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Adaptation of plasticity to predicted climates in Australian rainbowfishes (*Melanotaenia*) across climatically defined bioregions

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Abstract

Resilience to environmental stressors due to climate warming is influenced by local adaptations, including the capacity for plastic responses. The recent literature has focussed on genomic signatures of climatic adaptation, however little work has been done to address how plastic capacity may be influenced by biogeographic history and evolutionary processes. Here, we investigate phenotypic plasticity as a target of climatic selection, hypothesising that lineages that evolved under warmer climate will exhibit greater plastic adaptive resilience to thermal stress. This was tested using common garden experiments to compare gene expression regulation within and among a temperate, a subtropical and a desert ecotype of Australian rainbowfish. Individuals from each ecotype were subjected to contemporary and projected summer thermal conditions for 2070, and their global patterns of gene expression were characterized using liver transcriptomes. Critical thermal maximums were also determined for each ecotype to assess thermal tolerance. A comparative phylogenetic expression variance and evolution model framework was used to assess plastic and evolved changes in gene expression. Similar changes in both the direction and the magnitude of expressed genes were found within ecotypes. Although most expressed genes were identified in all ecotypes, 532 genes were identified as candidates subject to ecotype-specific directional selection. Twenty-three of those genes showed signal of adaptive (i.e. genetic-based) plastic response to future increases in temperature. Network analyses demonstrated centrality of these genes in thermal response pathways, along with several highly conserved hub genes thought to be integral for heat stress responses. The greatest adaptive resilience to warming was shown by the subtropical ecotype, followed by the desert and temperate ecotypes. Our findings indicate that vulnerability to climate change will be highly influenced by biogeographic factors, and we stress the need for integrative assessments of climatic adaptive traits for accurate estimations of population and ecosystem responses.

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47 **Introduction**

48 Characterizing mechanisms underpinning variation in ecological adaptation can assist in
49 identifying biogeographic patterns of vulnerability and resilience to environmental change.
50 Climate change has promoted numerous range shifts and local extinctions due to exposure of
51 populations to conditions outside their zones of tolerance (Grabherr, Gottfried, & Pauli, 2009;
52 Parmesan et al., 1999; Thomas & Lennon, 1999; Wiens, 2016). However, it is expected that
53 some populations will be able to persist *in situ* if they are not already living at the edge of their
54 tolerance limits; or, if they are able to acclimatise or adapt outside their current range of
55 tolerance (Catullo, Ferrier, & Hoffmann, 2015; Hoffmann & Sgro, 2011; Stillman, 2003;
56 Sunday, Bates, & Dulvy, 2011, 2012). Species' distributions are strongly influenced by thermal
57 conditions in their native climates; it is expected that tolerance ranges and vulnerability to
58 change will also be influenced by biogeographic factors (Addo-Bediako, Chown, & Gaston,
59 2000; Calosi, Bilton, Spicer, Votier, & Atfield, 2010; Cohet, Vouidibio, & David, 1980;
60 Compton, Rijkenberg, Drent, & Piersma, 2007). Exploring how molecular mechanisms
61 influence thermal resilience and, ultimately, the evolution of divergent thermal phenotypes, is
62 an important step for inferring responses to a warming environment (Komoroske, Connon,
63 Jeffries, & Fangue, 2015). While evidence suggests that plastic regulation of gene expression
64 plays an important role in ecological adaptation, the effects of selection on plasticity are so far
65 poorly understood and successfully untangling them is likely to require integrative approaches
66 (Gilad, Oshlack, & Rifkin, 2006; Jin et al., 2001; McCairns & Bernatchez, 2012).

67

68 Plasticity can be described as a change in expressed phenotype as a function of the
69 environment, and occurs through direct effects of the environment on allelic expression, as well
70 as changes in interactions among loci (Nonaka, Svanbäck, Thibert-Plante, Englund, &
71 Brännström, 2015; Scheiner, 1993). Here, we focus on plasticity as the ability or tendency of

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an individual to up- or down-regulate genes in response to the environment. For many genes, this occurs primarily at the level of transcription, and a complexity of plastic responses (i.e. adaptive, maladaptive or neutral) have been observed in regard to individual fitness (Ghalambor, McKay, Carroll, & Reznick, 2007; Gibert, Debat, & Ghalambor, 2019; Marden, 2008). For instance, plasticity can act as a buffer against environmental pressures, providing short-term advantage but potentially dampening the effects of natural selection (Ghalambor et al., 2007; Grenier, Barre, & Litrico, 2016). Plastic responses have also been found to increase the potential for colonisation of new areas and provide greater likelihood of adaptive radiation (Muschick, Barluenga, Salzburger, & Meyer, 2011; Pfennig et al., 2010; Wellband & Heath, 2017; Wund, 2012). Plasticity itself can be a target of selection if genotypes differ in their sensitivity to environmental variation (Fusco & Minelli, 2010), including selection driving rapid adaptive evolution of genes exhibiting non-adaptive plasticity (Ghalambor et al., 2015).

In the context of climate, studies of gene expression can inform about the functional pathways relevant for persistence under a given condition, as well as the likely targets of selection (Komoroske et al., 2015; Reed, Schindler, & Waples, 2011). This is especially important where phenotypes of ecological relevance are not obvious, and may be difficult to distinguish using traditional approaches (Nevins & Potti, 2007; Nosil, 2012). While a variety of methods have been developed to detect evidence of ecological adaptation at the genomic level, relatively few studies have so far attempted to find signals of selection acting on gene expression. Challenges include controlling for the large range of internal and external environmental variables influencing expression (Conesa et al., 2016; De Wit et al., 2012), as well as the effects of genetic distance which are typically expected to account for much of the variation in transcription observed between lineages (Dunn, Luo, & Wu, 2013).

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Climatically defined bioregions provide a scale at which environmental variation drives meaningful differences in evolutionary and ecological processes (Fine, 2015; Jetz & Fine, 2012). The ability of populations to persist under climate warming is predicted to vary geographically (Aitken & Whitlock, 2013; Catullo et al., 2015; Ghalambor, Huey, Martin, Tewksbury, & Wang, 2006; Polato et al., 2018; Sorte, Jones, & Miller, 2011; Thomas et al., 2004), making climatic bioregions valuable systems for comparative studies of adaptation. For instance, the climatic variability hypothesis (CVH) predicts a positive relationship between breadth of thermal tolerance and the level of climatic variability experienced by organisms as latitude increases (Janzen, 1967). Studies of climate change impacts are increasingly seeking to integrate spatial modelling (e.g. climatic envelopes) to uncover associations between landscape features and evolutionary processes such as temperature adaptation (Araújo, Whittaker, Ladle, & Erhard, 2005; Cianfrani, Satizábal, & Randin, 2015; Summers, Bryan, Crossman, & Meyer, 2012). While a majority of species distribution models are primarily correlative, there has been an urgent call for an increase in mechanistic approaches for predicting species' responses to climate change (Bay et al., 2017; Cavaleri, Reed, Smith, & Wood, 2015; Comte & Olden, 2016; Evans, Merow, Record, McMahon, & Enquist, 2016; Mathewson et al., 2017). Mechanistic approaches have the advantage of explaining the underlying processes associated with observed trends, minimising the risk of flawed extrapolation (Dormann et al., 2012; Kearney & Porter, 2009). On a molecular scale, this is important for disentangling the respective effects of adaptation and phylogenetic signal, as well as the interactions between these two factors (Comte & Olden, 2016; Hoffmann, Chown, & Clusella- Trullas, 2013).

Freshwater ecosystems are of particular interest when studying the impacts of climate change due to their vulnerability. They are dependent on the interrelated influences of temperature and

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121 precipitation, and their spatially fragmented and often linear nature reduces opportunities for
122 organismal dispersal. Freshwater fishes represent an important component of vertebrate
123 diversity and, as ectotherms, are especially vulnerable to thermal changes (Deutsch et al.,
124 2008). The subjects of this study are Australian rainbowfishes of *Melanotaenia* (family
125 *Melanotaeniidae*), a freshwater genus with historical origins in tropical southern New Guinea
126 (McGuigan, Zhu, Allen, & Moritz, 2000). *Melanotaenia* spp. of the 'australis' clade (Unmack
127 *et al.* 2013) provide an ideal model system to study climatic-driven adaptive evolution and to
128 address predictions from the CVH for freshwater ecosystems. The clade contains a minimum
129 of eight largely allopatric species that recently radiated into tropical, subtropical, desert and
130 temperate regions of mainland Australia (Unmack *et al.* 2013). Species of this clade show
131 adaptive phenotypic divergence due to selection linked to the hydrological environment
132 (McGuigan, Chenoweth, & Blows, 2005; McGuigan, Franklin, Moritz, & Blows, 2003), as
133 well as adaptive genomic divergence associated with hydroclimatic variation (Brauer, Unmack,
134 Smith, Bernatchez, & Beheregaray, 2018; McGuigan et al., 2005; McGuigan et al., 2003) In
135 terms of gene expression, common garden experiments in a subtropical 'australis' species (*M.*
136 *duboulayi*) have tested the effect of 2070-projected summer temperatures on short-term (Smith,
137 Bernatchez, & Beheregaray, 2013) and long-term (McCairns, Smith, Sasaki, Bernatchez, &
138 Beheregaray, 2016) transcriptional responses. Both studies indicated a capacity for plastic
139 response to future climates, and enabled identification of candidate genes for thermal
140 adaptation (McCairns et al., 2016; Smith et al., 2013). In addition, the transgenerational
141 experiment in *M. duboulayi* revealed pedigree-based evidence for heritability of observed
142 plastic responses (McCairns et al., 2016).

143

144 Our work focuses on three closely related 'australis' species, *M. splendida tatei*, *M. fluviatilis*
145 and *M. duboulayi*. Their ranges show a striking concordance with three major contemporary

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146 climatic bioregions of the Australian continent (Fig. 1), suggesting that their evolution has been
147 influenced by selective pressures associated with climatic regimes. For this reason, we refer to
148 them herein as climatic 'ecotypes', *sensu* Engelhard, Ellis, Payne, ter Hofstede, and Pinnegar
149 (2010). We used an experimental approach to compare short-term transcriptional responses to
150 a projected future temperature in subtropical, temperate and desert rainbowfish ecotypes. In
151 addition, physiological tolerance to thermal stress was assessed by empirically estimating the
152 critical thermal maximum of each ecotype. We hypothesise that ecotype resilience in future
153 climates will be dependent on the biogeographic region in which a given ecotype has evolved.
154 As such, we also predict to find evidence for adaptation of plastic responses to temperature
155 among ecotypes. To test this, we applied a comparative phylogenetic expression variance and
156 evolution model framework to detect transcriptional responses subject to ecotype-specific
157 directional selection. This enabled us to explore how divergent selection on gene expression
158 may have contributed to differences in thermal tolerance and to adaptive evolution in these
159 climatically defined ecotypes.

160

161 **Methods**

162 *Ecotype range, sampling and temperature experiments*

163 The evolution of divergent expression of genes related to thermal tolerance was assessed in
164 three closely related species of Australian rainbowfishes (Unmack *et al.* 2013). These are the
165 Crimson spotted rainbowfish (*Melanotaenia duboulayi*) – a species with a subtropical
166 distribution along coastal catchments of eastern Australia, the Murray River rainbowfish (*M.*
167 *fluviatilis*) – a temperate species found in the inland Murray-Darling Basin, and the desert
168 rainbowfish (*M. splendida tatei*) – a species found in arid and semi-arid catchments of central
169 Australia (Figure 1). *Melanotaenia duboulayi* individuals were collected using bait traps and

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hand nets from the upper section of the Brisbane River, near the township of Fernvale in Queensland (subtropical; 27°26'37.39"S, 152°40'12.76"E). *Melanotaenia fluviatilis* individuals were collected from the mid section of the Murray River, close to the town of Gol Gol in New South Wales (temperate; 34°10'50.3"S 142°13'16.8"E) using a seine net. *Melanotaenia splendida tatei* individuals were collected from Algebuckina Waterhole in South Australia (desert; 27°51'53.9"S 135°53'57.1"E) using fyke nets. Between 42 and 60 individuals were collected at each locality. The fish were transported live to the Flinders University animal rearing facility and acclimatised at 21°C for a minimum of 60 days prior to the start of temperature trials. Individuals from each species were maintained in single sex aquaria (~20 fish/ 100L) at 21°C under conditions of 12 h light/12 h dark and fed once daily on a mixture of blood worms and fish pellets. To assess short-term responses to contemporary (21°C) and 2070-projected (33°C) average Australian summer temperatures, individuals of each species were randomly assigned to a treatment or a control group (n = 6 per group, per species). Temperature in these 'climate-change treatment' groups was increased by 2°C per day over a period of six days towards the target of 33°C, and then maintained for 14 days. The temperature (33°C) is the projected average summer temperature for Australia's east coast in 2070 based on a high emission scenario (RCP8.5) of the International Panel on Climate Change (CSIRO, 2016; Smith et al., 2013). Control groups were kept at 21°C for the duration of the experiment. Fish were euthanized in an overdose of AQUI-S® solution (50% isoeugenol) and immediately dissected to extract the liver. Sampling procedures took place in the same period of the day, between 9:00 am and 11:00 am. Only adult males of similar length were used to control for sex and age-related effects on transcription responses. Liver tissue was incubated at 4°C for 12 hr in RNAlater (Ambion) as per manufacturer's instructions before storage at -80°C. In addition to being a

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194 relatively homogenous tissue, liver was selected because metabolic conditioning and gene
195 expression is known to respond to heat stress (McCairns et al., 2016; Smith et al., 2013).

196

197 *RNA extraction, Illumina libraries preparation and sequencing*

198 Total RNA was extracted from each individual liver tissue sample using the Ambion
199 Magmax™-96 total RNA isolation kit (Life Sciences) according to the manufacturer's
200 instructions and following Smith et al (2013). Integrity and concentration were evaluated
201 with an RNA Nano assay kit on an Agilent Bioanalyzer 2100 (Agilent Technologies) and
202 purity assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Normalised
203 quantities of total RNA were then used to prepare 36 separate Illumina sequencing libraries
204 with the TruSeq™ RNA sample preparation kit (Illumina) using the adapter indices supplied
205 by the manufacturer (Illumina MID tags 2, 4–7, 12–16, 18, 19) and following Gates, Sandoval-
206 Castillo, Bernatchez, and Beheregaray (2017). Individual libraries were normalised and pooled
207 together in groups of 12 samples. The resulting three pools were each sequenced in separate
208 100 bp paired-end lanes in an Illumina HiSeq2000 at Génome Québec Innovation Centre in
209 Montreal, Canada.

210

211 *Read filtering,* de novo *assembly and annotation*

212 Sequence data were demultiplexed by individual and trimmed of indexing adaptors. Low-
213 quality (Q<20) bases were trimmed, then remnant adapter sequences, low quality reads, and
214 reads shorter than 45 bp were removed using TRIMMOMATIC V0.36 (Bolger, Lohse, &
215 Usadel, 2014). Four transcriptomes were assembled *de novo* with the program TRINITY
216 V2.5.1 (Grabherr et al., 2011) using a pipeline described in Gates et al. (2017). One
217 transcriptome for each ecotype was assembled using both experiment and control groups of

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each ecotype, then a *Melanotaenia* transcriptome was assembled using the samples of all ecotypes combined. The success of the completed transcriptome assembly was evaluated using read content statistics (% raw reads present), contig length distribution (N50), annotation-based metrics (% full length transcripts) and Benchmarking Universal Single-Copy Orthologs (BUSCO) software (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015) (Table S1). The open reading frames (ORFs) of a minimum length of 100 peptides were extracted from the assembled *Melanotaenia* transcriptome using the script TRANSDECODER V3.0 (Haas et al., 2013) and identified as candidate protein coding regions. Then, where two or more transcripts showed 80% or higher similarity, all but the longest transcript were removed to generate a non-redundant set of transcripts; herein referred to as 'unigenes'. The whole transcriptome was functionally annotated using the command blastx (Altschul, Gish, Miller, Myers, & Lipman, 1990) to query uni-genes against the UniprotKB protein database (using a 1×10^{-2} *e*-value cut-off; Consortium, 2014) to identify homology to known proteins. In addition, any transcript showing 50% or higher similarity to any bacteria, fungus or virus genes were removed from further analysis.

Transcript quantification and differential expression analysis

To test for differential expression (DE) between experimental groups and among ecotypes, reads for each sample were mapped back to the predicted protein coding regions using BOWTIE2 V2.2.7 (Langmead & Salzberg, 2012), then gene-level abundance estimations were performed with RSEM V1.2.19 (Li & Dewey, 2011). To enable comparison of expression level among samples, read count estimations were cross-sample-normalized using the trimmed mean of M-values method (TMM; Robinson and Oshlack, 2010). Normalized count data were then used as input for the program DESeq2 V1.10.1 (Love, Huber, & Anders, 2014). We used a

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242 conventional threshold (e.g. McCulloch et al., 2019) specifying that transcripts with a
243 minimum log2 fold change of two between any two groups (i.e. experiment vs control, ecotype
244 vs ecotype) were considered differentially expressed at a false discovery rate of 5%.

245

246 *Gene expression plasticity and divergent selection*

247 We implemented the Expression Variance and Evolution Model (EVE) (Rohlfs & Nielsen,
248 2015) using the transcriptome of three ecotypes of *Melanotaenia* to identify transcripts
249 potentially under divergent selection for expression levels. Briefly, the model uses a
250 phylogenetic tree and expression data to estimate a parameter β that represents the ratio of
251 among-lineage expression divergence to within-lineage expression diversity. This ratio should
252 be approximately constant over most genes if no divergent selection is acting between lineages.
253 For each transcript (i), the EVE model assesses the null hypothesis that independent transcript
254 β_i is not significantly different to a shared β_s for all transcripts; if β_i is higher than β_s, the model
255 assumes that transcript i is subject to lineage-specific directional selection on expression level.
256 Following Brauer, Unmack, and Beheregaray (2017), we considered transcripts to be under
257 divergent selection when βi was significantly higher than βs at a false discovery rate of 10%.

258

259 To calculate the expected expression covariance between lineages under shared and
260 independent evolutionary history scenarios, we constructed a phylogenetic tree, using genome-
261 wide SNP (single nucleotide polymorphisms) data from 12 samples of each ecotype. These
262 data were obtained using reduced-representation sequencing methods (ddRAD) in previous
263 studies of population genomics of the three ecotypes (Brauer et al. 2018, Attard et al. in review;
264 Smith et al. in review; Supplementary Information X). The software PyRAD V3.0.6 (Eaton,
265 2014) was used to align the genome-wide sequences and RAXML V8.2.1 (Stamatakis, 2014)

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was used to perform a maximum-likelihood phylogenetic analysis, with the GTRGAMMA model and 1000 bootstrap replicates. The final concatenated dataset for the 36 rainbowfishes was based on 529 loci and 44681 bp. The consensus phylogenetic tree was used as the input phylogeny for the EVE analysis.

Gene ontology enrichment analysis and pathway network analysis

We performed an enrichment analysis on the DE genes and on the EVE candidate genes relative to all genes, using the R package TOPGO v2.34 (Alexa & Rahnenfuhrer, 2010). For this analysis, terms were considered to be enriched if they were significant in both Fisher's classic and weight tests with a P=<0.01. Moreover, to understand the relative importance of candidate and shared plastic genes in the response of rainbowfishes to heat stress, a network analysis was conducted using CYTOSCAPE V3.7 (Shannon et al., 2003). First, a protein interaction network was created from the entire DE gene set by drawing edges between genes with physical and functional interactions reported for humans and with orthologous functions in zebrafish in the STRING database (Szklarczyk et al., 2016). The relative importance of a protein is correlated with its connectivity in an interactive network. We calculated the node degrees as an estimator of protein connectivity. Then we identified highly connected genes (hubs) as those with a node degree greater than or equal to the sum of the mean plus twice the standard deviation of the node degree distribution (Rakshit, Rathi, & Roy, 2014).

Determination of thermal tolerance (CT_{MAX})

We determined the thermal tolerance of each ecotype via short-term CT_{MAX} experiments following Becker and Genoway (1979). To control for sex and age-related effects, we collected 10 females of each ecotype from the same populations used for the transcriptomic experiments.

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After acclimation to 21°C for a minimum period of 60 days, each fish was placed individually in a 5 L glass beaker containing 3L of water at 21°C. Water temperature was increased at a rate of approximately 1°C every 3 minutes (rate of 0.33°C/min) using a digital water bath SWBD (Stuart®). We stopped the experiment and recorded the temperature when the fish showed both motor disorganization and loss of equilibrium for a period of one minute. Motor disorganization was considered when the swimming pattern was evidently changed with respect to their original condition; loss of equilibrium was determined when the fish turned upside down while swimming. Thermal limit for a given ecotype was obtained by averaging over 10 independent replicates. An ANOVA test was used to assess statistical differences in CT_{MAX} among ecotypes.

Results

Transcriptome sequencing and assembly

Illumina sequencing of the 36 individual libraries produced over 848 million paired-end reads (2 x 100 bp). After trimming and quality filtering, 741 million reads (87.4%) were retained (Table S1) for the *de novo* assemblies of each of the three ecotypes as well as the combined assembly for the genus *Melanotaenia*. The *Melanotaenia* assembly resulted in a total of 457,235 contigs ('Trinity transcripts'), and 269,386 genes ('Trinity genes') from which 37,160 ORFs were detected and 34,815 uni-genes were identified (Table S1). Based on all transcript contigs, an N50 of 1702 and an average contig length of 904 were achieved. Assembly completeness assessment using BUSCO found a high percentage of genes in common with fish gene datasets (e.g. 89.2% for *Melanotaenia*, Fig. S1). All downstream analyses were based on the reference set of 34,815 *de novo* assembled uni-genes.

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314

315 *Differential expression analysis*

316 Of the 34,815 uni-genes, over 81% (28,483) were present in all three *Melanotaenia* ecotypes,
317 with low percentages of uni-genes exclusive to each ecotype (Fig. 2A). Comparison of gene
318 expression profiles among ecotypes and between climate-change treatments identified 2,409
319 differentially expressed (DE) uni-genes. Expression profiles of these genes showed a strong
320 phylogenetic pattern with individuals showing the highest correlation of transcription
321 responses within ecotypes, followed by high correlation between experiment and control
322 groups within each ecotype (Fig. 2B, see also below). On the other hand, when gene expression
323 was compared between experimental treatments, only 236 DE uni-genes were identified (Fig.
324 3A). Of these, 16 uni-genes were DE between treatments in two or more ecotypes (Fig. 3B),
325 indicating shared plasticity for those genes. In contrast, unique plastic responses to projected
326 summer temperatures were observed for the temperate ecotype in 27 uni-genes, the desert
327 ecotype in 84 uni-genes and the subtropical ecotype in a much higher 109 uni-genes. This
328 indicates a strong effect of phylogeny on plastic gene expression but may also represent the
329 effects of divergent selection and adaptation to unique climatic ecoregions.

330

331 *Divergent selection on gene expression*

332 The RAXML majority-rule consensus tree provided strong support for reciprocal monophyly
333 of each ecotype (i.e. each named taxon), with all individuals from each ecotype clustering
334 within a single clade (Fig. 1B). The topology is consistent with previous studies (McGuigan et
335 al., 2000) that indicated a sister relationship between the temperate (*M. fluviatilis*) and the
336 subtropical (*M. duboulayi*) ecotypes. This consensus tree was used as the input phylogeny for
337 the EVE analysis (Fig. 1B). Of the 34,815 uni-genes assessed with the EVE, 532 were

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identified as candidates subject to lineage-specific directional selection on expression level (FDR 10%). These were the genes that showed greater expression variance among rather than within lineages after controlling for phylogenetic effects. The dendrogram of individuals based on these genes was consistent with phylogenetic patterns (Fig. S2) which showed greater differentiation between subtropical and desert ecotypes. Out of these 532 candidate genes, 23 were also identified as differentially expressed between treatments (Fig. 4A). The expression profiles of these genes are more similar between the same treatment for subtropical and temperate ecotypes, than between among treatments within the same ecotype. This was not the case for the desert ecotype, for which control and experiment are differentiated, yet cluster together. Only one of these 23 EVE candidate genes is DE between treatments in all ecotypes. This suggests that the plastic gene expression responses for these 23 genes are under divergent selection for resilience to heat stress among ecotypes, with the greatest differences between desert and the other two ecotypes.

Functional annotation, enrichment analysis and network analysis

Uni-genes aligned to UniprotKB resulted in 25,315 protein hits, of which 24,276 (96%) were assigned to a total of 293,781 GO terms (Supplementary Information Table S6). Enrichment analysis of GO terms assigned to the 236 DE uni-genes between experiment and control (Fig. 3A) found terms for five molecular functions (MF), 13 biological processes (BP) and five cellular components (CC) significantly enriched ($p < 0.01$; Table S3). The same enrichment analysis using the 23 EVE candidates for divergent selection that were also identified as DE between treatments (Fig. 4A) found three MF, four BP and two CC terms significantly enriched ($p<0.01$; Table S4).

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362 The protein network analysis identified six genes with high degree of interaction, all of which
363 were heat shock proteins. These hub genes included the only uni-gene identified as DE in all
364 three ecotypes, and as a candidate for divergent selection by the EVE model (Fig. 4B; Table
365 S5). In addition, the 16 uni-gene candidates for shared plasticity found to be DE between
366 treatments in two or more ecotypes, as well as the 23 EVE candidate genes related to heat stress
367 response between treatments, showed higher average node degrees compared with the rest of
368 the DE genes (Table S5). This suggests an important role of these genes in plastic and adaptive
369 heat stress responses of rainbowfish, respectively.

370

371 *Empirical thermal tolerance (CT_{MAX})*

372 Thermal tolerance was significantly different among ecotypes ($p = <0.001$, Table S2, Fig 5)
373 with the highest CT_{MAX} shown by the subtropical ecotype (38.0°C; CI=37-38.6°C), followed
374 by the desert ecotype (37.2°C; CI=36.1-37.6°C) and finally the temperate ecotype (34.9°C;
375 CI=33.1-36.5°C). Interestingly, the inferred estimates of CT_{MAX} across ecotypes were
376 correlated with the number of DE uni-genes between climate change treatments displayed by
377 each ecotype ($r= 0.998$, Fig. 5).

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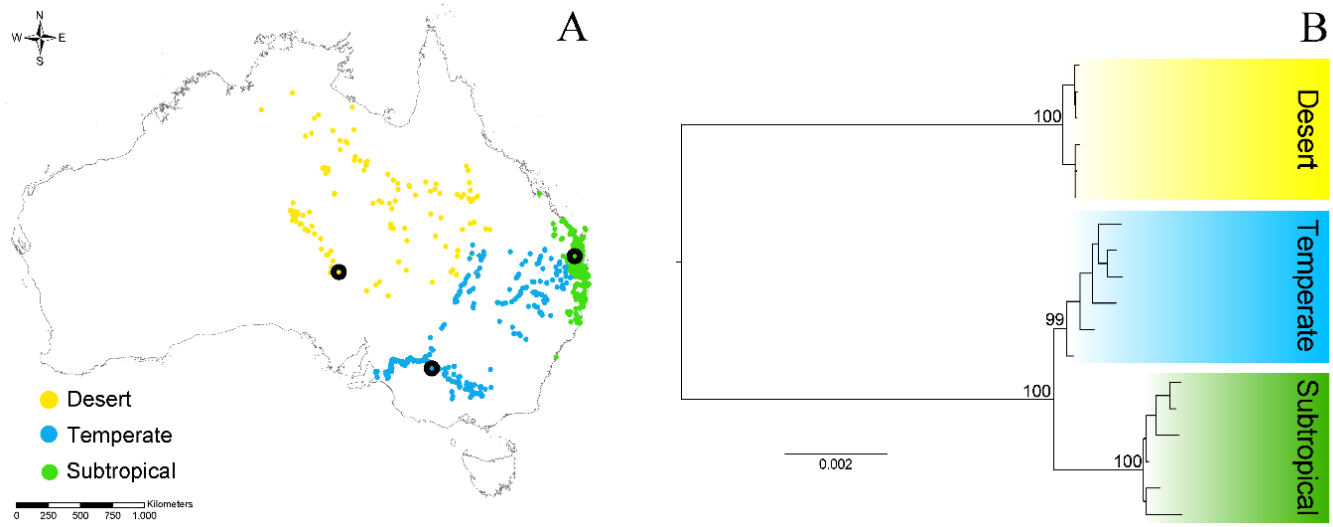

Fig. 1 **A)** Spatially-validated records and range of the three *Melanotaenia* ecotypes, black circles show sampling localities for the transcriptomic and physiological experiments. **B)** Maximum Likelihood tree depicting evolutionary relationships among 36 individuals of the three ecotypes based on ddRAD sequences of 529 loci and 44681 bp. Numbers above nodes denote bootstrap support values.

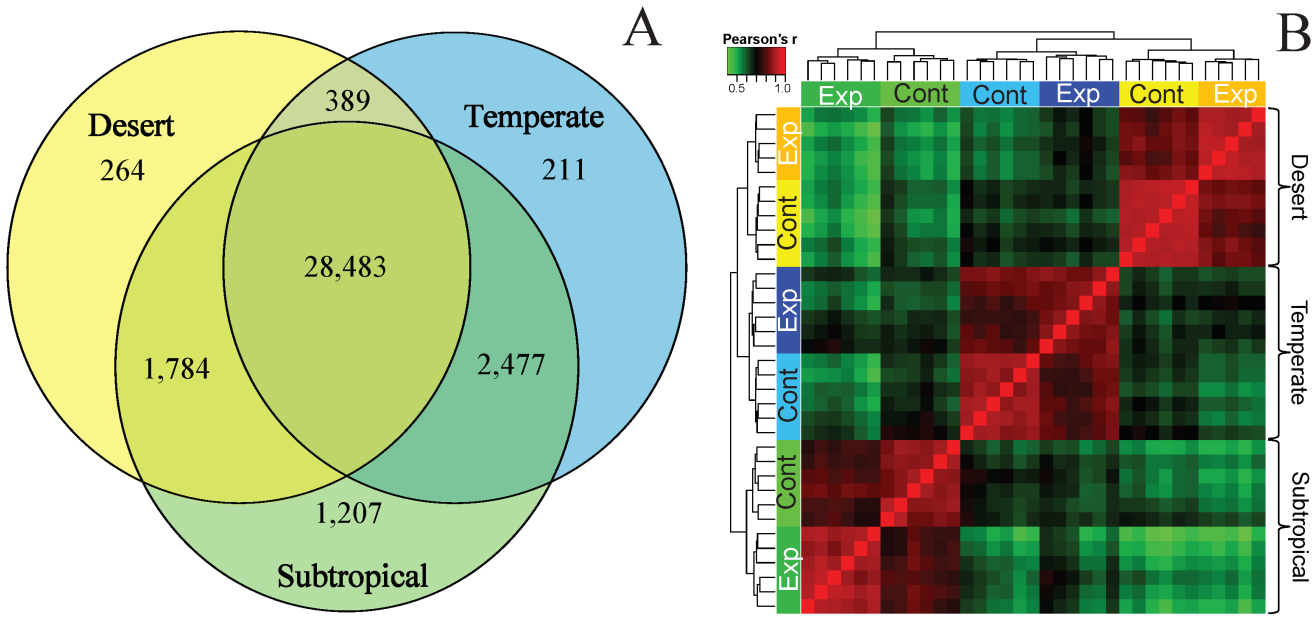

Fig. 2 **A)** Venn diagram of uni-genes identified in each ecotype of *Melanotaenia* as well as shared among ecotypes (based on a total of 34,815 uni-genes). **B)** Heatmap summarizing correlation among ecotypes in $\log_2$ gene expression profiles. This analysis was based on 2,409 differentially expressed transcripts. Coloured bars under the sample dendrograms represent the climate-change experiment (Exp) and control (Cont) groups.

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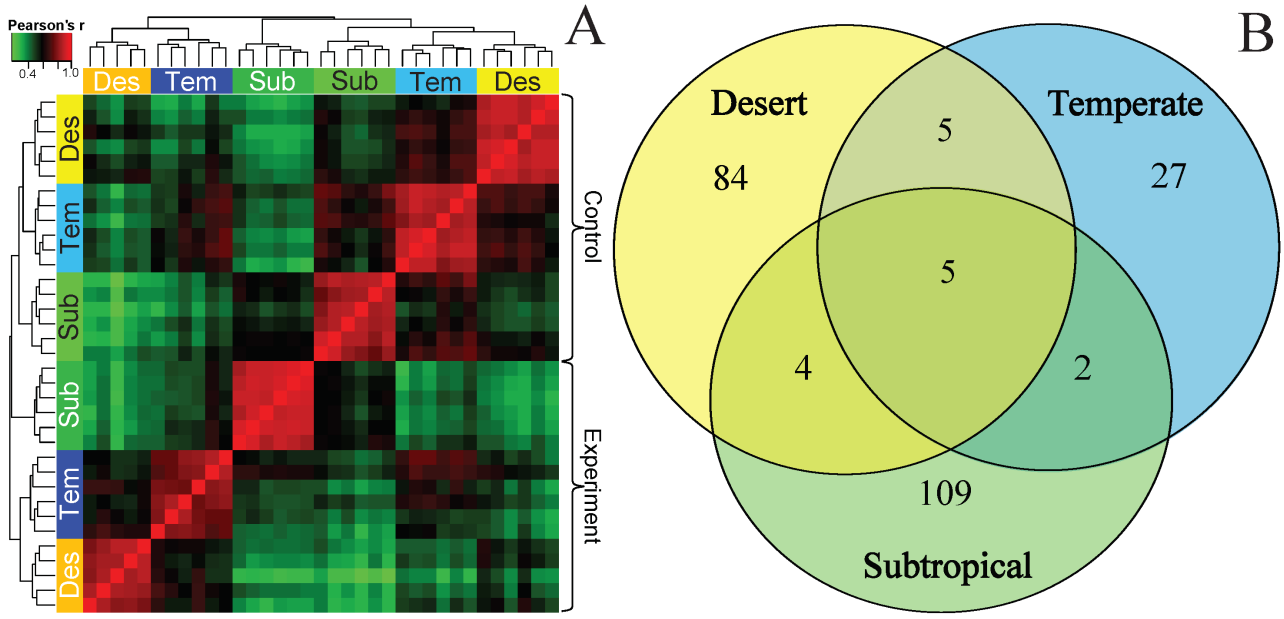

Fig. 3 **A)** Heatmap summarizing correlation between treatments (Control vs Experiment) in log2 gene expression profiles. This analysis was based on 236 differentially expressed uni-genes identified between Control and Experiment samples. Coloured bars under the sample dendrograms represent the ecotypes, with climate-change experiment groups represented by dark colour variation and control groups represented by light colour variation. **B)** Venn diagram of differentially expressed uni-genes shared between ecotypes.

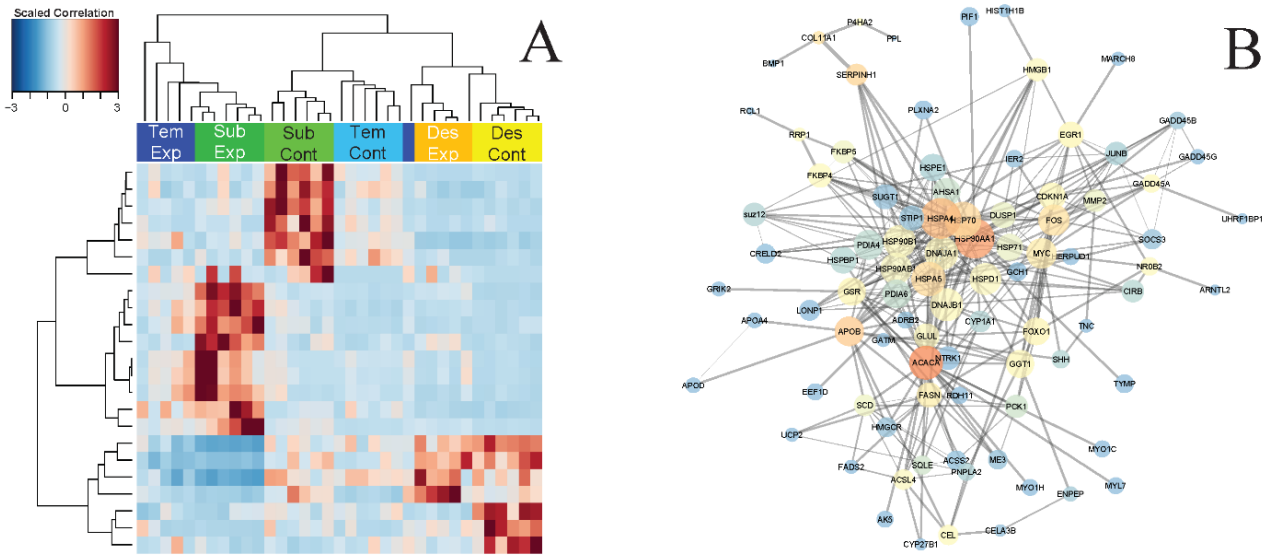

Fig. 4 **(A)** Hierarchical clusters of 23 transcripts identified as candidates for divergent selection on expression level and also showing significant differential expression between control and experiment. Color bars indicate the ecotype of the samples. TemCont=Temperate control (20°C), TemExp= Temperate experimental (33°C), SubCont=Subtropical control (20°C), SubExp= Subtropical experimental (33°C), DesCont= Desert control (20°C), DesExp= Desert experimental (33°C). **(B)** Protein interaction network containing 137 heat stress associated proteins lined via 1114

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interactions. Size of node is proportional to it centrality in the network, color of node indicate the relative number of interaction it is directly involved (blue lower – red higher number of interactions), both color and size of the node indicate realtive importance of the protein in *Melanotaenia* heat stress response.

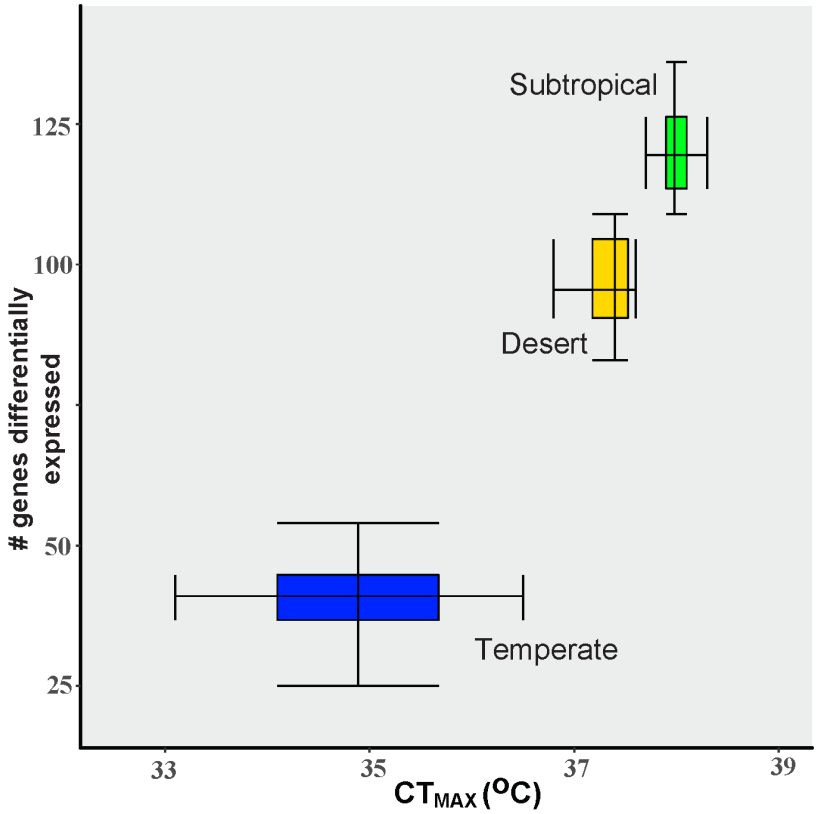

Fig. 5 Association between CT_{MAX} and number of genes differentially expresed in response to heat stress in three ecotypes of *Melanotaenia* (r=0.998). Box plots display the uppper and lower quartiles, whiskers represent 95 and 5 percentiles, and their intersections represent the median.

Discussion

We compared transcriptional plasticity to projected future temperatures and physiological tolerance to thermal stress in three contrasting climatic ecotypes of Australian rainbowfish: temperate, desert and subtropical. Within ecotypes, individuals exhibited very similar changes in both the direction and the magnitude of expressed genes. On the other hand, despite most expressed genes being identified in the transcriptomes of all ecotypes (i.e. 81% of the 34,815 uni-genes), response mechanisms to predicted thermal stress differed remarkably among

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426 ecotypes. Interestingly, both the plastic responses and tolerance to thermal increases varied in
427 a biogeographically determined manner. Subtropical rainbowfish showed both the highest
428 transcription response and tolerance to thermal stress amongst ecotypes, temperate rainbowfish
429 showed the lowest responses for both mechanisms, and desert rainbowfish showed
430 intermediate transcriptional responses and physiological tolerance. Although all species
431 mounted substantial responses to 2070-projected summer temperatures, a striking result was
432 that transcriptional changes common to all three ecotypes were limited to only five genes, thus
433 confirming variation in plastic responses to thermal stress among ecotypes.

434

435 Such divergence in plastic responses between ecotypes is consistent with lineage-specific
436 adaptation resulting from contrasting selective pressures across the climatically-defined
437 bioregions, but can also be associated with neutral mechanisms of evolution (Dunn et al., 2013;
438 Whitehead, 2012; Whitehead & Crawford, 2006). For this reason, we incorporated a
439 phylogenetic model (EVE) to control for the effects of neutral drift on gene expression (Rohlfs
440 & Nielsen, 2015). This approach identified a large suite of candidates for divergent selection
441 on gene expression between ecotypes, of which a subset of 23 genes also showed significant
442 ecotype-specific response to thermal manipulation (Fig. 4A). We consider these as strong
443 candidates for adaptive (i.e. genetic-based) plastic response to future increases in temperature.
444 Network analyses demonstrated centrality of these genes in thermal response pathways, while
445 also identifying several highly conserved hub genes. These genes appear to be of fundamental
446 importance for modulating thermal response pathways and adaptive potential in the three
447 ecotypes. Together, these results show that while integral expression responses can be
448 conserved among lineages, the tendency for divergence in response to thermal stress is high.
449 This divergence not only exceeds neutral expectations but corresponds to differences in thermal

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450 tolerance across climatically defined bioregions, speaking to the importance of biogeographic
451 history in considerations of climate-adaptive potential.

452

453 *Adaptive mechanisms contribute to gene expression plasticity among ecotypes*

454 Shifts in gene expression regulation have for a long time been hypothesised to contribute to
455 adaptive diversity (King & Wilson, 1975). However, the evolution of plastic responses by
456 natural selection has been infrequently documented in empirical studies, particularly in natural
457 populations (but see Whitehead and Crawford (2006); McCairns and Bernatchez (2010);
458 Kenkel and Matz (2016); Roelofs et al. (2009); Kingsolver and Buckley (2017)). Although a
459 diversity of mechanisms regulate gene expression (Orphanides & Reinberg, 2002), substantial
460 empirical evidence supports heritability of expression responses (Roberge, Guderley, &
461 Bernatchez, 2007; Schadt et al., 2003; Whitehead & Crawford, 2006), as recently demonstrated
462 in the subtropical Australian rainbowfish (*M. duboulayi*) (McCairns et al., 2016). As such,
463 plasticity is likely to be subject to the same broad evolutionary processes as other heritable
464 traits. For instance, under directional selection, limited expression polymorphism is expected
465 within ecotypes, while extensive divergence is expected between ecotypes (Hodgins-Davis &
466 Townsend, 2009). Under stabilising selection, expression regulation is predicted to be highly
467 consistent within and across ecotypes (Ghalambor et al., 2007; Hodgins-Davis & Townsend,
468 2009). Meanwhile, under neutral evolution, patterns of gene expression are expected to
469 correlate with evolutionary divergence (Whitehead & Crawford, 2006), which in our case was
470 assessed using a phylogenomic-based approach. Our comparative analyses suggested that all
471 of the above mechanisms have influenced gene expression responses to projected thermal stress
472 in rainbowfishes. This fits with our understanding of thermal tolerance adaptation in ectotherms

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473 as highly complex, and involving multiple levels of biological organisation (Cossins, 2012;
474 Pritchard & Di Rienzo, 2010).

475

476 The majority of DE genes under 2070-projected temperature manipulation exhibited patterns
477 of variation that could be associated with phylogenetic distance (Fig. 1B; Fig. 2B). This
478 demonstrates that plastic responses to future climates can be highly constrained by
479 demographic history, even among groups which are as recently diverged as Australian
480 rainbowfishes (Unmack, Allen, & Johnson, 2013). Nonetheless, we were able to reject neutral
481 scenarios as the most parsimonious explanation for the variation in a large subset of DE genes.
482 In the case of the EVE candidates for directional selection to thermal stress, there was very
483 little expression polymorphism within ecotypes, but high levels of divergence between
484 ecotypes. We suggest that regulatory differences in these genes have helped to facilitate
485 persistence of rainbowfish ecotypes within their respective thermal environments, bringing
486 each closer to a local phenotypic optimum. While evidence of ecological selection on plasticity
487 is rare, an example includes the gene expression divergence of a soil-dwelling hexapod
488 (*Orchesella cincta*) in populations subsisting in contaminated mine sites (Roelofs et al., 2009).
489 This was correlated with heavy metal tolerance, which resulted in a heritable increase in metal
490 excretion efficiency. Similarly, Brennan, Galvez, and Whitehead (2015) demonstrated a shift
491 in salinity-specific expression responses in populations of killifish (*Fundulus heteroclitus*) in
492 populations adapted to habitats of contrasting salinity.

493

494 The centre of the gene interaction network for the three rainbowfish ecotypes consisted largely
495 of heat shock proteins, which play an important role in thermal responses in a wide variety of
496 taxa (Chen, Farrell, Matala, & Narum, 2018; Feder & Hofmann, 1999). Patterns of plastic

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497 responses to temperature were most likely to be shared among ecotypes in these central 'hub'
498 genes (Table S5). This indicates a conserved functional role, which may have been retained
499 through purifying and possibly stabilising selection. Hub genes influence the expression and
500 activity of genes downstream in an expression network, and tend to be highly conserved in
501 their expression between lineages (Evans, 2015). In genome-wide studies of model organisms,
502 the deletion of hub genes is more likely to be deleterious than for non-hub genes. This can be
503 due to either compromised network structure, or simply because they are more likely to be
504 involved in essential interactions (He & Zhang, 2006). However, the fact that the EVE
505 candidates for divergent expression among ecotypes also exhibited greater average
506 connectedness than other DE genes (Table S5) suggests the importance of these genes in the
507 respective ecological adaptations they have likely facilitated. In fact, three EVE candidates
508 were also identified as hub genes, and one of these shows plasticity in all ecotypes
509 (HSP90AA1). A change in expression in one or a few hub genes could therefore translate to a
510 completely different stress response pathway. Indeed, enrichment analyses indicate that
511 functions as diverse as metabolism, immune response, oxidative stress response, DNA damage
512 response, signal transduction and other stress responses are contributing to local adaptation
513 among temperate, desert and subtropical ecotypes.

514

515 While the transcriptomic methods used here directly address mechanisms for thermal response,
516 we are not yet able to infer specific fitness effects of divergent expression patterns in warming
517 climates. Despite this, the number of genes regulated in response to warming differed markedly
518 between ecotypes, with the greatest number responding in the most heat-resilient subtropical
519 ecotype (CT_{MAX} 38.0°), and the smallest number responding in the least heat-resilient
520 temperate ecotype (CT_{MAX} 34.9°). Similarly, previous work comparing montane and desert
521 redband trout (*Oncorhynchus mykiss gairdneri*) found that the more resilient desert trout

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522 regulated a larger number of genes than the less resilient montane trout in response to acute
523 warming conditions (Garvin, Thorgaard, & Narum, 2015; Narum & Campbell, 2015). While
524 the absolute number of transcripts regulated in a given condition can depend on many factors,
525 including differences in constitutive expression or qualitative differences such as amino acid
526 or regulatory changes (Somero, 2010), it is possible that a larger number of regulated genes
527 may often reflect a more sophisticated plastic response to environmental stressors. In the case
528 of rainbowfish, it is too early to say whether the observed increase in number of DE genes
529 represents a more specialised adaptation to heat by the subtropical rainbowfish compared to
530 desert and temperate ecotypes. However, the association between thermal tolerance and
531 number of regulated transcripts does provide further evidence to support adaptive differences
532 in the potential for expression-mediated phenotypic plasticity.

533

534 *Physiological thermal tolerance is specific to ecotype*

535 It is generally assumed that organisms are adapted to or have the ability to acclimate to the
536 temperatures normally encountered in their habitat range (Ghalambor et al., 2006). Thus, it has
537 been proposed that organisms that evolved in warmer climates will have higher thermal
538 tolerances than those in cool climates (Vernberg, 1959), and that those which have evolved in
539 variable climates will have greater acclimation capacities and tolerance ranges than those in
540 more stable climates (Janzen, 1967). Here, we found that critical thermal maximum (CT_{MAX})
541 differed significantly among ecotypes, with the temperate ecotype displaying the lowest
542 average thermal tolerance (34.9°C), the desert ecotype displaying intermediate thermal
543 tolerance (37.2°C), and the subtropical ecotype displaying the highest thermal tolerance
544 (38.0°C). Consistent with several studies assessing relationships between thermal tolerance and
545 latitude (Addo-Bediako et al., 2000; Aguilar-Kirigin & Naya, 2013; Deutsch et al., 2008;

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546 Ghalambor et al., 2006; Magozzi & Calosi, 2015; Sunday et al., 2011), the observed pattern of
547 rainbowfish CT_{MAX} is an increase with proximity to the equator. However, in this instance
548 CT_{MAX} does not coincide with average maximum summer temperatures (or even average yearly
549 temperatures) experienced in the climate of origin, with the hottest Australian temperatures
550 found in the central deserts as opposed to the north-eastern subtropical region (Table S2, BOM
551 2014).

552

553 Perhaps counterintuitively, this finding is consistent with previous research which has
554 emphasised the importance of temperature variability in relation to an organism's upper limits
555 of thermal tolerance (Ghalambor et al., 2006; Magozzi & Calosi, 2015; Sunday et al., 2011).
556 Although wider ranges of tolerance have been found at higher latitudes, these have been largely
557 attributed to lower critical thermal minimums of the temperate organisms studied (Addo-
558 Bediako et al., 2000; Ghalambor et al., 2006). Meanwhile, higher thermal tolerances have been
559 observed in tropical regions, but with an inverse relationship to tolerance breadth (Comte &
560 Olden, 2016). This has led to the use of the term 'climate specialists' to describe tropical
561 species, with an evolutionary trade-off suggested to exist between upper thermal tolerance and
562 the capacity to acclimate to a wide range of temperatures (Comte & Olden, 2016; Deutsch et
563 al., 2008; Payne & Smith, 2017; Tewksbury, Huey, & Deutsch, 2008).

564

565 Due to this apparent trade-off, our findings may highlight an unforeseen risk for desert taxa.
566 The low humidity of desert environments allows rapid heat transfer, exposing organisms to
567 some of the most extreme temperature variations both diurnally and annually. Of the three
568 ecotypes assessed, the desert rainbowfish is currently faced with temperatures closest to its
569 CT_{MAX} (Table S2; BOM, 2014), and its ability to adapt to extremely high temperatures may be

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570 compromised by the need to maintain a large window of tolerance. It is already common for
571 ambient temperatures at the desert rainbowfish's sampling location (BOM, 2014) to exceed its
572 CT_{MAX}, though larger water bodies are unlikely to reach such extremes due to the fluctuation
573 in diurnal temperatures (~15°C/day) and slow rates of heat exchange between air and water
574 (Livingstone & Lotter, 1998). However, desert environments are predicted to experience more
575 extreme heat waves and longer droughts under climate change scenarios (IPCC, 2014). This is
576 likely to not only increase the length of time in which organisms are exposed to thermal stress
577 conditions, but decrease the overall volume of aquatic refugia, making them more susceptible
578 to ambient temperatures (Magoulick & Kobza, 2003). In such circumstances, typical
579 behavioural responses such as seeking shade or cool-water sites created by deeper water or
580 inflowing tributaries may be unable to compensate for these effects (Breau, Cunjak, & Peake,
581 2011; Magoulick & Kobza, 2003). Our data support a high risk for desert species relative to
582 the other ecotypes, which is consistent with at least one species distribution modelling approach
583 (Wiens, Stralberg, Jongsomjit, Howell, & Snyder, 2009) and a functional trait analysis (Vale
584 & Brito, 2015), but not with a broader study using the vegetation sensitivity index (Seddon,
585 Macias-Fauria, Long, Benz, & Willis, 2016). This lack of consensus is symptomatic of the
586 current poor understanding of desert ecosystems and disparate approaches used to examine
587 potential impacts of global change in these regions (Maestre, Salguero-Gomez, & Quero,
588 2012), highlighting the need for an integrated reassessment of dryland vulnerability to climate
589 change.

590

591 *Conclusions and perspectives*

592 Climate change is creating a discord between some organisms' physiologies and their
593 environments. In order to predict the likelihood of range shifts, population declines or local

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extinctions, it is useful to understand the distribution of adaptive diversity, including that of adaptive plasticity. However, despite extensive empirical studies about standing genetic variation and its effects on climate related traits, the concept of adaptive plasticity remains relatively unaddressed. Here, we compared transcriptional and physiological responses to projected future temperatures in three contrasting climatic ecotypes of Australian rainbowfish, to test the effects of ecotype-specific directional selection on plasticity. Our results supported the hypothesis that the capacity for plastic response to climate will vary biogeographically, even within a closely related group. Moreover, by controlling for the effects of phylogeny, we were able to infer that divergent selection on gene expression has contributed to observed differences in plastic capacity among ecotypes. By demonstrating immediate response mechanisms to thermal stress, as well as evidence for ecological selection on these mechanisms among lineages, our results emphasise the key role of plasticity in both short- and long-term climatic adaptation. While it is too early to link the observed transcriptional responses to direct measures of fitness, the proxy provided by the assessment of thermal tolerance provides a strong indication of ecological relevance. This has wide-ranging implications both for broad biogeographic assessments of climate impacts, as well as for more focussed predictions of species distribution changes which are only now beginning to account for intra-taxonomic adaptive variation. This study represents a stride towards a more holistic understanding of climatic adaptive potential in natural populations.

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