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# Clownfish larvae exhibit faster growth, higher metabolic rates and altered gene expression under future ocean warming

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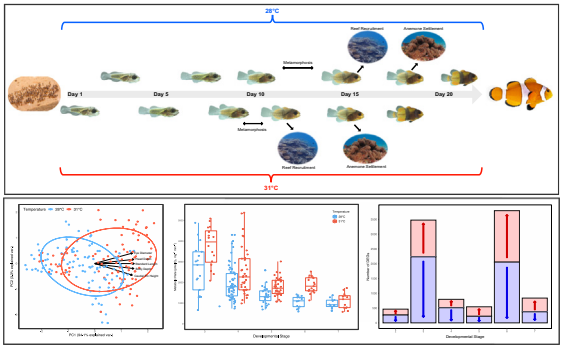
## HIGHLIGHTS

- Clownfish larvae were reared at temperatures associated with future ocean warming.
- Larvae display faster growth and development, and higher metabolic rates at +3 °C.
- Larvae have >450 differentially expressed genes throughout development at +3 °C.
- Altered expression of genes involved in heat-stress and epigenetic modifications
- Clownfish development will be altered at physiological and molecular level at +3 °C.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Increasing ocean temperatures have been demonstrated to have a range of negative impacts on coral reef fishes. However, despite a wealth of studies of juvenile/adult reef fish, studies of how early developmental stages respond to ocean warming are limited. As overall population persistence is influenced by the development of early life stages, detailed studies of larval responses to ocean warming are essential. Here, in an aquaria-based study we investigate how temperatures associated with future warming and present-day marine heatwaves (+3 °C) impact the growth, metabolic rate, and transcriptome of 6 discrete developmental stages of clownfish larvae (*Amphiprion ocellaris*). A total of 6 clutches of larvae were assessed, with 897 larvae imaged, 262 larvae undergoing metabolic testing and 108 larvae subject to transcriptome sequencing. Our results show that larvae reared at +3 °C grow and develop significantly faster and exhibit higher metabolic rates than those in control conditions. Finally, we highlight the molecular mechanisms underpinning the response of larvae from different developmental stages to higher temperatures, with genes associated with metabolism, neurotransmission, heat stress and epigenetic reprogramming differentially expressed at +3 °C. Overall, these results indicate that clownfish development could be altered under future warming, with developmental rate, metabolic rate, and gene expression all affected. Such changes may lead to altered larval dispersal, changes in settlement time and increased energetic costs.

## 1. Introduction

Global anthropogenic emissions of carbon dioxide are driving a range of changes to the earth's climate (IPCC, 2021). Increasing oceanic temperatures are amongst the most prominent of these changes, with average ocean temperatures predicted to increase by +3 °C by 2100 (IPCC, 2021). In addition to the gradual increase in temperature, anomalous

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short term increases in ocean temperature (marine heatwaves; MHW) are already occurring (Wernberg et al., 2013), and are predicted to increase in frequency, intensity, and duration under future climate change (Frolicher et al., 2018). This increase in MHW intensity has been linked to ecosystem disruptions and increased mortality of multiple marine organisms, with recent heatwaves in the Mediterranean Sea, Western Australia and Great Barrier Reef having a range of negative impacts (Garrabou et al., 2009; Hughes et al., 2017; Wernberg et al., 2013).

As ectotherms inhabiting environments close to their maximum thermal limit, coral reef fish are one group of organisms particularly threatened by ocean warming and heatwaves (Smale et al., 2019). This has been demonstrated in previous studies with impaired physiology (Portner and Farrell, 2008; Rummer et al., 2014), altered behaviour (Biro et al., 2010; Malavasi et al., 2013) and reduced reproductive output (Donelson et al., 2010; Miller et al., 2015) all observed at increased temperatures. If realized, these effects will inevitably lead to declines in coral reef fish, reducing their ability to provide vital ecosystem services (Pratchett et al., 2014; Cinner et al., 2020). However, the response of reef fish to ocean warming varies with geographic location, as fish inhabiting low-latitude reefs close to their thermal limit, display stronger responses than those of higher latitude populations (Rummer et al., 2014). In fact, such warming may benefit populations of reef fish inhabiting higher latitude marginal reefs (Lin et al., 2022; Feary et al., 2014). Whilst previous studies have primarily focused on adult/juvenile coral reef fish, less research has focused on the response of early developmental stages to warming. This is due to the challenges associated with studying early developmental stages, which include the collection of pelagic larval stages, maintenance of ex-situ brood stock and the fragility of early-stage larvae (Burggren and Blank, 2009; Peck and Moyano, 2016). Nevertheless, as the persistence of reef fish populations is dependent upon the development, dispersal, recruitment and survival of early life stages (Selkoe and Toonen, 2011; Shima et al., 2020) it is imperative that we investigate how these stages will fare under future ocean warming.

Despite the challenges, previous studies have begun to elucidate how larval fish respond to future climate change. Fish larvae generally display faster development and reduced larval durations when exposed to higher temperatures (Raventos et al., 2021; O'Connor et al., 2007; Robitzch et al., 2016), likely driven by the increased rate of physiological processes at elevated temperatures (Munday et al., 2008). The ecological consequences of faster larval growth and development remain uncertain, however for coral reef fish this could reduce dispersal potential and genetic mixing (Raventos et al., 2021; Lo-Yat et al., 2011; Munday et al., 2009) or improve larval survival, as stages susceptible to predation will conclude earlier (Munday et al., 2009; O'Connor et al., 2007), provided such larvae can secure sufficient food to meet increased metabolic demands (Munday et al., 2009). This faster larval development at increasing temperatures is often observed within the natural thermal range of the species. However, when exposed to temperatures outside their natural thermal range (e.g. future warming scenarios), larval developmental rates can be unaffected (McLeod et al., 2013; McLeod et al., 2015), highlighting a potentially opposing response. In addition to altered developmental rates, larval metabolic rates can be impacted by increasing ocean temperatures. For example, larval seabream (Pimentel et al., 2020), kingfish (Laubenstein et al., 2018) and European seabass (Howald et al., 2022) all display increased metabolic rates at temperatures associated with future warming. Although studies of isolated responses are valuable, limited studies of coral reef fish combine growth and metabolic measurements, thus the complex relationship between these two larval traits at increased temperatures remains uncertain.

Transcriptomic analyses can be used to further probe the response of reef fish larvae to warming, with larvae of several species of fish displaying altered gene expression at increased temperatures (Anastasiadi et al., 2017; Goikoetxea et al., 2021; Han et al., 2021). Genes displaying altered expression include those involved with stress response such as glucocorticoid and corticotropin-releasing hormone receptors (Anastasiadi et al., 2017; Goikoetxea et al., 2021), whilst heat shock proteins (HSPs) display both increased expression (Madeira et al., 2016b) or no change (Madeira et al., 2016b; Anastasiadi et al., 2017). Metabolic genes also display a varying response in larvae with metabolic pathways both heavily upregulated (Han et al., 2021) and unaffected (Madeira et al., 2016b). Such variability suggests the molecular response to warming may be species-specific and dependent upon levels of temperature increase. There is also transcriptomic evidence that developmental processes can be affected, as many genes involved in muscle formation (myogenin), DNA methylation (DNA methyltransferases) and growth (somatotropin) are upregulated at higher temperatures (Anastasiadi et al., 2017; Madeira et al., 2016b). Although valuable, these insights come from only a few studies as molecular assessments of the effects of warming on fish larvae are limited, with stage-specific data even rarer. Moreover, investigations into the transcriptomic response of coral reef fish larvae to warming are yet to be conducted. This scarcity of data means the molecular mechanisms underpinning the previously described physiological impacts of warming on fish larvae are currently unknown.

The clownfish *Amphiprion ocellaris* is a well-recognized species of reef fish that lives in association with host anemones and is popular within the tourism and aquarist industries (Fautin and Allen, 1992). As benthic spawners (Leis and McCormick, 2002) clownfish lay eggs near their anemone host, with a 6–8 day embryonic period beginning after fertilization (Salis et al., 2021). Following hatching, *A. ocellaris* begin a 10–15 day larval period in which they disperse into the pelagic ocean (Roux et al., 2019). Development is characterized by 7 stages, culminating in metamorphosis into small juveniles that seek a new host anemone (Roux et al., 2019). Studies assessing the impact of future warming on clownfish indicate that the response will be complex and temperature dependent. For example, cellular diagnostic approaches using stress biomarkers indicate that juvenile *A. ocellaris* experience thermal stress at 30 °C. However, studies also show that neither body condition index nor survival are negatively affected by this temperature (Madeira et al., 2016a; Madeira et al., 2017a; Evangelista et al., 2020). Yet, juvenile *A. ocellaris* exhibit increased mortality (Madeira et al., 2021) at 32 °C and reduced aerobic scope at 35 °C (Velasco-Blanco et al., 2019), suggesting the potential existence of a thermal threshold. In the most comprehensive assessment of temperature effects on clownfish larvae, McLeod et al. (2013) showed that *A. percula* have an increased pelagic larval duration (PLD), reduced development rates and higher metabolic rates at 32.2 °C. Such a result agrees with previous studies showing increased metabolic rates in reef fish at higher temperatures (Portner and Farrell, 2008), however observations of slower growth/longer PLD contradict the generally accepted prediction of faster growth/shorter PLD at higher temperatures (Raventos et al., 2021; O'Connor et al., 2007). These complex and contrasting results highlight the need for further investigations into how clownfish larvae respond to temperature increase.

To date, no study of coral reef fish larvae has combined physiological measurements of growth and metabolism with transcriptome-wide measurements at increased temperatures. Furthermore, previous research has often focused on a single timepoint of larval development, thus excluding multiple developmental stages that differ significantly from each other (Roux et al., 2019). Therefore, studies encompassing multiple developmental stages will provide a more complete understanding of how warming will impact reef fish throughout development. Here, we investigate how discrete developmental stages of *A. ocellaris* larvae respond to future warming by exposing them to control (28 °C) and elevated (31 °C) temperature treatments. Throughout 20 days of exposure to these temperatures we measured daily growth and development via microscopic imaging, metabolic rate via respirometry, and gene expression via RNA-sequencing. We hypothesized that (i) larvae reared at 31 °C would grow and develop faster than those raised at 28 °C, (ii) larvae of all developmental stages would exhibit higher metabolic rates at 31 °C, (iii) larval gene expression profiles would differ between temperature treatments.

## 2. Methods

### 2.1. A. ocellaris breeding pairs

Larvae used in this study were sourced from four breeding pairs of *A. ocellaris* collected from around Okinawa, Japan and housed in four

260 L open system tanks at Okinawa Institute of Science and Technology marine station (Table S1). Owing to the continuous water supply, water temperature within the tanks followed the natural seasonal cycle of Okinawa (Loya et al., 2001). Two breeding pairs were housed with the anemone *Heteractis magnifica* (collected from reefs in Okinawa) and two breeding pairs were housed with terracotta pots (Table S1) (Roux et al., 2021). Breeding pairs were fed twice daily (~9 am and ~5 pm) with a mixed diet of frozen shrimp (Kyorin Clean Brine Shrimp and Kyorin Clean White Shrimp), fish flakes (Marine Pros Red) and dried krill (TetraKrill).

### 2.2. Larval hatching and experimental treatments

Following breeding pair spawning, eggs remained with the parents at ambient temperature throughout development (6–8 days). On the predicted day of hatching eggs were transferred to 35 L float glass larval tanks, kept in complete darkness by black window film and a plastic lid. A total of six clutches of eggs were utilized in this study. Owing to differences in spawning patterns between breeding pairs, not all clutches were studied at the same time. Following hatching, half of the larvae were transferred to a separate larval tank resulting in a total of two experimental tanks per larval clutch, with these two tanks randomly assigned a temperature treatment of 28 °C or 31 °C. A temperature of 28 °C was designated as the control, as this is the average temperature of Okinawan oceans in summer (Loya et al., 2001) (Mean temperature of 27.71 °C in July, August, September – *Unpublished data*). A temperature of 31 °C was selected to represent summer temperatures in Okinawa under future ocean warming, based on IPCC SSP3-7.0 predictions (IPCC, 2021). Furthermore, temperatures of +3 °C has been reached during previous marine heatwaves (Wernberg et al., 2013), and may therefore be experienced by reef ecosystems in the present day. After dividing larvae between the two experimental tanks, temperature was gradually increased (0.5 °C every 2 h) to the target temperature of 31 °C.

### 2.3. Larval rearing

Larvae were reared in a closed system and thus 40 % of the tank water was replenished with fresh seawater each day, at a rate of 500 mL/min. Water temperature within each experimental tank was regulated by a REI-SEA thermocontroller (TC-101) and two 150 W (Kotobuki) heaters that maintained temperature within +/− 0.4 °C of the target temperature. Temperature was measured twice daily with a handheld digital thermometer (Fisherbrand, Traceable) temperature probe (Tables S2-S7). Larval *A. ocellaris* were reared following the best practices detailed in Roux et al. (2021). Each larval clutch was reared for a total of 20 days, with sampling for growth and development measurements, metabolic testing and RNA-sequencing conducted during this period. Of the 6 clutches sampled, we aimed to conduct each type of measurement on at least 3 separate clutches, thus ensuring sufficient replication and genotypic variability for each measurement. See Table S8 for a detailed summary of the measurements conducted for each clutch.

### 2.4. Growth and development measurements and transcriptome sampling

Larvae from clutches 1–4 were collected from 2 to 20 dph for microscopic imaging. At 1 dph temperature within the 31 °C treatment was still being increased, and thus 1 dph and stage 1 larvae were excluded from all sampling and analysis. A total of 3 larvae were randomly sampled per temperature treatment each day and euthanized via an overdose of Tricaine Methanesulfonate (MS222) (400 mg/L) (Sigma Aldrich). Larvae remained in MS222 while images were taken on a Zeiss discovery V20 stereoscope. Following imaging, larvae were transferred to RNAlater (Sigma Life Sciences) and stored at −20 °C. In addition to daily imaging, at 20 dph the remaining larvae in experimental tanks from clutches 1, 2, 4, 5, and 6 (minimum 16 larvae, maximum 25 larvae) were imaged using the same approach (clutch 3 was excluded from 20 dph imaging due to insufficient larvae remaining). Images were analyzed using ImageJ to identify larval stage and measure standard length (SL), eye diameter (ED), head depth (HD), body depth (BD) and caudal fin height (CFH) (according to Roux et al. (2019)).

### 2.5. Nucleic acid extraction and sequencing

RNA was extracted from 108 larvae, as the aim was to sequence 6 larval stages (S2–7) of 3 clutches (C1–3) from 2 temperatures (28 °C and 31 °C), with a total of 3 replicates for each stage, temperature, and clutch combination. RNA was extracted using Maxwell® RSC simplyRNA Tissue Kit (Promega, USA) and the associated Maxwell® RSC Instrument, following the manufacturers guidelines. The quality and quantity of extracted total RNA was assessed using an Invitrogen QuBit Flex Fluorometer and an Agilent TapeStation 4200. RNA libraries were prepared from total RNA using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA). Following, 150 bp paired end RNA sequencing was conducted on an Illumina NovaSeq 6000 platform. Library preparation and sequencing were conducted at Okinawa Institute of Science and Technology sequencing center.

### 2.6. Metabolic rate measurements

Measurements of metabolic rate were conducted on larvae at varying dph from clutches 4–6 (Table S9). Prior to metabolic measurements no live food was added to larval tanks for 14 h, and larvae were transferred to a 5 L temperature-controlled holding tank, where they were starved for 1 h prior to metabolic testing. Metabolic trials were conducted using a Loligo Systems 1700 μL microplate oxygen sensing system and the associated Loligo Systems MicroResp software. Seawater temperature within this system was regulated by placing the microplate within a SANYO (MIR-162) incubator, set to either 28 °C or 31 °C. Following the 1-hour starvation period larvae were transferred to a 50 mm petri dish filled with seawater and placed inside of the incubator to acclimate for 5 min. Following this initial acclimation period, larvae were transferred to a single 1700 μL well in the microplate system. Larvae were kept within these wells for another 2-minute acclimation period before oxygen concentration recording began. Across clutches 4–6 a total of 133 larvae at 28 °C and 129 larvae at 31 °C underwent metabolic testing. This number was sufficient to produce $n > 12$ for each stage, treatment combination. Metabolic trials were conducted from stage 3 onwards, due to the sensitivity of stage 2 larvae. Oxygen consumption rates were normalized by the dry weight of larvae to give a final $MO_2$ value (pmol $O_2$ $mg^{-1}$ $min^{-1}$). For additional information on metabolic testing see supplementary methods and Fig. S1.

### 2.7. Statistical analysis

For C1–4 differences in stage, SL, ED, HD, BD and CFH between the two treatments, from 2 to 20 dph were assessed using a generalized linear mixed effects model with dph and treatment as fixed factors, and parent ID, clutch, and experimental tank as random factors, with clutch nested inside parent ID. As all morphometric measurements (SL, ED, HD, BD, CFH) were not normally distributed, these response variables were log-transformed. All morphometric measurements for C1, 2, 4, 5, 6 at 20 dph were analyzed using a correlation matrix based principal component analysis (PCA). Following the PCA, PC1 was extracted, square root transformed and used as a response variable in a generalized linear mixed effects model with treatment as a fixed factor, and parent ID, clutch, and experimental tank as random factors, with clutch nested inside parent ID. The impact of temperature on larval metabolic rate was assessed using a linear mixed effects model with stage and treatment as fixed factors and parent ID, clutch, and experimental tank as random factors, with clutch nested inside parent ID. As metabolic rate was not normally distributed it was log-transformed for analysis. Assumptions of all models were checked via visual inspection of residuals and standard model diagnostic tests conducted using DHARMa v0.4.4. All analyses were performed in R version 3.6.1.

### 2.8. Bioinformatic analysis

Following RNA-sequencing the number of reads per sample ranged from 41,000,000 to 100,000,000. Reads were trimmed using Trimmomatic

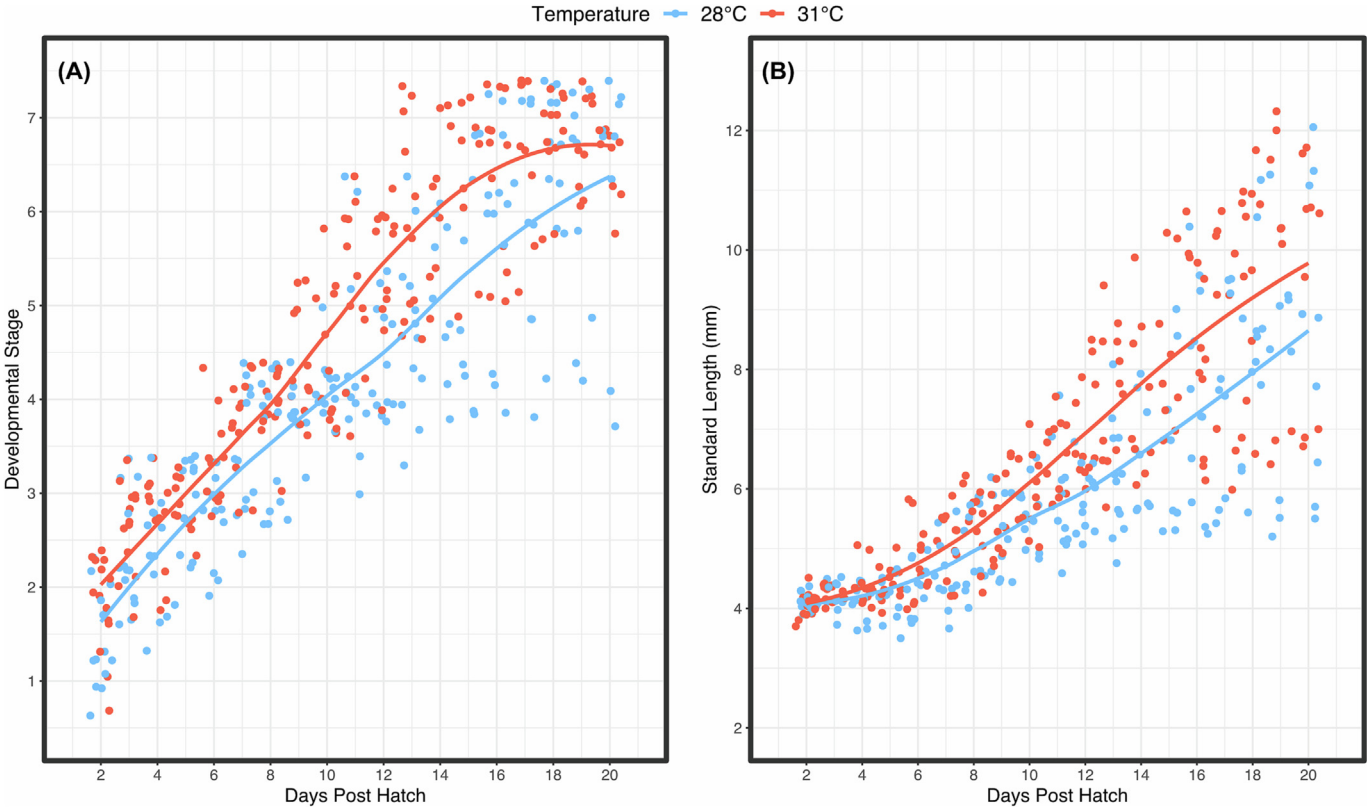


**Fig. 1.** Developmental stage (A) and standard length (B) of *A. ocellaris* larvae measured from 2 to 20 dph at two temperature regimes. Measurements are taken from clutches 1–4.

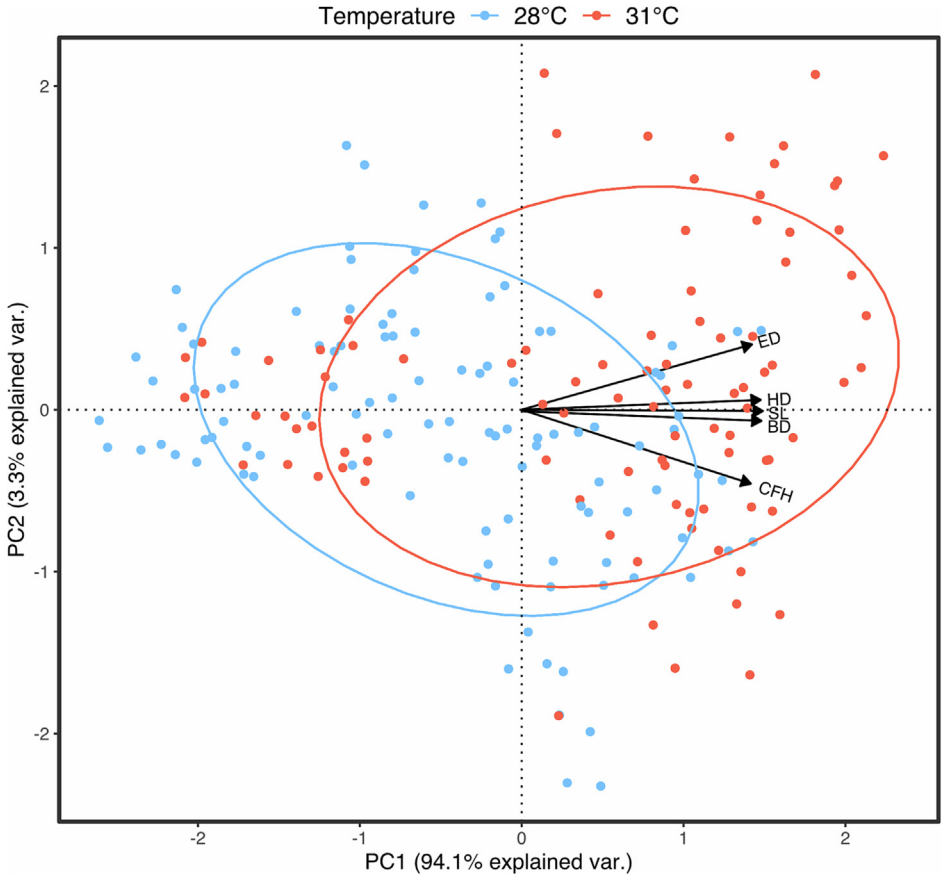


**Fig. 2.** Principal component analysis of morphometric measurements (ED: eye diameter, HD: head depth, SL: standard length, BD: body depth, CFH: caudal fin height) for larval *A. ocellaris* at 20 dph. Measurements are from clutches 1, 2, 4, 5 and 6.

v0.39 (Bolger et al., 2014) with the parameters: "TruSeq3-PE.fa:2:30:10:8: keepBothReads LEADING:3 TRAILING:3 MINLEN:36.". Quality trimmed reads were mapped to a chromosome-scale *A. ocellaris* genome (Ryu et al., 2022) using HISAT2 v2.2.1 (Kim et al., 2019), and the resulting SAM files were converted to BAM files using SAMtools v1.10 (Li et al., 2009). BAM files and an *A. ocellaris* gene annotation were used as input for StringTie v2.1.4 (Pertea et al., 2016) to quantify expression levels. Gene-specific raw reads counts were generated from the StringTie output using the python script prepDE.py3 (Pertea et al., 2016), and differential gene expression between treatments and developmental stages was quantified using DESeq2 v1.26.0 (Love et al., 2014). Following gene expression analysis, the "enricher" function of the ClusterProfiler package (Yu et al., 2012) and Gene Ontology (GO) terms (Consortium, 2004), including the cellular component, molecular function and biological process categories were used to perform functional enrichment analysis on groups of differentially expressed genes. For visual representation of expression, DESeq2 normalized gene counts were extracted, averaged for each stage and temperature group, and scaled by each genes mean and standard deviation.

## 3. Results

### 3.1. Morphology

The temperature at which the larvae were reared affected both the developmental stage ($F = 98.334$, $p < 0.001$) and standard length ($F = 104.14$, $p < 0.001$) of larvae, with larvae displaying accelerated growth and development at 31 °C (Fig. 1, Table S10). For example, on average larvae developing at 31 °C reached stage 4 after 8 days of development, whereas larvae developing at 28 °C reached this stage after 10 days. Moreover, after 18 days of development larvae raised at 31 °C were on average 16 % longer than those raise at 28 °C. Similar trends of accelerated growth and development were observed for all other morphometric parameters measured (Fig. S2). Principal component analysis showed separation of 20 dph larvae reared at 28 °C and 31 °C (Fig. 2), with separation primarily occurring along the PC1 axis, that explained 94.1 % of variation. Here PC1 was positively correlated with all morphometric variables (SL, ED, HD, BD and CFH), with each variable displaying higher values at 31 °C and roughly equal loadings for PC1 (0.434–0.457). Thus, trends of accelerated growth and development at 31 °C are reinforced in 20 dph morphometric measurements of larvae ($F = 48.096$, $p < 0.001$, Table S11).

### 3.2. Metabolic rate

Weight normalized metabolic rates of *A. ocellaris* decreased from stage 3 to stage 7 ($F = 162.030$, $p < 0.001$) in both temperatures (Fig. 3, Table S12). Larvae reared at 31 °C had a higher mean metabolic rate than those reared at 28 °C for all developmental stages, resulting in an overall effect of temperature on metabolic rate ($F = 91.927$, $p < 0.001$). Although the effect of temperature did not vary with developmental stage, increases in mean metabolic rates at 31 °C ranged from 76.6 % (stage 6) to 13.94 % (stage 7). Additionally, the parents of the larvae influenced larval metabolic rates ($F = 7.536$, $p < 0.001$).

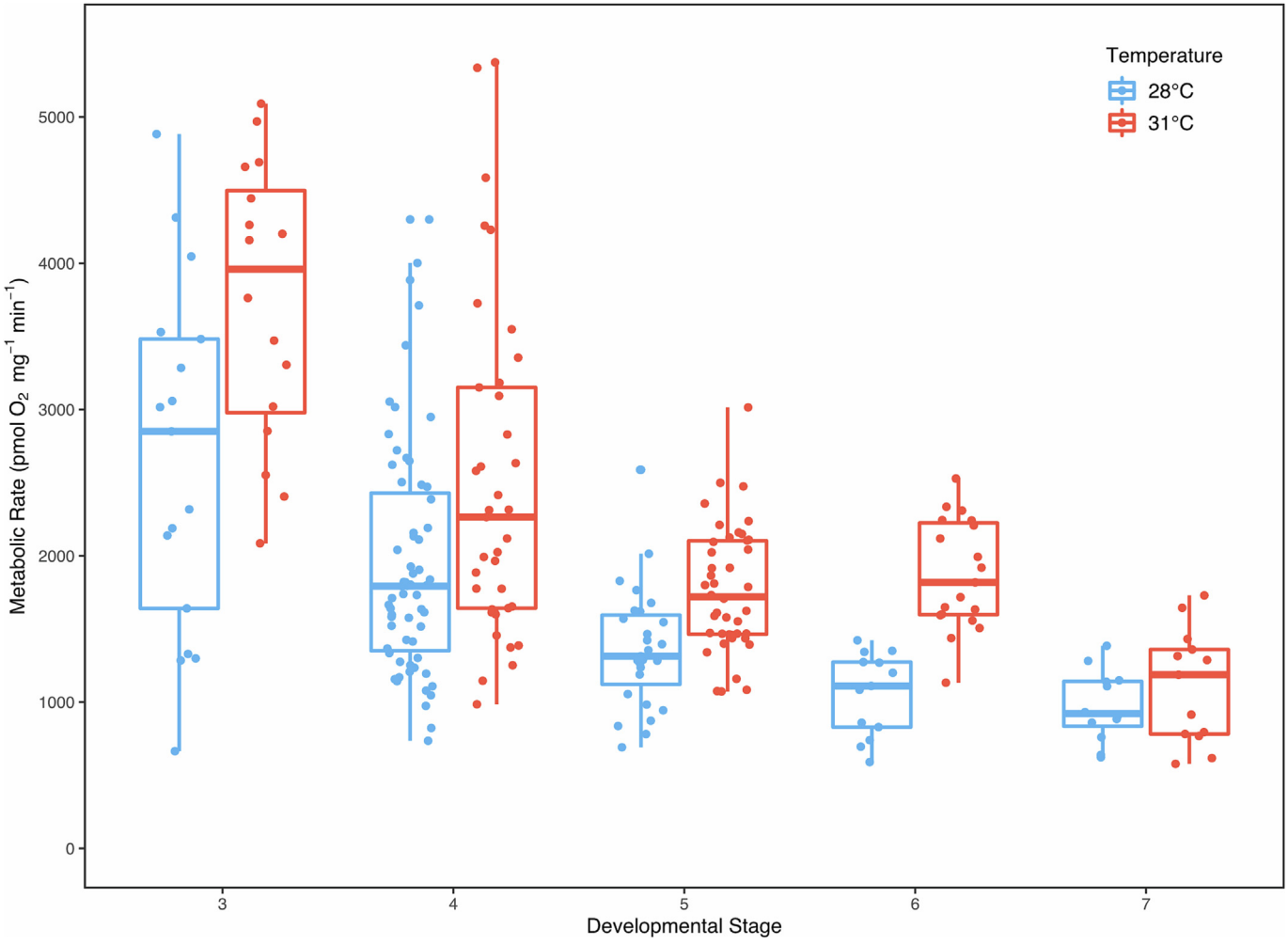


**Fig. 3.** Metabolic rate of five developmental stages of *A. ocellaris* larvae measured at 28 °C and 31 °C. Measurements are normalized by the dry weight of larvae. Measurements are from clutches 4, 5 and 6.

### 3.3. Differential gene expression

See Table S13 for number of reads generated per sample. Stage 2, 4, 5, 7 larvae displayed lower numbers of differentially expressed genes (DEGs) (464–838) at 31 °C, with stage 3 and 6 larvae displaying a higher number of DEGs (3475 and 3794 respectively) (Fig. 4). GO enrichment analysis of stage-specific differentially expressed gene sets highlighted a range of enriched GO categories, with stage 2 showing the lowest number of enriched GO categories (7) (Table S14) and stage 6 the highest (292). In stage 3 larvae genes associated with "mRNA processing", "translation" and "regulation of mitotic cell cycle phase transition" were the most enriched, whilst other enriched categories include "chromatin remodeling", "methylation", "cellular response to oxidative stress" and "regulation of cellular amino acid metabolic process" (Table S15). In stage 4 larvae genes associated with "mRNA processing", "translation" and "proton transmembrane transport" were the most enriched, whilst many categories related to metabolism such as "mitochondrial matrix", "ATP metabolic process" and "oxidative phosphorylation" were also enriched (Table S16). In stage 5 larvae genes associated with "translation" and "angiogenesis" were the most enriched, with other enriched categories including "liver development", "proton transmembrane transport" and "transcription regulator complex" (Table S17). The most enriched GO categories in stage 6 larvae were "cell division", "mRNA processing" and "DNA repair" with other enriched categories including "regulation of mitotic cell cycle phase transition", "histone binding" and "regulation of cellular response to heat" (Table S18). In stage 7 GO categories related to neurotransmitter activity, including "neurotransmitter transport", "glycinergic synapse" and "sodium ion transmembrane transport" were enriched, with other enriched categories including "motor activity" and "muscle contraction" (Table S19).

As GO categories such as "regulation of cellular response to heat" and "unfolded protein binding" were enriched in multiple developmental stages, we investigated the expression of 10 HSP genes (*dnajc2, serpinh1b, hsp90aa1, hspa8b, hspb1, hsp70, hsbp1b, hspa8, hsp90ab1, hsc70*) at 31 °C (Fig. 5). These 10 HSP genes were identified and selected as they displayed significant upregulation at 31 °C in at least one of the six developmental stages analyzed. Stage 5 larvae displayed the strongest upregulation of these HSP genes with a total of 6 genes upregulated at 31 °C, whilst stage 2 larvae displayed a log2foldchange > 1 for *dnajc2, serpinh1b, hsp90aa1, hspa8b, hspb1*at 31 °C. Contrastingly, stage 3 larvae did not upregulate any HSPs and stage 4 larvae only upregulated (log2foldchange < 1) 2 HSPs at 31 °C. Additionally, as multiple GO categories related to epigenetic modifications were enriched in stage 3 and 6 larvae, we plotted the mean scaled counts of 38 genes at 28 °C and 31 °C across stages 2–7 (Fig. 6). The 38 genes were selected based on them being significantly downregulated in either stage 3 or stage 6 larvae at 31 °C. These 38 genes were separated into 6 separate functional groups (DNA methylation, histone lysine methylation, histone arginine methylation, histone deacetylation, histone binding and chromatin modification) based on the gene annotation in (Ryu et al., 2022) (Table S20). Increases in the mean scaled count of these 38 genes in stages 3 and 6 larvae highlight the activation/upregulation of these 38 genes at 28 °C. However, at 31 °C activation/upregulation of these genes was not observed at stages 3 and 6.

## 4. Discussion

Here, we demonstrate that *A. ocellaris* larvae exhibit accelerated growth and development when reared at temperatures associated with future ocean warming and marine heatwaves. This trend was observed in both daily measurements and 20 dph measurements of larvae. Together, these

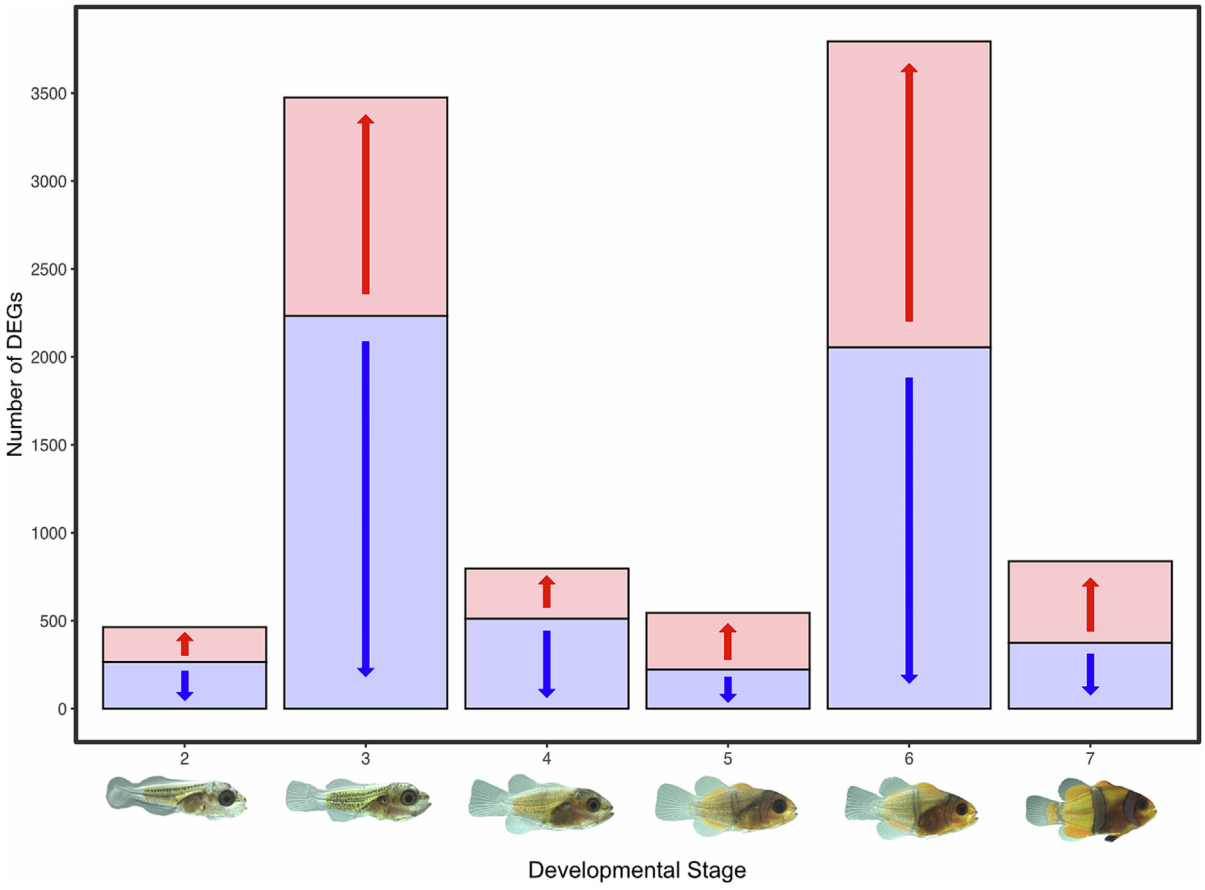


**Fig. 4.** Number of differentially expressed genes (DEGs) at 31 °C (compared to 28 °C) in *A. ocellaris* larvae of varying developmental stages. Up arrow indicates upregulated genes, down arrow indicates down regulated genes.

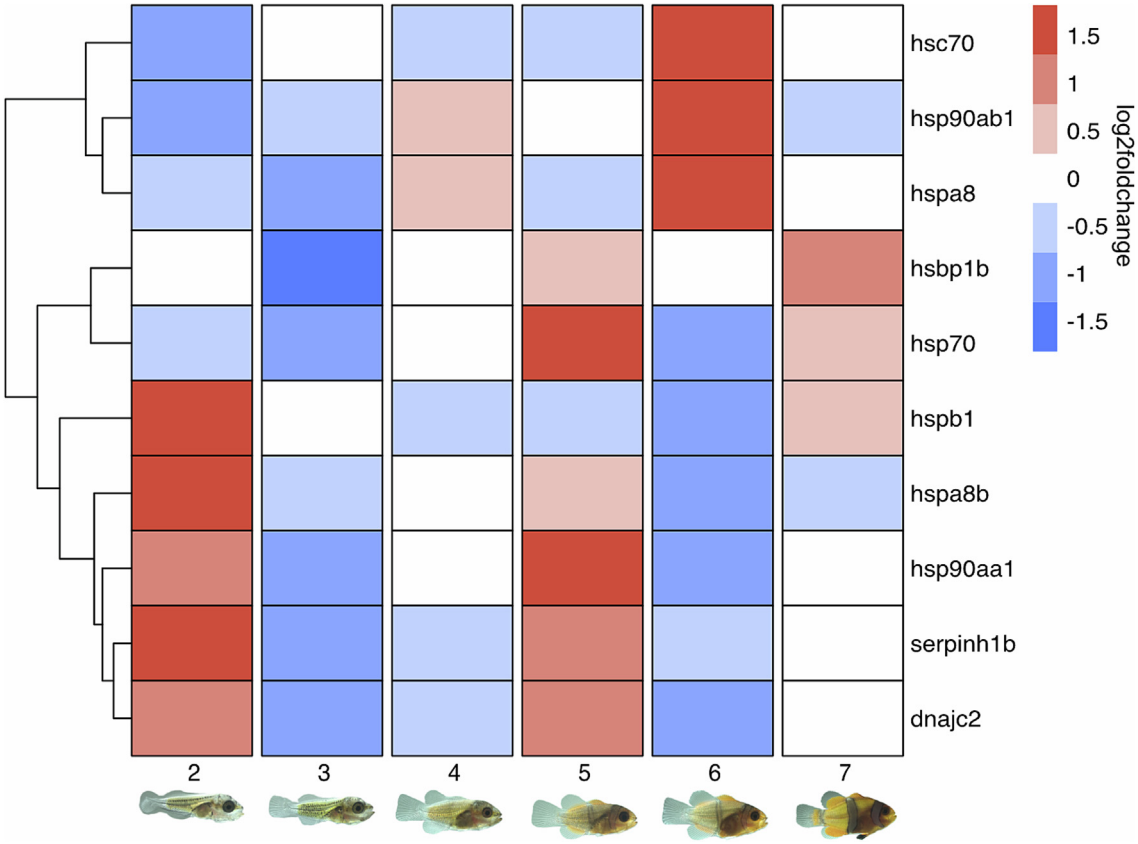


**Fig. 5.** Heatmap displaying the log2foldchange of 10 heat shock proteins at 31 °C (compared to 28 °C). Results are displayed for 6 stages of *A. ocellaris* larval development.

measurements represent the growth and development of 635 larvae from 6 separate clutches and 4 different genotypes, thus providing a robust assessment of how ocean warming, and marine heatwaves could impact the development of larval reef fish. This trend is in line with previous studies that show larvae of both temperate and tropical fish display accelerated development at higher temperatures (Raventos et al., 2021; O'Connor et al., 2007). For example, Robitzch et al. (2016) show that 3 species of reef fish (*Dascyllus* sp.) exhibit a decline in PLD at increasing temperatures in the Red sea, whilst another species of anemonefish (*Amphiprion melanopus*) had a larval duration 25 % shorter when raised at 28 °C compared to 25 °C (Green and Fisher, 2004). Contrastingly, larvae of the closely related clownfish species *A. percula* exhibited slower growth and development at temperatures associated with future warming (+1.5 °C and + 3 °C) (McLeod et al., 2013). Here, the authors postulate that larval development rates may not increase at temperatures beyond those naturally experienced by the population, potentially due to the energetic tradeoff between somatic growth and thermal stress (Clarke and Fraser, 2004), highlighting the fact that predictions of faster development are based on studies of fish within their natural temperature range (Munday et al., 2009; O'Connor et al., 2007). Further work by McLeod et al. (2015) showed that the damselfish *Pomacentrus moluccensis* and the wrasse *Halichoeres melanurus* display faster larval development up to temperatures of 28–29 °C, however at temperatures beyond 29 °C larval growth rates either stabilized or declined. Thus, providing further evidence that faster larval growth may not always occur under ocean warming (McLeod et al., 2015). Nevertheless, the results presented here, as well as those of recent studies, indicate that some species of fish will undergo faster larval development under future warming (Watson et al., 2018; Huss et al., 2021; Shelley and Johnson, 2022). A trend that has also been demonstrated to occur for larva of other taxa, such as coral (Figueiredo et al., 2014; Heyward and Negri, 2010).

Faster larval growth and development will have a range of ecological consequences for populations of *A. ocellaris*. For example, larvae are likely to undergo key developmental process such as metamorphosis, reef recruitment and anemone settlement earlier, thus reducing time spent in the pelagic environment. This could lead to reduced predation and increased larval survival, potentially leading to increases in population size (O'Connor et al., 2007). Alternatively, it may restrict larval dispersal, thus reducing population connectivity between reefs (Munday et al., 2009). However, further studies are required to understand the ecological consequences of such accelerated development, as the postulated impacts of accelerated development have opposing implications for population persistence. Moreover, predictions of how warming and larval development will interact must consider the negative impacts of ocean warming on other physiological processes such as reproduction (Donelson et al., 2010; Spinks et al., 2021), as outcomes of these processes also impact population persistence. For example, the anemonefish *Amphiprion melanopus* was observed to reduce reproductive output at +1.5 °C, with reproduction ceasing altogether at +3 °C, thus highlighting the potential limitations reproductive output may have in the future.

Faster larval growth at higher temperatures is driven by an increase in the speed of physiological processes and elevated metabolic rates (McLeod et al., 2013; Munday et al., 2008), with increased oxygen consumption, heart rate, and metabolic enzymatic activities observed (Pimentel et al., 2020). We find further evidence of this link as *A. ocellaris* larvae reared at 31 °C exhibited higher metabolic rates throughout development, as well as faster growth. Increased metabolic rates at higher temperatures are observed for multiple life stages of fish, as juvenile/adult reef fish display increased resting metabolic rates (RMR) under future ocean warming (Donelson et al., 2012; Nilsson et al., 2009). Elevated RMR in juvenile/adult fish is driven by increased cellular activity and the increased cost of maintaining cellular homeostasis at higher temperatures (Clarke and Fraser, 2004; Munro and Treberg, 2017), which is hypothesized to reduce the energy available for non-essential functions such as reproduction and growth. This principle is extended to larvae by McLeod et al. (2013), as they attribute the slower development of *A. percula* larvae at increased temperatures to elevated routine oxygen consumption rates and the

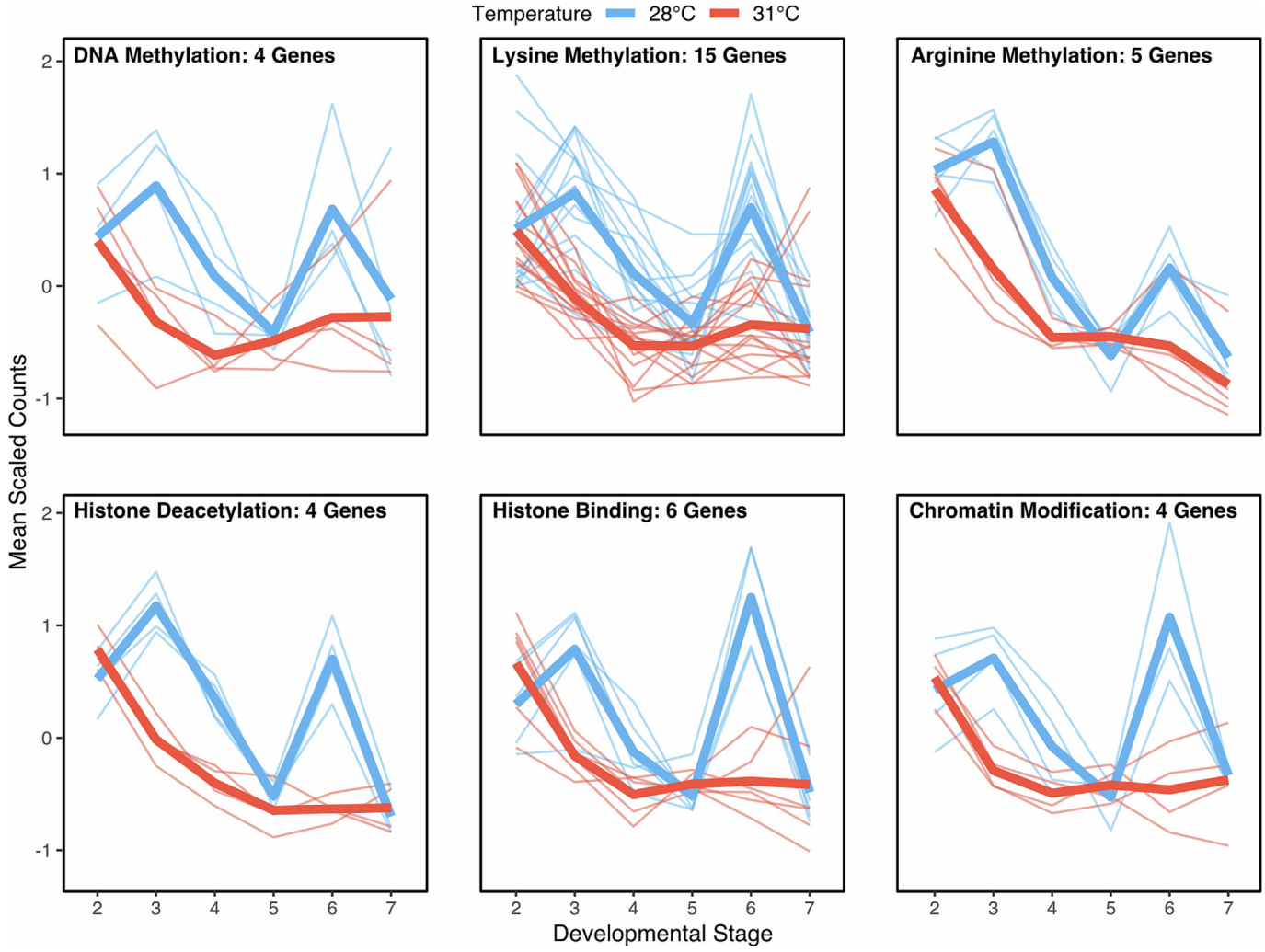


**Fig. 6.** Mean scaled gene counts of 38 genes associated with epigenetic modifications. Gene counts are displayed for larvae reared at 28 °C and 31 °C, and for 6 stages of development. Plots are split by the type of epigenetic modification associated with each gene. These modifications include DNA methylation, 4 types of histone modification: 1) lysine methylation, 2) arginine methylation, 3) histone deacetylation, 4) histone binding and chromatin modifications. Thick line shows the average trend of genes, for each type of epigenetic modification.

subsequent reductions in energy availability. Thus, observations of faster larval growth coupled with elevated metabolic rates such as in this study, highlight the importance of food availability in satisfying these increased metabolic demands at higher temperatures. Here, larvae were fed multiple times per day, ensuring that larvae were never food limited. Increasing food intake likely allowed larvae to satisfy the increased energy demands of higher temperatures (Logan and Buckley, 2015), whilst also providing enough energy to fuel the faster growth driven by increased metabolic rates (Munday et al., 2008). If larvae were reared at 31 °C in a food limited environment, we would likely have observed suppressed growth and elevated metabolic rates, as increased maintenance costs would restrict the energy available for growth. A study by Shelley and Johnson (2022) observed such a trend as larvae of the California grunion *Leuresthes tenuis* exhibited increased growth rates with increasing temperature in a high-food environment, but in a low-food environment exhibited decreased growth rates with increasing temperature. These measurements were coupled with observations of increased food intake and energy expenditure at higher temperatures, thus directly linking the availability of food to growth rates (Shelley and Johnson, 2022). As trends of faster larval development are observed across latitudinal gradients in nature (McLeod et al., 2015), it appears that food availability is sufficient to support increased energetic demands within natural temperature ranges. Thus, if larval food supplies are maintained under future ocean warming, fish larvae will likely experience the higher energetic costs, elevated metabolic rates, and faster growth rates observed in this study. However, if future larval food supplies are disrupted by future ocean warming (Gregg and Rousseaux, 2019; Heneghan et al., 2021), larvae may experience higher energetic costs, elevated metabolic rates, but slower growth rates and decreased survivorship (Lo-Yat et al., 2011).

In addition to altered physiological processes, *A. ocellaris* larvae displayed altered gene expression at increased temperatures throughout development. This is evidenced by the number of DEGs at 31 °C for each within stage comparison, with at least 464 genes differentially expressed in all developmental stages. Here, stage 3 and stage 6 larvae exhibited a much stronger molecular response at 31 °C, with 3475 and 3784 DEGs respectively. For stage 3 larvae, this large difference may be related to the onset of metamorphosis at stage 4, which brings with it a large shift in the molecular program (Roux et al., 2022). Therefore, although pairwise comparisons for each stage were conducted independently here, it may be that faster development of larvae at 31 °C resulted in the activation of molecular processes associated with metamorphosis earlier, thus giving rise to the high number of DEGs in stage 3. Stages 6/7 represent another significant developmental period, as it is during these stages that *A. ocellaris* are settling to a host anemone and beginning juvenile life. Therefore, earlier activation of genes naturally involved in settlement and anemone inhabitation may underpin this high number of DEGs observed in stage 6 larvae

at 31 °C. Alternatively, these results coupled with the large increase in metabolic rate and the high number of enriched GO categories in stage 6 larvae at 31 °C may indicate that larvae are more susceptible to higher temperatures during this developmental stage. However, as genes associated with heat stress were not upregulated more in stage 6 larvae at 31 °C (compared to other stages), such a hypothesis may be unfitting.

GO categories associated with metabolic processes were enriched in stages 3, 4 and 5, indicating multiple genes associated with metabolism were differentially expressed. Altered expression of metabolic genes at higher temperatures has previously been reported for multiple species of fish (Jeffries et al., 2016; Madeira et al., 2017b; Logan and Buckley, 2015). However, these studies often report upregulation of genes involved in metabolic processes, whereas here we primarily observed downregulation of such genes, indicating a decoupling of the phenotypic and molecular response. This result is likely underpinned by the complexity of metabolism and the various pathways that can be used to generate ATP, with previous studies reporting dynamic metabolic alterations, such as switches to lipid metabolism at higher temperatures (Li et al., 2021; Veilleux et al., 2018). As studies coupling metabolic rate measurements with transcriptomic assessments are currently limited, the relationship between elevated metabolic rates and the dysregulation of metabolic genes remains unclear. This may be attributed to variability amongst individuals, or to adjustments beyond gene expression, such as post-translational modifications or altered enzymatic activity (Madeira et al., 2016c). DEGs in stage 7 displayed unique enrichment for multiple categories relating to neurotransmission and synapse functioning, with genes in these categories downregulated at 31 °C. Elevated temperatures can lead to altered behaviour phenotypes in coral reef fish (Allan et al., 2015; Warren et al., 2017; Allan et al., 2017), with downregulation of genes involved in neuronal functioning implicated in such changes (Toni et al., 2019; Nonnis et al., 2021; Bernal et al., 2022). As stage 7 *A. ocellaris* are juveniles inhabiting a reef and anemone environment (Roux et al., 2019) their behaviour can vary from the innate developmental program they adhere to in stages 1–6. Thus, the alterations to neurotransmission and synapse functioning observed here may lead to behavioural changes.

Heat-shock proteins (HSPs) are involved in protein folding/unfolding and protein stabilization, and thus provide cells with greater thermal tolerance (Liu et al., 2013; De Maio, 1999). Here, we tracked the expression of 10 HSPs at 31 °C, observing sporadic upregulation of these genes throughout development (Fig. 5). This variable regulation of HSP synthesis reflects previous studies of fish, that show both upregulation (Logan and Buckley, 2015) and no change (Veilleux et al., 2015) in the expression of HSPs at elevated temperatures. Here, stage 2 and stage 5 larvae displayed the greatest upregulation of HSPs, with 5 and 6 of 10 HSPs upregulated respectively. This result indicates that *A. ocellaris* larvae are experiencing heat stress very early in development, as stage 2 can be reached at only 2 dph. Interestingly, stage 3 and stage 4 larvae display minimal upregulation of the 10 HSPs, potentially indicating reduced heat stress during these stages. However, previous studies have demonstrated that elevated HSP production can be followed by suppressed HSP synthesis, as sufficient HSPs would already exist within the cells (Didomenico et al., 1982). Thus, it may be that elevated HSP synthesis during stage 2, leads to suppression in stages 3 and 4 larvae. Overall, the activation of multiple HSPs including those belonging to the *hsp70* and *hsp90* families which have well described roles in protein chaperoning and stabilization during heat exposure indicates these larvae experience molecular heat stress at 31 °C (Feder and Hofmann, 1999).

In addition to altered HSP expression, GO enrichment analysis identified multiple DEGs associated with epigenetic modifications, such as those involved in DNA and histone methylation, histone deacetylation and binding, and chromatin modification. These categories consisted of 38 genes and included well characterized DNA methyltransferases (*dnmt1* and *dmap1*) (Lyko, 2018), lysine methyltransferases (*kmt2a, kmt2c,* and *kmt5ab*) (Black et al., 2012), arginine methyltransferases (*prmt1, prmt5, pmrt7*) (Stopa et al., 2015), histone deacetylases (*hdac3, hdac4, hdac5*) (Turner, 2000), histone binding proteins (*nasp*) (Nabeel-Shah et al., 2014), and chromatin remodelers (*atrx*) (Park et al., 2004). Differential expression of these genes was underpinned by their activation and upregulation during stages 3 and 6 at 28 °C, suggesting *A. ocellaris* larvae undergo natural epigenetic reprogramming during these developmental stages. Such reprogramming is known to occur during development, with DNA methylation and histone modification integral processes (Feng et al., 2010; Kucharski et al., 2008). Altered epigenetic reprogramming during larval exposure to elevated temperatures has been observed in multiple fish species (Skjærven et al., 2014; Anastasiadi et al., 2017; Fellous et al., 2015), with experimental evidence linking the altered expression of methyltransferases such as *dnmt1* to patterns of DNA methylation (Anastasiadi et al., 2017). Thus, inactivation of these mechanisms at 31 °C indicates that the natural epigenetic reprogramming that occurs during *A. ocellaris* development is dampened at elevated temperatures. On the other hand, it may be that the timing of epigenetic reprogramming is simply altered, rather than dampened at 31 °C, with such modifications occurring earlier/later in development. However, as we did not observe the reverse trend (e.g., increased expression of genes involved in epigenetic reprogramming at 31 °C) in any other stages we believe this is unlikely. However, without transcriptomic assessments of more fine scale larval stages (e.g., daily), such a hypothesis cannot be excluded. Nevertheless, as epigenetic marks acquired during development can influence the gene expression of later developmental stages (Feng et al., 2010), these disruptions at 31 °C may underpin the molecular and phenotypic differences previously described for later life stages of coral reef fish.

Overall, this study demonstrates that larvae of the clownfish *A. ocellaris* will experience an altered developmental program under future ocean warming or if hatching during a marine heatwave. Firstly, faster larval growth and development at +3 °C will result in *A. ocellaris* reaching key developmental stages earlier, potentially influencing larval survival and dispersal in nature. Secondly, higher metabolic rates, likely driven by increased cellular activity and faster growth rates highlight the increased energetic costs at +3 °C, that may influence survival if food is limited. Finally, larval transcriptome profiles reveal stage-specific differences in gene expression at +3 °C, including upregulation of HSPs throughout development and a dampening of natural epigenetic reprogramming mechanisms. Ultimately, these alterations to *A. ocellaris* larval development at higher temperatures may impact future population persistence.

## CRediT authorship contribution statement

BM, TR1 (Timothy Ravasi), TR2 (Taewoo Ryu) conceived the study. MI and EK collected wild *A. ocellaris* breeding pairs. BM was responsible for experimental set-up, larval husbandry, daily microscopic imaging, image analysis, RNA extractions and data analysis. BM and JJ conducted metabolic measurements. BM conducted bioinformatic analysis with TR2. BM wrote the manuscript with help from all authors. All authors read and approved the final manuscript.

## Data availability

Data is available from repositories specified in the manuscript.

## Declaration of competing interest

The authors declare no competing interest.

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## Data accesibility statement

All transcriptomic sequencing reads have been deposited in the NCBI database under the BioProject ID: PRJNA874849. The data that support the findings of this study are openly available in the Dryad Repository: https://doi.org/10.5061/dryad.95x69p8pj.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162296.

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