Hickey, A. J. R., Fitzgibbon, Q. P., & Smith, G. (2020). Thermal sensitivity
929 links to cellular cardiac decline in three spiny lobsters. *Scientific Reports*, *10*(1), 202.
930 https://doi.org/10.1038/s41598-019-56794-0

931 Pan, T., Li, T., Yang, M., Jiang, H., Ling, J., & Gao, Q. (2024). Cardiac transcriptome and
932 histology of the heart of the male Chinese mitten crab (*Eriocheir sinensis*) under high-
933 temperature stress. *Fishes*, *9*(3), 92. https://doi.org/10.3390/fishes9030092

934 Pandey, A., Rajesh, M., Baral, P., Sarma, D., Tripathi, P. H., Akhtar, M. S., Ciji, A., Dubey, M.
935 K., Pande, V., Sharma, P., Kamalam, B. S. (2021). Concurrent changes in thermal tolerance
936 thresholds and cellular heat stress response reveals novel molecular signatures and markers of
937 high temperature acclimation in rainbow trout. *Journal of Thermal Biology*, *102*, 103124.
938 https://doi.org/10.1016/j.jtherbio.2021.103124

939 Pardhe, B. D., Kwon, K. P., Park, J. K., Lee, J. H., & Oh, T.-J. (2022). H2O2-driven
940 hydroxylation of steroids catalyzed by cytochrome P450 CYP105D18: Exploration of the
941 substrate access channel. *Applied and Environmental Microbiology*, *89*(1), e01585-22.
942 https://doi.org/10.1128/aem.01585-22

943 Parra, M., Stahl, S., & Hellmann, H. (2018). Vitamin B6 and its role in cell metabolism and
944 physiology. *Cells*, *7*(7), 84. https://doi.org/10.3390/cells7070084

945 Pichaud, N., Ekström, A., Hellgren, K., & Sandblom, E. (2017). Dynamic changes in cardiac
946 mitochondrial metabolism during warm acclimation in rainbow trout. *Journal of Experimental*
947 *Biology*, *220*(9), 1674–1683. https://doi.org/10.1242/jeb.152421

948 Podrabsky, J. E., & Somero, G. N. (2004). Changes in gene expression associated with
949 acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish
950 *Austrofundulus limnaeus.* J*ournal of Experimental Biology*, *207*(13), 2237–2254.
951 https://doi.org/10.1242/jeb.01016

952 Pörtner, H. O., Mark, F. C., & Bock, C. (2004). Oxygen limited thermal tolerance in fish?:
953 Answers obtained by nuclear magnetic resonance techniques. *Respiratory Physiology &*
954 *Neurobiology*, *141*(3), 243-260. https://doi.org/10.1016/j.resp.2004.03.011

Power, A., Pearson, N., Pham, T., Cheung, C., Phillips, A., & Hickey, A. (2014). Uncoupling of oxidative phosphorylation and ATP synthase reversal within the hyperthermic heart. *Physiological Reports*, *2*(9), e12138. https://doi.org/10.14814/phy2.12138

Price, E. R., Sirsat, T. S., Sirsat, S. K. G., Kang, G., Keereetaweep, J., Aziz, M., Chapman, K. D., Dzialowski, E. M. (2017). Thermal acclimation in American alligators: Effects of temperature regime on growth rate, mitochondrial function, and membrane composition. *Journal of Thermal Biology*, *68*, 45–54. https://doi.org/10.1016/j.jtherbio.2016.06.016

Purohit, G. K., Mahanty, A., Suar, M., Sharma, A. P., Mohanty, B. P., & Mohanty, S. (2014). Investigating *hsp* gene expression in liver of *Channa striatus* under heat stress for understanding the upper thermal acclimation. *Biomedical Research International*, *2014*, 381719. https://doi.org/10.1155/2014/381719

Qian, X., Ba, Y., Zhuang, Q., & Zhong, G. (2014). RNA-Seq technology and its application in fish transcriptomics. *OMICS: A Journal of Integrative Biology*, *18*(2), 98–110. https://doi.org/10.1089/omi.2013.0110

Ragsdale, A., Ortega-Recalde, O., Dutoit, L., Besson, A. A., Chia, J. H. Z., King, T., Nakagawa, S., Hickey, A., Gemmell, N. J., Hore, T., & Johnson, S. L. (2022). Paternal hypoxia exposure primes offspring for increased hypoxia resistance. *BMC Biology*, *20(*1), 185. https://doi.org/10.1186/s12915-022-01389-x

R Core Team. (2023). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://R-project.org

Rebl, A., Verleih, M., Köbis, J. M., Kühn, C., Wimmers, K., Köllner, B., & Goldammer, T. (2013). Transcriptome profiling of gill tissue in regionally bred and globally farmed rainbow trout strains reveals different strategies for coping with thermal stress. *Marine Biotechnology*, *15*(4), 445–460. https://doi.org/10.1007/s10126-013-9501-8

Roussel, D., & Voituron, Y. (2020). Mitochondrial costs of being hot: Effects of acute thermal change on liver bioenergetics in toads (*Bufo bufo*). *Frontiers in Physiology*, *11*, 153. https://doi.org/10.3389/fphys.2020.00153

Sandoval-Castillo, J., Gates, K., Brauer, C. J., Smith, S., Bernatchez, L., & Beheregaray, L. B. (2020). Adaptation of plasticity to projected maximum temperatures and across climatically defined bioregions. *Proceeding of the National Academy of Sciences of the United States of America, 117(29)*, 17112-17121. https://doi.org/ 10.1073/pnas.1921124117

Schuckmann, K., Minière, A., Gues, F., Cuesta-Valero, F. J., Kirchengast, G., Adusumilli, S., Straneo, F., Ablain, M., Allan, R. P., Barker P. M., Beltrami, H., Blazquez, A., Boyer, T., Cheng, L., Church, J., Desbruyeres, D., Dolman, H., Domingues, C. M., García-García, A., … Giglio, D. (2023). Heat stored in the Earth system: 1960-2020: where does the energy go? *Earth System Science Data*, *15(4)*, 1675-1709. https://doi.org/10.5194/essd-15-1675-2023

Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *Journal of Experimental Biology*, *218*(12), 1856–1866. https://doi.org/10.1242/jeb.118851

Seebacher, F., White, C. R., & Franklin, C. E. (2015). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nature Climate Change*, *5*(1), 61–66. https://doi.org/10.1038/nclimate2457

Shama, L. N. S., Mark, F. C., Strobel, A., Lokmer, A., John, U., & Wegner, M. K. (2016). Transgenerational effects persist down the maternal line in marine sticklebacks: gene expression matches physiology in a warming ocean. *Evolutionary Applications*, *9*(9), 1096–1111. https://doi.org/10.1111/eva.12370

Shears, N. T., & Bowen, M. M. (2017). Half a century of coastal temperature records reveal complex warming trends in western boundary currents. *Scientific Reports*, *7*(1), 14527. https://doi.org/10.1038/s41598-017-14944-2

Shi, K-P., Dong, S-L., Zhou, Y-G., Li, Y., Gao, Q-F., & Sun, D-J. (2019). RNA-seq reveals temporal differences in the transcriptome response to acute heat stress in the Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. 30,* 169-178. https://doi.org/10.1016/j.cbd.2018.12.011

1008 Shin, S. C., Kim, S. J., Lee, J. K., Ahn, D. H., Kim, M. G., Lee, H., Lee, J., Kim, B., & Park, H.
1009 (2012). Transcriptomic and comparative analysis of three Antarctic notothenioid fishes. *PLoS*
1010 *One, 7*(8), e43762. https://doi.org/10.1371/journal.pone.0043762

1011 Smith, S., Bernatchez, L., & Beheregaray, L. B. (2013). RNA-seq analysis reveals extensive
1012 transcriptional plasticity to temperature stress in a freshwater fish species. *BMC Genomics, 14*,
1013 375. https://doi.org/10.1186/1471-2164-14-375

1014 Smyth, G. K. (2005). limma: Linear models for microarray data. In: R. Gentleman, V. J. Carey,
1015 W. Huber, R. A. Irizarry, & S. Dudoit (Eds.), *Bioinformatics and computational biology*
1016 *solutions using R and Bioconductor* (1st ed., pp. 397-420). Springer. https://doi.org/10.1007/0-
1017 387-29362-0_23

1018 Sokolova, I. M. (2023). Ectotherm mitochondrial economy and responses to global warming.
1019 *Acta Physiologica, 237*(4), e13950. https://doi.org/10.1111/apha.13950

1020 Sommer, A., Klein, B., & Pörtner, H. O. (1997). Temperature induced anaerobiosis in two
1021 populations of the polychaete worm *Arenicola marina* (L.). *Journal of Comparative Physiology*
1022 *B, 167*, 25-35. https://doi.org/10.1007/s003600050044

1023 Storey, J. D. (2002). A direct approach to false discovery rates. *Journal of the Royal Statistics*
1024 *Society: Series B, 64*(3), 479-498. https://doi.org/10.1111/1467-9868.00346

1025 The UniProt Consortium. (2015). UniProt: A hub for protein information. *Nucleic Acids*
1026 *Research, 43*(1), 204-212. https://doi:10.1093/nar/gku989

1027 Toni, M., Angiulli, E., Miccoli, G., Cioni, C., Alleva, E., Frabetti, F., Pizzetti, F., Grassi
1028 Scalvini, F., Nonnis, S., Negri, A., Tedeschi, G., & Maffioli, E. (2019). Environmental
1029 temperature variation affects brain protein expression and cognitive abilities in adult zebrafish
1030 (*Danio rerio*): A proteomic and behavioural study. *Journal of Proteomics, 204*, 103396.
1031 https://doi.org/10.1016/j.jprot.2019.103396

1032 Tripp-Valdez, M. A., Harms, L., Pörtner, H. O., Sicard, M. T., & Lucassen, M. (2019). De novo
1033 transcriptome assembly and gene expression profile of thermally challenged green abalone

(*Haliotis fulgens*: Gastropoda) under acute hypoxia and hypercapnia. *Marine Genomics*, *45*, 48–56. https://doi.org/10.1016/j.margen.2019.01.007

Tucić, M., Stamenković, V., & Andjus, P. (2021). The extracellular matrix glycoprotein tenascin C and adult neurogenesis. *Frontiers in Cell Development Biology*, *9*, 674199. https://doi.org/10.3389/fcell.2021.674199

Uno, T., Ishizuka, M., & Itakura, T. (2012). Cytochrome P450 (CYP) in fish. *Environmental Toxicology and Pharmacology*, *34*(1), 1–13. https://doi.org/10.1016/j.etap.2012.02.004

Veilleux, H. D., Ryu, T., Donelson, J. M., van Herwerden, L., Seridi, L., Ghosheh, Y., Berumen, M. L., Leggat, W., Ravasi, T., & Munday, P. L. (2015). Molecular processes of transgenerational acclimation to a warming ocean. *Nature Climate Change*, *5*(12), 1074–1078. https://doi.org/10.1038/nclimate2724

Venegas, R. M., Acevedo, J., Treml, E. A. (2023). Three decades of ocean warming impacts on marine ecosystems: A review and perspective. *Deep Sea Research Part II: Topical Studies in Oceanography*, *212*, 105318. https://doi.org/10.1016/j.dsr2.2023.105318

Vergauwen, L., Benoot, D., Blust, R., & Knapen, D. (2010). Long-term warm or cold acclimation elicits a specific transcriptional response and affects energy metabolism in zebrafish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *157*(2), 149–157. https://doi.org/10.1016/j.cbpa.2010.06.160

Voituron, Y., Roussel, D., Teulier, L., Vagner, M., Ternon, Q., Romestaing, C., Dubillot, E., & Lefrancois, C. (2022). Warm acclimation increases mitochondrial efficiency in fish: A compensatory mechanism to reduce the demand for oxygen. *Physiological and Biochemical Zoology*, *95*(1), 15–21. https://doi.org/10.1086/716904

Vornanen, M. (1996). Effect of extracellular calcium on the contractility of warm-and cold-acclimated crucian carp heart. *Journal of Comparative Physiology B*, *165*(7), 507–517. https://doi.org/10.1007/BF00387511

Wang, W., Liu, Z., Li, Z., Shi, H., Kang, Y., Wang, J., Huang, J., & Jiangapp, L. (2015). Effects of heat stress on respiratory burst, oxidative damage and *SERPINH1 (HSP47*) mRNA expression

in rainbow trout *Oncorhynchus mykiss*. *Fish Physiology and Biochemistry, 42*, 701-710. https://doi.org/ 10.1007/s10695-015-0170-6

Walesby, N. J., & Johnston, I. A. (1980). Temperature acclimation in brook trout muscle: Adenine nucleotide concentrations, phosphorylation state and adenylate energy charge. *Journal of Comparative Physiology B, 139*(2), 127–133. https://doi.org/10.1007/BF00691027

Watson, W. D., Miller, J. J. J., Lewis, A., Neubauer, S., Tyler, D., Rider, O. J., & Valjovič, L. (2020). Use of cardiac magnetic resonance to detect changes in metabolism in heart failure. *Cardiovascular Diagnosis & Therapy, 10*(3): 583-597. https://doi.org/10.21037/cdt.2019.12.13

Wellband, K. W., & Heath, D. D. (2017). Plasticity in gene transcription explains the differential performance of two invasive fish species. *Evolutionary Applications, 10*(6), 563-576. https://doi.org/10.1111/eva.12463

Wellenreuther, M., Barrett, P. T., & Clements, K. D. (2007). Ecological diversification in habitat use by subtidal triplefin fishes (Tripterygiidae). *Marine Ecology Progress Series, 330*, 235–246. https://doi.org/10.3354/meps330235

Wellenreuther, M., Syms, C., & Clements, K.D. (2008). Consistent spatial patterns across biogeographic gradients in temperate reef fishes. *Ecography*, *31*, 84-94. https://doi.org/10.1111/j.2007.0906-7590.05270

West, J. L., Bailey, J. R., Almeida-Val, V., Val, A. L., Sidell, B. D., & Driedzic, W. R. (1999). Activity levels of enzymes of energy metabolism in heart and red muscle are higher in north-temperate-zone than in Amazonian teleosts. *Canadian Journal of Zoology, 77*(5), 690–696. https://doi.org/10.1139/z99-016

Whitehouse, D. G., May, B., & Moore, A. L. (2019). Respiratory chain and ATP synthase. In *Reference Module in Biomedical Sciences*. Elsevier. https//doi.org /10.1016/B978-0-12-801238-3.95732-5

Willis, J. R., Hickey, A. J. R., & Devaux, J. B. L. (2021). Thermally tolerant intertidal triplefin fish (Tripterygiidae) sustain ATP dynamics better than subtidal species under acute heat stress. *Scientific Reports*, *11*(1), 11074. https://doi.org/10.1038/s41598-021-90575-y

Wodtke, E. (1981). Temperature adaptation of biological membranes. The effects of acclimation temperature on the unsaturation of the main neutral and charged phospholipids in mitochondrial membranes of the carp *(cyprinus carpio* L.). *Biochimica et Biophysica Acta – Biomembranes*, *640*(3), 698–709. https://doi.org/10.1016/0005-2736(81)90100-0

Yampolsky, L. Y., Zeng, E., Lopez, J., Williams, P. J., Dick, K. B., Colbourne, J. K., & Pfrender, M. E. (2014). Functional genomics of acclimation and adaptation in response to thermal stress in *Daphnia*. *BMC Genomics*, *15*(1), 859. https://doi.org/10.1186/1471-2164-15-859

Young, M. D., Wakefield, M. J., Smyth, G. K, & Oshlack, A. (2010). Gene ontology analysis for RNA-seq: Accounting for selection bias. *Genome Biology*, *11*, 14. https://doi.org/10.1186/gb-2010-11-2-r14

Zhao, H., Ke, H., Zhang, L., Zhao, Z., Lai, J., Zhou, J., Huang, Z., Li, H., Du, J., & Li, Q. (2022). Integrated analysis about the effects of heat stress on physiological responses and energy metabolism in *Gymnocypris chilianensis. Science of The Total Environment, 806*, 151252. https://doi.org/10.1016/j.scitotenv.2021.151252

Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., Babenko, V. A., Zorov, S. D., Balakireva, A. V., Juhaszova, M., Sollott, S. J., & Zorov, D. B. (2018). Mitochondrial membrane potential. *Analytical Biochemistry, 552*, 50-59. https://doi.org/10.1016/j.ab.2017.07.009

Supplemental Material

New Zealand triplefin sampling locations

Figure S1. A map of sampling sites across the East Otago Tāiāpure where the common triplefin (*F. lapillum*) and estuarine triple (*F. nigripenne*) study specimens were collected. Black dots indicate sampling locations. Map taken from Google Maps.

Long-term temperature monitoring data from sampling locations

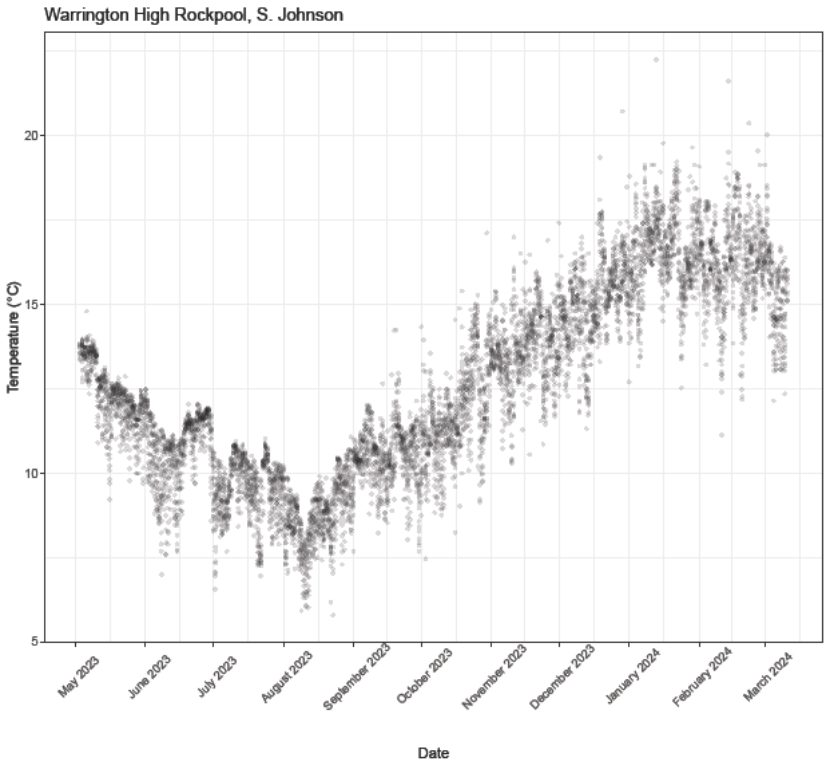

Figure S2. Long-term temperature monitoring data taken from a high-tide zone rockpool in Warrington, located on the East Coast of the South Island of New Zealand. Dots represent individual temperature recordings taken across a period of 11 months from May 2023 until March 2024.

Experimental controls for fluorescent probes

Before running assays, controls were conducted for the TMRM and the MgG probe. Controls for TMRM were completed by inundating a section of brain tissue with the probe and running an assay at room temperature. After a 5-minute run-in period, oligomycin was added at 2 uL to the tissue. This addition caused a rise in fluorescence intensity as oligomycin inhibits ATP synthesis, leading to membrane hyperpolarisation. After another 5 minutes with the oligomycin, Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added at 5 uL to the tissue. As CCCP acts as a

mitochondrial uncoupling agent by inhibiting oxidative phosphorylation, the addition of CCCP caused membrane potential to depolarise and, therefore, fluorescence to decline. These same two reagents were added to tissue inundated with MgG following the same protocols as for TMRM. Due to the nature of these reagents, when added to the MgG probe, the CCCP caused no change in fluorescence due to having no effect on the amount of ATP present, whilst oligomycin caused fluorescence to rise, indicating a decline in the amount of ATP.

Effect of acclimation time on brain mitochondrial performance

Results

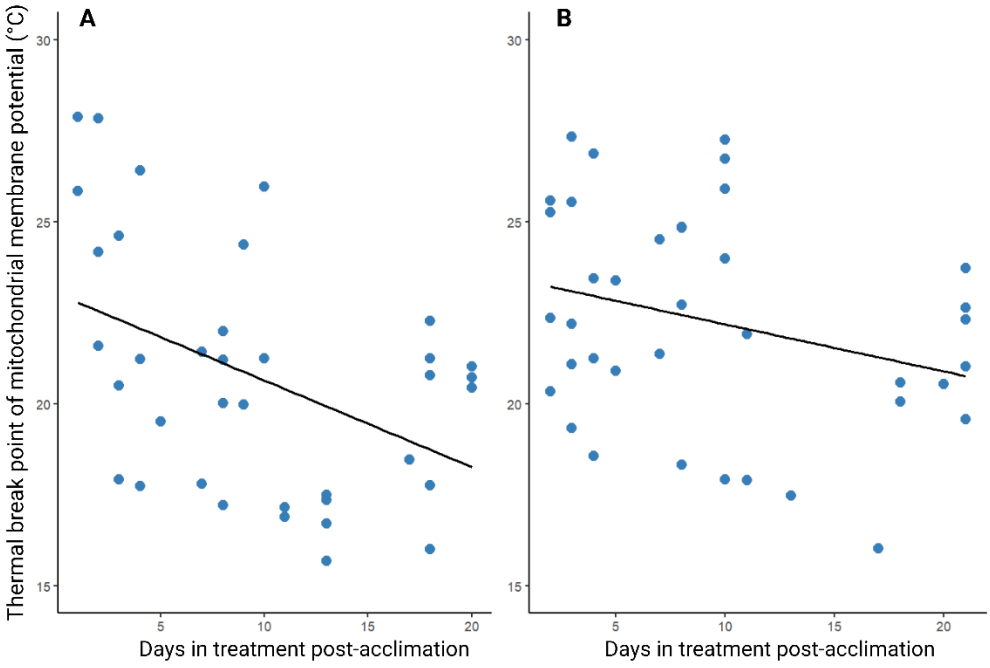

Figure S3. The influence of acclimation period on thermal breakpoints of mitochondrial membrane potential in the brain tissue of (A) estuarine triplefin, *Forsterygion nigripenne* and (B) common triplefin, *Forsterygion lapillum* based on the length of time fish spent within the experimental temperature treatments after a four-week acclimation period, before being assayed.

n = 37 *F. nigripenne* and *n* = 39 *F. lapillum*, total *n* = 76. Thermal break points were derived from individual fish using SegReg (95% CI).

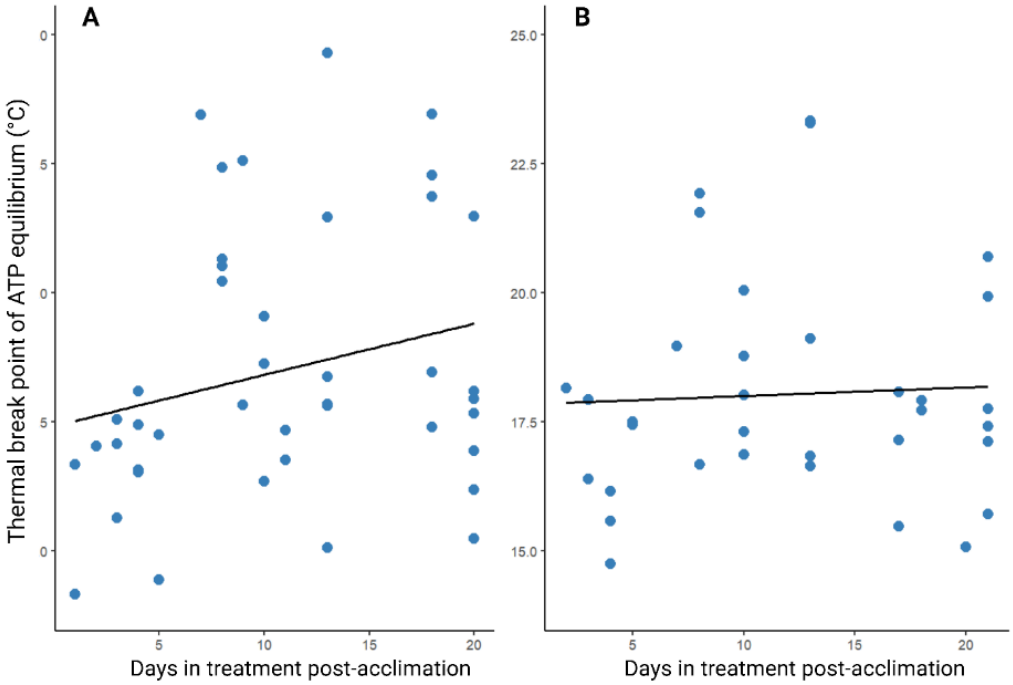

Figure S4. The influence of acclimation period on thermal breakpoints of ATP equilibrium in the brain tissue of (A) estuarine triplefin, *Forsterygion nigripenne* and (B) common triplefin, *Forsterygion lapillum* based on the length of time fish spent within the experimental temperature treatments after a four-week acclimation period, before being assayed. *n* = 42 estuarine triplefin and *n* = 34 common triplefin, total *n* = 76. Thermal break points were derived from individual fish using SegReg (95% CI).

Effect of condition factor (K) on brain mitochondrial performance

A condition factor (K) was calculated for each fish using the following equation:

$$K = \frac{100W}{L^3}$$

W is the weight measured in grams, whilst *L* represents the standard length of each fish in cm. ANOVA tests were run using the statistical software R version 4.3.0 (R Core Team, 2023) to compare condition factors between acclimation treatments for both species. There were no significant differences in condition factor across temperature treatments for either species

(estuarine triplefin: $F_{3,44} = 0.47$, $p = 0.70$; common triplefin: $F_{3, 48} = 1.20$, $p = 0.32$), and we did not use this variable in any further analyses.

Sex effects on brain mitochondrial performance

Results

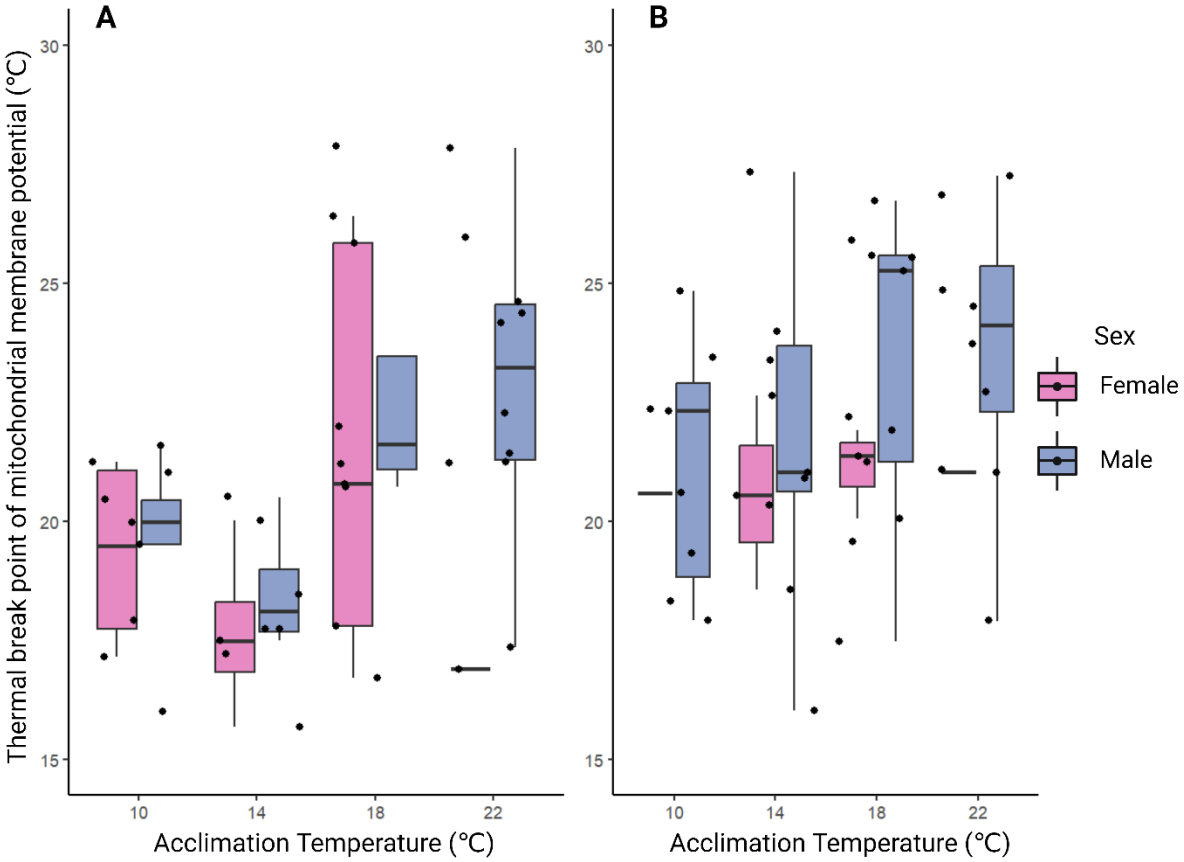

Figure S5. Thermal breakpoints of mitochondrial membrane potential in the brain tissue of (A) Estuarine triplefin, *Forsterygion nigripenne*, and (B) Common triplefin, *Forsterygion lapillum*, male and female fish after an acclimation period of four to eight weeks in one of four experimental temperature treatments: 10°C (Control), 14°C, 18°C and 22°C. Sample sizes for estuarine triplefins ranged from n = 9-11 fish per treatment and for common triplefins from n = 8-12 fish per treatment. Total n = 76 fish. Thermal break points were derived from individual fish using SegReg (95% CI). Dots represent individual break points, whilst boxes depict the distribution of the data for each temperature treatment. Different coloured boxes represent the different sexes with sample sizes for estuarine triplefin ranging from n = 1-5 females per

treatment, *n* = 4-10 males per treatment and for the common triplefin from *n* = 1-3 females per treatment, *n* = 7-9 males per treatment.

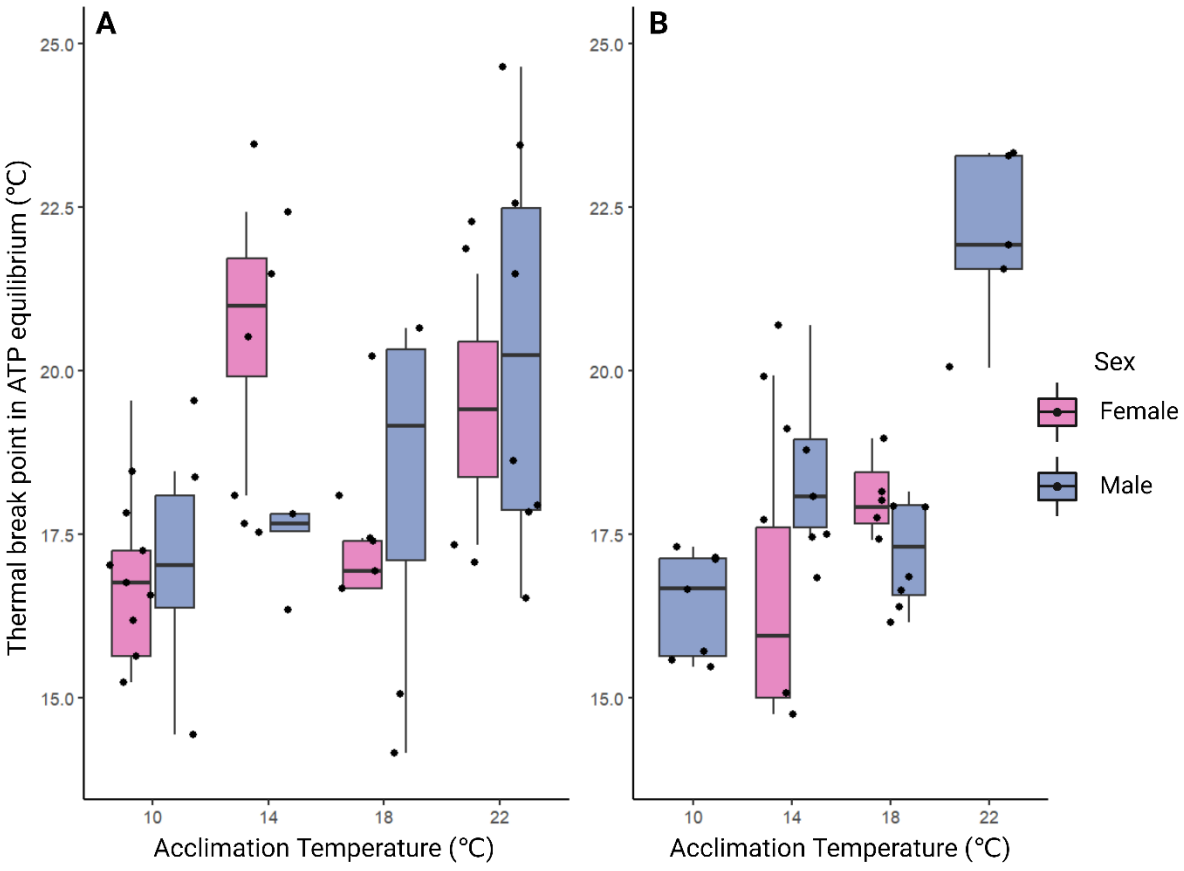

Figure S6. Thermal breakpoints of ATP equilibrium in the brain tissue of (A) Estuarine triplefin, *Forsterygion nigripenne,* and (B) Common triplefin, *Forsterygion lapillum,* male and female fish after an acclimation period of four to eight weeks in one of four experimental temperature treatments: 10°C (Control), 14°C, 18°C and 22°C. Sample sizes for estuarine triplefins ranged from *n* = 9-12 fish per treatment and for common triplefins ranged from *n* = 5-11 fish per treatment. Total *n* = 76 fish. Thermal break points were derived from individual fish using SegReg (95% CI). Dots represent individual break points, whilst boxes depict the distribution of the data for each temperature treatment. Different coloured boxes represent the different sexes with sample sizes for estuarine triplefin ranging from *n* = 2-5 females per treatment, *n* = 4-10 males per treatment and for the common triplefin from *n* = 0-4 females per treatment, *n* = 5-8 males per treatment.

Multi-dimensional scaling plot of brain transcriptomes

Results

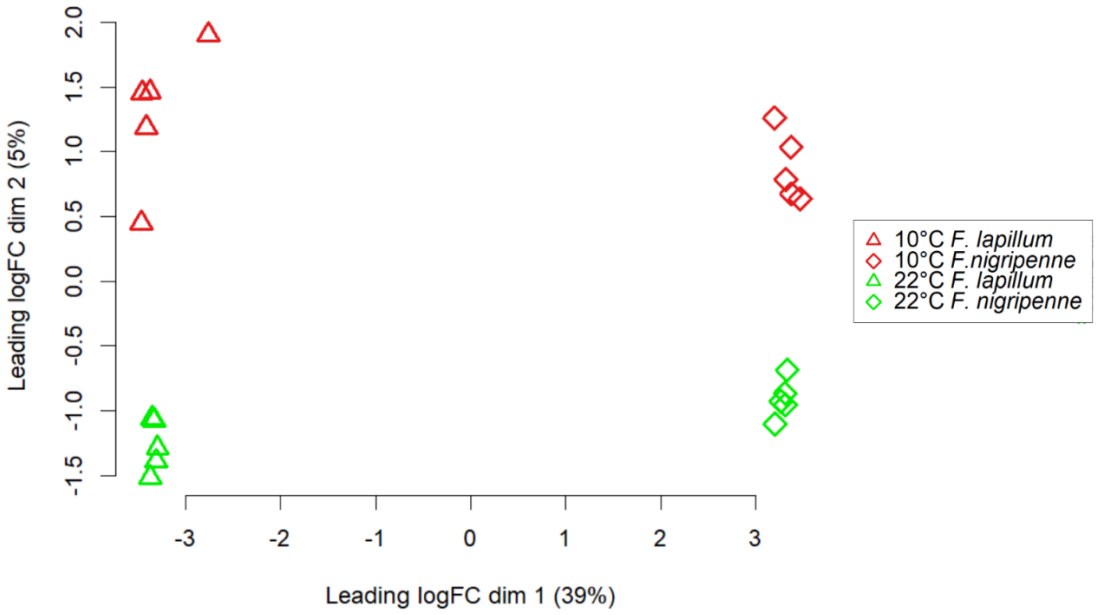

Figure S7. Multi-dimensional scaling plot of variation in gene expression data amongst RNA-seq samples taken from the brain tissue of common triplefins, *Forsterygion lapillum* (triangles) and estuarine triplefins, *Forsterygion nigripenne* (diamonds) from the South Island of New Zealand. Fish were exposed to 10℃ control temperatures (red circles, n = 10 sample) or 22℃ elevated temperatures (green circles, n = 10 samples) for an acclimation period of eight weeks (total n = 20 samples). Distance between samples indicates dissimilarity of gene expression.

Significant Gene Ontology Terms

Results

Table S1. Table of all significantly overrepresented gene ontology terms observed in the brain tissue of estuarine triplefin, *Forsterygion nigripenne*, and common triplefin, *Forsterygion lapillum* after being experimentally exposed to warm 22 ℃ temperatures compared to control (10 ℃) temperatures (n = 5 samples per tissue and species). Fish were exposed for an acclimation period of eight weeks. Significant terms are those which passed the false discovery rate threshold (q-value) of 0.05. Table indicates within which warm acclimated species and tissues enriched gene ontology terms were observed.

Gene Ontology ID	***Gene Ontology Term***	***Species***	***q-value***
GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	Estuarine triplefin	0.03458
GO:0003341	Cilium movement	Common triplefin	0.02938
GO:0003700	DNA-binding transcription factor activity	Estuarine triplefin	0.01823
GO:0004497	Monooxygenase activity	Estuarine triplefin	0.0382
GO:0004565	Beta-galactosidase activity	Estuarine triplefin	0.01823
GO:0005576	Extracellular region	Common triplefin	0.0286
		Common triplefin	0.01437
GO:0005615	Extracellular space	Estuarine triplefin	0.02742
GO:0005879	Axonemal microtubule	Common triplefin	0.01922
GO:0005886	Plasma membrane	Estuarine triplefin	0.00186
GO:0005911	Cell-cell junction	Estuarine triplefin	0.03458
GO:0005929	Cilium	Common triplefin	0.00127
GO:0006690	Icosanoid metabolic process	Estuarine triplefin	0.03458
GO:0006952	Defense response	Common triplefin	0.04245
GO:0006954	Inflammatory response	Common triplefin	0.01922
GO:0008395	Steroid hydroxylase activity	Estuarine triplefin	0.01823
GO:0009648	Photoperiodism	Common triplefin	0.02938
GO:0009888	Tissue development	Estuarine triplefin	0.02352
GO:0015925	Galactosidase activity	Estuarine triplefin	0.01823
GO:0016712	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	Estuarine triplefin	0.03458
GO:0016742	Hydroxymethyl-, formyl- and related transferase activity	Common triplefin	0.03629
GO:0019228	Neuronal action potential	Estuarine triplefin	0.02137
GO:0019842	Vitamin binding	Estuarine triplefin	0.03358
GO:0030054	Cell junction	Estuarine triplefin	0.03713
GO:0030170	Pyridoxal phosphate binding	Estuarine triplefin	0.02352
GO:0030368	Interleukin-17 receptor activity	Common triplefin	0.01922
GO:0030431	Sleep	Estuarine triplefin	0.03458
GO:0031514	Motile cilium	Common triplefin	0.00744
GO:0031974	Membrane-enclosed lumen	Common triplefin	0.01922
GO:0038024	Cargo receptor activity	Estuarine triplefin	0.03458
GO:0042383	Sarcolemma	Estuarine triplefin	0.03458

GO:0043233	Organelle lumen	Common triplefin	0.01922
GO:0045124	Regulation of bone resorption	Estuarine triplefin	0.03458
GO:0045780	Positive regulation of bone resorption	Estuarine triplefin	0.03458
GO:0046852	Positive regulation of bone remodelling	Estuarine triplefin	0.03458
GO:0048029	Monosaccharide binding	Common triplefin	0.01922
GO:0050900	Leukocyte migration	Common triplefin	0.04535
GO:0070013	Intracellular organelle lumen	Common triplefin	0.01922
GO:0070279	Vitamin B6 binding	Estuarine triplefin	0.02461
GO:0070330	Aromatase activity	Estuarine triplefin	0.03458
GO:1900029	Positive regulation of ruffle assembly	Estuarine triplefin	0.0382
GO:2000338	Regulation of chemokine (C-X-C motif) ligand 1 production	Common triplefin	0.01922
GO:2000340	Positive regulation of chemokine (C-X-C motif) ligand 1 production	Common triplefin	0.01922

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