Comparative Biochemistry and Physiology, Part A 274 (2022) 111318

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Metabolic rates, feed intake, appetite control, and gut transit of clownfish *Amphiprion ocellaris* exposed to increased temperature and limited feed availability

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ARTICLE INFO

Edited by Michael Hedrick

Keywords:
Clownfish
Temperature
Food availability
Food intake
Appetite
Gene expression

ABSTRACT

Episodes of elevated temperature, combined with lower feed availability, are among the predicted scenarios of climate change representing a challenge for coral reef fish. We investigated the response of clownfish (*Amphiprion ocellaris*) to a scenario in which it received a single meal to satiety after 48 h fasting at 32 °C (climate change scenario) and 28 °C (control). We analysed the metabolic rate (MR), feed intake, gut transit, and expression of selected brain neuropeptides and one receptor believed to be involved in appetite control. Fish at 32 °C ingested 17.9% less feed and had a faster gut transit than did fish at 28 °C. MR in the unfed fish was 31% higher at 32 °C compared to 28 °C. In the fed fish, postprandial MR at 28 °C was 30% higher compared to that of unfed fish, while at 32 °C it was only 15% higher. The expression of *agrp1* did not differ between unfed and refed fish. The levels of both *pomca* and *mc4r* increased immediately after the meal and subsequently declined, suggesting a possible anorexic role for these genes. Notably, this pattern was accelerated in fish kept at 32 °C compared with that in fish kept at 28 °C. The dynamics of these changes in expression correspond to a faster gut transition of ingested feed at elevated temperatures. For both *agrp2* and *pomcb* there was an increase in expression following feeding in fish maintained at 32 °C, which was not observed in fish kept at 28 °C. These results suggest that low feed availability and elevated temperature stimulate anorexigenic pathways in clownfish, resulting in significantly lower feed intake despite the temperature-induced increase in metabolic rate. This may be a mechanism to ameliorate the decrease in aerobic scope that results from higher temperatures.

1. Introduction

Climate change is predicted to result in a continuous increase in temperature, as well as more frequent and variable extreme events, including episodes of small-scale acute warming or heat waves (IPCC, 2021). Exposure of fish to such changes in temperature causes acute stress (Alfonso et al., 2021) and dramatic shifts in physiology, metabolism, and behaviour (Kovacevic et al., 2019). Acute exposure to elevated water temperature not only affects homeostasis but may also alter the response to other stressors, compromising the long-term coping capacity of fish (Alfonso et al., 2021). Species that have evolved to inhabit stable ecosystems, such as coral reefs, are typically stenothermic, and their capacity to cope with changes in temperature beyond their narrow natural thermal zones is limited. As such, any slight increase in temperature results in a temporal reduction in organism performance (Dowd et al., 2015). Therefore, studies on the physiological responses of coral reef fish exposed to acute changes in temperature are critical to understanding the adaptation capacity of species under fluctuating environmental conditions. In the present study, we used clownfish (*Amphiprion ocellaris)*, a canonical representative of coral reef residents, which is frequently used as a model to investigate the impact of climate change on survival, development, reproduction, and reef fish welfare (Chambel et al., 2015; Fobert et al., 2021; Madeira et al., 2017; Nguyen et al., 2019a). It is also an interesting species in that it tolerates warmer water better than other species in the subfamily Amphiprioninae. As such, it has the potential for thermal acclimation to harsher global warming scenarios if given time to acclimate (Madeira et al., 2016, 2017; Pham et al., 2021).

Because of their poikilothermic, ectothermic nature, most fish, including clownfish, maintain the same temperature as the surrounding

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https://doi.org/10.1016/j.cbpa.2022.111318
Received 29 June 2022; Received in revised form 7 September 2022; Accepted 7 September 2022
Available online 14 September 2022

water, and body temperature will thus vary closely with water temperature fluctuations (Beitinger and Fitzpatrick, 1979). Consequently, water temperature consistently determines the rates of virtually all biochemical reactions, physiological processes, and metabolic pathways. Therefore, temperature is a key factor that affects the minimum and maximum metabolic rates and thereby the aerobic scope (Volkoff and Rønnestad, 2020). Importantly, some species are particularly vulnerable to the effects of increased water temperature. Fish that live close to their upper thermal optimum may have a limited capacity for thermal acclimation and may show a significant reduction in aerobic scope following a slight increase in temperature (Dowd et al., 2015; Munday et al., 2012). The aerobic scope of marine fish is predicted to determine their thermal tolerance. In line with this, a previous study found that the aerobic scope of two thermally sensitive coral reef species (cardinalfishes *Ostorhinchus cyanosoma* and *O. doederleini*) was reduced by nearly half at 31 °C compared to 29 °C, and virtually all capacity for additional oxygen uptake was exhausted by 33 °C (Nilsson et al., 2009). In contrast, three thermally tolerant species (*Dascyllus anuarus, Chromis atripectoralis*, and *Acanthochromis polyacanthus*) retained over half their aerobic scope at 33 °C (Nilsson et al., 2009).

Increased temperatures affect metabolic rates, feed intake (energy input), digestion, and nutrient utilisation, and the challenge is to meet increasing energy demands (Nguyen et al., 2019b; Yúfera et al., 2019). Physiological responses are typically activated to reimplement homeostasis and depend on whether fish are given time to adjust to the elevated water temperature. Generally, within the thermal tolerance limit, higher temperatures result in increased feed intake, digestion, and gut transition rates. However, when temperatures approach the upper thermal tolerance limit (i.e. beyond the optimum), there is typically a reduction in feed intake and eventually a complete loss of appetite: that is, anorexia (Chen et al., 2019). While processing and assimilating the ingested feed, some energy is lost via digestion and metabolic handling of absorbed nutrients in a process called specific dynamic action (SDA) (Secor, 2009), which represents the postprandial metabolic rate (MR). Higher temperatures tend to induce higher SDA up to a certain point, but in general also shorten the duration of SDA (Volkoff and Rønnestad, 2020). Thus, temperature not only affects whole-body metabolism and aerobic scope, but also influences the allocation and trade-off regarding the energy budget for routine activities, growth, development, and feed intake via SDA (Metcalfe et al., 2016; Nilsson et al., 2009; Volkoff and Rønnestad, 2020). Global warming is predicted to cause lower primary production of plankton (Fu et al., 2016), a key source of feed for fish. An acute increase in water temperature may create harsh synergetic situations in which fish physiological plasticity is challenged by limited availability of feed to meet increasing metabolic demands.

While the effect of increased temperature can be dire, long-term acclimation to tolerable temperatures will enable fish to adapt by adjusting their MR and reallocating the energy budget or changing their behaviour to tolerate or even avoid thermal threats (Alfonso et al., 2021). Indeed, *A. ocellaris* adapted well to 30 °C temperature after 28 days (Madeira et al., 2016), although thermal stress biomarkers, e.g. heat shock protein 70 kDa and total ubiquitin, showed a temporal increase after 7 days of exposure to the elevated temperature. However, little is known about the potential impact of acute thermal changes on the feeding and ingestion energy of this species. When there is an acute change in environmental conditions, the response in metabolism and physiology is more substantial compared with slow and steady long-term acclimation (Schmidt-Nielsen, 1997). Fish prioritise the allocation of energy to balance the trade-off between survival and growth (McLeod and Clark, 2016). This is particularly relevant because climate change strongly affects plankton communities, such that reef fish may experience periodic or prolonged limitations of available feed resources (McLeod et al., 2013) combined with increased metabolic demands.

Acclimation to altered water temperatures also includes adjustments in feed consumption and digestion; however, the underlying mechanisms have not been thoroughly described. In mammals, feed intake and appetite are believed to be controlled by neural circuits, mainly in the hypothalamus, releasing neuropeptides with opposing functions: orexigenic, that stimulate hunger and feed intake (e.g. neuropeptide Y, NPY; agouti-related-protein, AgRP), and anorexigenic: that stimulate satiety and inhibit feed intake (e.g. Pro-opiomelanocortin, POMC; cocaine- and amphetamine-regulated transcript, CART) (Rønnestad et al., 2017; Volkoff, 2016). Central to this regulatory network is also the melanocortin-4-receptor (MC4R). MC4Rs are located on higher-order integrating neurons, where these neuropeptides act as competitive ligands, and thus either stimulate or inhibit appetite. A range of studies indicate that this system is conserved in many teleosts, while little information exists in clownfish (Pham et al., 2021).

The appetite-controlling pathways respond to energy deficits and peripheral signals via blood hormones, metabolites, or nerves (e.g. *n. vagus*) from a range of peripheral organs and tissues (e.g. filling and composition of the digestive tract) to regulate appetite, digestion, and energy metabolism (Rønnestad et al., 2017; Soengas, 2021; Soengas et al., 2018; Volkoff and Rønnestad, 2020). Goldfish (*Carassius auratus*) exposed to seasonal variations in temperature consumed more feed at a higher temperature (28 °C vs. 15 °C), and in parallel, showed a lower expression level of anorexigenic factors (Pomc, Cart, cholecystokinin Cck, and Melanin-concentrating hormone Mch), but higher levels of orexin, a neuropeptide associated with second-order appetite-controlling neurons (Chen et al., 2019). However, little data are available on how these factors are involved in the regulation of appetite when temperature is beyond the optimum. Short-term fluctuations in temperature (6.5 °C in 4 h) reduced feed intake in Atlantic salmon parr (Folkedal et al., 2012). Although appetite-controlling genes were not targeted, changes in gene expression in fish analysed by microarray in response to long-term acclimation to gradually changing temperature differed from those observed with short-term fluctuations in temperature (Podrabsky and Somero, 2004). For instance, these authors showed that genes involved in changes in fatty acid saturation may be more involved in long-term acclimation and response to fluctuating temperatures, whereas genes involved in cholesterol metabolism may be more critical for short-term acclimation to fluctuating temperatures. This indicates that the time permitted to adopt a change in temperature is important.

The objective of the current study was to assess the metabolic rate, feed intake, and gut transit of clownfish exposed to a fast increase in temperature combined with limited feed availability (one meal after fasting). We also analysed the gene expression of four selected brain neuropeptides: agouti-related-protein 1 & 2 (*agrp1*, *agprp2*), pro-opiomelanocortin a & b (*pomca*, *pomcb*), and one receptor: melanocortin-4-receptor *mc4r* to assess their role in modulating appetite and feeding behaviour in response to such challenging conditions.

2. Materials and methods

2.1. Ethics statement

This study adhered to the National Regulations for Ethical Guidelines for the Use of Animals in Research in Vietnam. All authors have FELASA Category C accreditation and have implemented the best practice for animal use in research.

2.2. Animals, experimental design, and sampling

Juveniles purchased from a commercial hatchery were kept in a large glass tank connected to a water-recirculating system at Nha Trang University prior to distribution to the experimental tanks. The fish were acclimatized to the environment for two weeks with the following water parameters: temperature 28 °C ± 0.5, salinity at 32–33 g L^{-1}, pH at 8.0–8.1, and NH_3 below 0.01 mg L^{-1}. Fish were hand-fed to satiety three times per day (at 08:00, 12:00, and 15:00) with extruded pellets (Win-FAST- 600–800 μm; SPAROS Lda., Olhão, Portugal). This feed was used throughout the whole experiment. After the acclimation period, fish

were sorted to a uniform size (ca. 315 mg body weight) and randomly distributed into 12 experimental tanks (0.4 × 0.5 × 0.5 m, 80 L water) (n = 25 fish per tank). Two groups of six tanks were connected to separate recirculation systems with biofilters and independent temperature control. The temperature was maintained using thermal controllers (JBL ProTemp 300 W, Neuhofen, Germany). Fish were allowed two more days for acclimation to the new tanks while the water conditions were maintained constant, as described above. Feed continued to be carefully offered to ensure satiety of all fish and to prevent waste and uneaten pellets.

At the start of the trial, all fish were fasted for 24 h before the temperature in one group (six tanks) was elevated at a rate of 1 °C for 6 h and thus reached 32 °C within 24 h (See **Supplemental Fig. S1**). The selected temperatures of the trial were 28 °C and 32 °C for the control and projected temperatures, respectively, by the end of the century for the NhaTrang region (IPCC, 2021). At the end of the 48-h period, a single meal was administered to three of the tanks in each temperature treatment (28 °C and 32 °C), while the other three tanks were kept unfed for the remainder of the trial (Unfed). According to the feeding time in the refed groups, fish in all treatments were sampled at five time points (n = 4–5 individuals for each time point per tank) 30 min before feeding (t = − 0.5 h), and then 30 min (t = 0.5 h), one and a half hours (t = 1.5 h), three hours (t = 3 h), and six hours (t = 6 h) after termination of feeding. The fish sampled were measured for body weight (BW) and length (BL), and the head was collected in RNAlater for subsequent dissection and qPCR analysis of appetite-controlling factors, as described by (Pham et al., 2021). In addition, the digestive tract was dissected and visually characterized for segmental filling to assess the gut transit rate (see below), and the content feed/digesta were emptied into a pre-weighed vial. Feed intake was expressed as the feed weight (%) of the fish BW. The body weight of the fish sampled after feeding was corrected by subtracting the weight of the feed in the digestive tract. In parallel with the sampling described above, oxygen consumption was measured in fish from all four groups at time = − 0.5 and 1 h.

2.3. Gut transit rate

The gut transit rate was based on a semi-quantitative assessment using visual observation for the presence of food/digesta at five sampling intervals from −0.5 to 6 h after feeding. The gut was divided into three compartments: stomach, midgut, and hindgut. Filling in each compartment (fed groups, n = 5 at each temperature at each time point) was based on visual assessment of abundance of content (filling) of the respective compartment and graded from − (empty) to ++++ (full). Newly ingested feed could easily be identified due to the pink clour of the pellets. Based on this assessment, five distinct phases of filling each compartment were defined (**Supplementary Fig. S2**). Phase 1 was characterized by a completely empty GI tract (any remaining contents was completely white and the wall of the digestive tract was thick and contracted). In Phase 2, the food fully occupied the stomach only (visibly stretched) while the intestine was completely empty. In phase 3, the stomach was still relatively full, and small amount of digesta had entered the midgut (any remnants in hindgut was white). In Phase 4, stomach filling was lower (and less stretched), and there was significant digesta in the midgut and often in the hindgut (hindgut was pink). In Phase 5, the stomach was almost empty (significantly lower volume and much less stretch), but digesta was still present in the midgut and hindgut. No other phases were observed during the 6 h sampling period after feeding.

2.4. Metabolic rate

Metabolic rate, assessed as oxygen uptake (MO_2), was analysed using closed respirometry (FireStingO_2, Pyro Science, Aachen, Germany). Oxygen concentrations were analysed in 20-ml gas-tight vials using fibre-optic oxygen sensors connected to one of the four channels of an optical oxygen meter. The oxygen meter registers the signals emitted by the integrated sensor stripes in the chamber walls using the REDFLASH technology. The chambers were connected to the fibre cables using adapter rings and were submerged in the same water bath to maintain the temperature of the experimental tanks from which fish were collected. Signals were displayed using the Pro Oxygen Logger software (Pyro Science).

Fish were individually transferred into a respirometry vial that was previously filled with temperature-controlled sterilised seawater and saturated with air. After the vials were closed off and immersed in the water bath, behaviour and oxygen levels were observed to ensure that the fish did not exhibit abnormal behaviour and oxygen levels declined steadily (see McLeod et al., 2013). Results from respirometry with fluctuating oxygen levels were excluded. After an initial adaptation period of approximately 10 min, measurements were performed five times at 1-min duration at 15-min intervals or until the oxygen content in each respirometer was reduced by 20%. In parallel, control treatments in vials without fish were conducted to calculate the background respiration at each temperature (which never exceed 1%). Calculations of the decline in oxygen content ($mgO_2\ L^{-1}$) in the vials were based on the slope of the linear regression between the average oxygen content over five time points, and then subtraction of the background. The metabolic rate MO_2 (expressed as $mgO_2\ kg^{-1}\ h^{-1}$) was calculated according to the following formula:

$$MO_2 = \frac{(\Delta O_{\exp} - \Delta O_{blank}) \times V}{BW} \times 3600 \times 1000$$

ΔO_{exp} and ΔO_{blank} ($mgO_2L^{-1}s^{-1}$) are slopes of linear regressions of oxygen density in experimental respirometry and control respirometry (without fish) against incubation time (s). V is the volume of the respirometry chamber (mL), and BW (g) is the wet body weight of the fish.

2.5. Gene expression analysis

Prior to total RNA extraction, the eyes were removed from the head to prevent contamination due to high levels of neuropeptides such as NPY. RNA was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol. Genomic DNA was removed using an Ambion TURBO DNA-free kit (Life Technologies, CA, USA). RNA quality and quantity were assessed using a NanoDrop One spectrophotometre (Thermo Fisher Scientific, Waltham, MA, USA). To examine RNA integrity, 25% of the total samples were analysed using an Agilent 1000 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RIN scores ranged from 9 to 10. Quality-checked RNA (3 μg) was subjected to cDNA synthesis using the SuperScript™ IV First-Strand Synthesis System Kit (Invitrogen, USA) and oligo-dT priming (Oligo T $(dT)_{20}$) according to the manufacturer's protocol. No-enzyme controls were used to monitor the presence of genomic DNA. qPCR analysis was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Samples were run in triplicate using 20 ng of template (cDNA, 40×, diluted) and iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA). Controls included an inter-run calibrator, no template, and no reverse transcriptase. Essay efficiency was monitored using a 2-fold dilution series based on a pool of random samples (n = 20). Thermal cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min, repeated 40 times. All runs included quality control of the melting curves. Details on primer sequences, amplicon size, and data analysis for each gene are provided (Pham et al., 2021). The qPCR efficiencies ranged between 90 and 107%. Data are presented as log2 normalized gene expression data.

2.6. Data analysis

Linear models for the effects of temperature and feeding status on body weight, feed intake, and oxygen consumption (response variables)

were tested. When no interaction was found between the two factors, the interaction was eliminated from the model. The difference in variance in the response variables was then explained by the total independent effects of the two factors (ANOVA, oxygen consumption). When only one factor showed an effect, the difference in the mean was tested using a *t*-test (feed intake). Relative quantitation of gene expression data were log transform (base = 2) prior to statistical analysis. The effects of temperature, feeding status, and time related to feeding on gene expression were presented using linear mixed-effect models, including tanks as random factors. Analysis of variance (ANOVA) between groups and followed by Tukey post hoc tests (using emmeans package) for pairwise comparisions within groups (e.g., different time points within each treatment, different treatments in the same time point) were applied on the model. Shapiro –Wilk test and Fligner -Killeen test were performed to check the normality and homogeity of the data set for applied parametric analysis. Statistical significance was obtained when the *p*-value was <0.05. Graphs and analyses were performed using RStudio, version 1.1.456.

3. Results

3.1. Growth, feed intake, and metabolic rate (MO_2)

Fish body weight, feed intake, and MO_2 are presented in Table 1. Fish body weight did not differ among the treatments ($p > 0.05$). In the refed groups, feed intake was 6.4 ± 0.3 and 7.8 ± 0.3% of BW at 32 °C and 28 °C, respectively, and thus significantly lower (17.9%) at 32 °C compared to 28 °C (*t*-test, $p < 0.01$). MO_2 in the unfed fish was 735.3 ± 24.4 and 561.3 ± 24.5 $mgO_2\ kg^{-1}\ h^{-1}$ at 32 °C and 28 °C, respectively; thus, it was 31% higher in fish kept at 32 °C ($p < 0.003$, ANOVA). In the refed fish at 28 °C, the MO_2 was 730.0 ± 48.0 $mgO_2\ kg^{-1}\ h^{-1}$, which was 30.0% higher compared to the unfed fish ($p = 0.01$, ANOVA), while at 32 °C it was 841.6 ± 53.9 $mgO_2\ kg^{-1}\ h^{-1}$, which was 15.2% higher (p = 0.01, ANOVA). There was no interaction between temperature and feeding status on oxygen consumption.

3.2. Gut transit

Feed transit through the digestive tract was described as a distinct phase based on assessment of the filling of each segment (Table 2, **Supplementary Fig. 2)**. In unfed fish, the digestive tracts of all fish were completely empty (that is, phase 1). In refed fish, after feeding ($t = 0.5$ h), all fish were in phase 2, with all the ingested food located in the stomach only, and with a full stomach. Digestive tracts sampled from fish at 32 °C at times = 1.5, 3, and 6 h postprandial were at phases 3, 4, and 5, respectively, while the same timepoints were in phases 2, 3, and 4, respectively, at 28 °C, indicating a faster rate of gastric evacuation and gut transit at elevated temperature.

3.3. Gene expression

Gene expression data are presented in Fig. 1. In unfed fish, there were no differences in the expression of *agrp1, agprp2*, or *mc4r* between different temperatures or time points.

Table 2
Characterization of filling of the digestive tract in clownfish refed a single meal at 28 °C and 32 °C after 48 h of fasting. Assessment based on viscera dissected from 5 fish in each treatment at each time point −0.5, 0.5, 1.5, 3 and 6 h in response to refeeding time. Filling of each compartment was characterized as (++++) to (+++), (++), (+) and (−) that represent the relative fullness from highest (completely full) to empty (−). Phases are defined according to fullness level of the stomach. Dissected tracts representative for each phase is shown in Supplementary Fig. 2.

Time (hours)	Temp	Stomach	Anterior gut	Posterior gut	Phase
−0.5	28 °C	−	−	−	1
	32 °C	−	−	−	**1**
Feeding (at 0 h)					
0.5	28 °C	++++	−	−	2
	32 °C	++++	−	−	**2**
1.5	28 °C	++++	−	−	2
	32 °C	+++	+	−	**3**
3.0	28 °C	+++	+	−	3
	32 °C	++	++	+	**4**
6.0	28 °C	++	++	+	4
	32 °C	+	+	+	**5**

In the fed group, at 32 °C, there was a similar trend for all genes with a peak of mRNA levels 0.5 h after the meal, followed by a gradual decrease over time until 6 h ($p = 0.009$, <<0.001, 0.02, 0.02, and 0.0001 for *agrp1, agrp2, pomca, pomcb,* and *mc4r*, respectively). Among the genes, *pomca* and *mc4r* showed similar expression patterns at both temperatures after feeding. Expression of these two genes reached a peak at 0.5 and 1.5 h for fish kept at 32 °C and 28 °C, respectively, followed by a significant drop at 1.5 h and 3 h at 32 °C and 28 °C, respectively (Fig. 1).

After feeding, expression of for *agrp1* (at time = 6, $p = 0.02$), *agrp2* (at time = 0.5, $p = 0.04$), and *mc4r* (at time = 0.5, $p = 0.03$) were higher in fish kept at 32 °C than at 28 °C.

For fish at 32 °C, the refed group analysed immediately after feeding ($t = 0.5$) had higher levels of *agrp2* (p = 0.02), *pomca* (p = 0.03), *pomcb* (p = 0.03), and *mc4r* (p = 0.02) than the unfed group. No similar differences were observed for *agrp1*.

4. Discussion

In this study, we investigated the effects of increased temperature combined with limited feed availability on metabolic rate, appetite control, feed intake, and gut transit in clownfish.

Given that metabolic rate is known to be affected by temperature (Nguyen et al., 2019b; Yúfera et al., 2019), the observed increase in MO_2 in clownfish exposed to 32 °C compared to fish kept at 28 °C (Table 1) was expected, demonstrating a higher metabolic demand in clownfish at elevated temperatures.

To further investigate the effects of acute temperature increase on clownfish, we compared the MO_2 of fed and unfed fish at the two temperatures (Table 1). The MO_2 of unfed clownfish represents the standard metabolic rate (SMR), also known as the routine MR (RMR). SMR represents the cost of living, including normal resting swimming activity

Table 1
Body weight, feed intake and metabolic rate (MO_2) in clownfish at 28 °C and 32 °C. The Refed group was fed one meal after 48 h fasting, while Unfed control group were continuously fasted (Unfed). Data presented as Mean ± SEM, different superscripts indicate significance between feeding status (lowercase letters) and temperatures (uppercase letters) ($p < 0.05$). Ns: no significant difference ($p > 0.05$).

Temperature	28 °C		32 °C		*P* value		
Feeding	Unfed	Refed	Unfed	Refed	Temperature	Feeding	Interaction
Body weight; BW (mg)	310.0 ± 9.0	319.1 ± 8.6	327.3 ± 9.5	307.7 ± 10.4	ns	ns	ns
Feed intake (% BW)		7.8b ± 0.3		6.4a ± 0.3	$p = 0.004$, t-test		
*MO_2 (mgO_2/kg(BW)/h	561.3^{aB} ± 24.5	730.0^{bB} ± 48.0	735.3^{aA} ± 24.4	841.6^{bA} ± 53.9	$p = 0.003$, ANOVA	p = 0.01	ns

* MO_2 of Unfed fish represent routine metabolic rate (RMR), while MO_2 of Refed fish represent standard dynamic action (SDA).

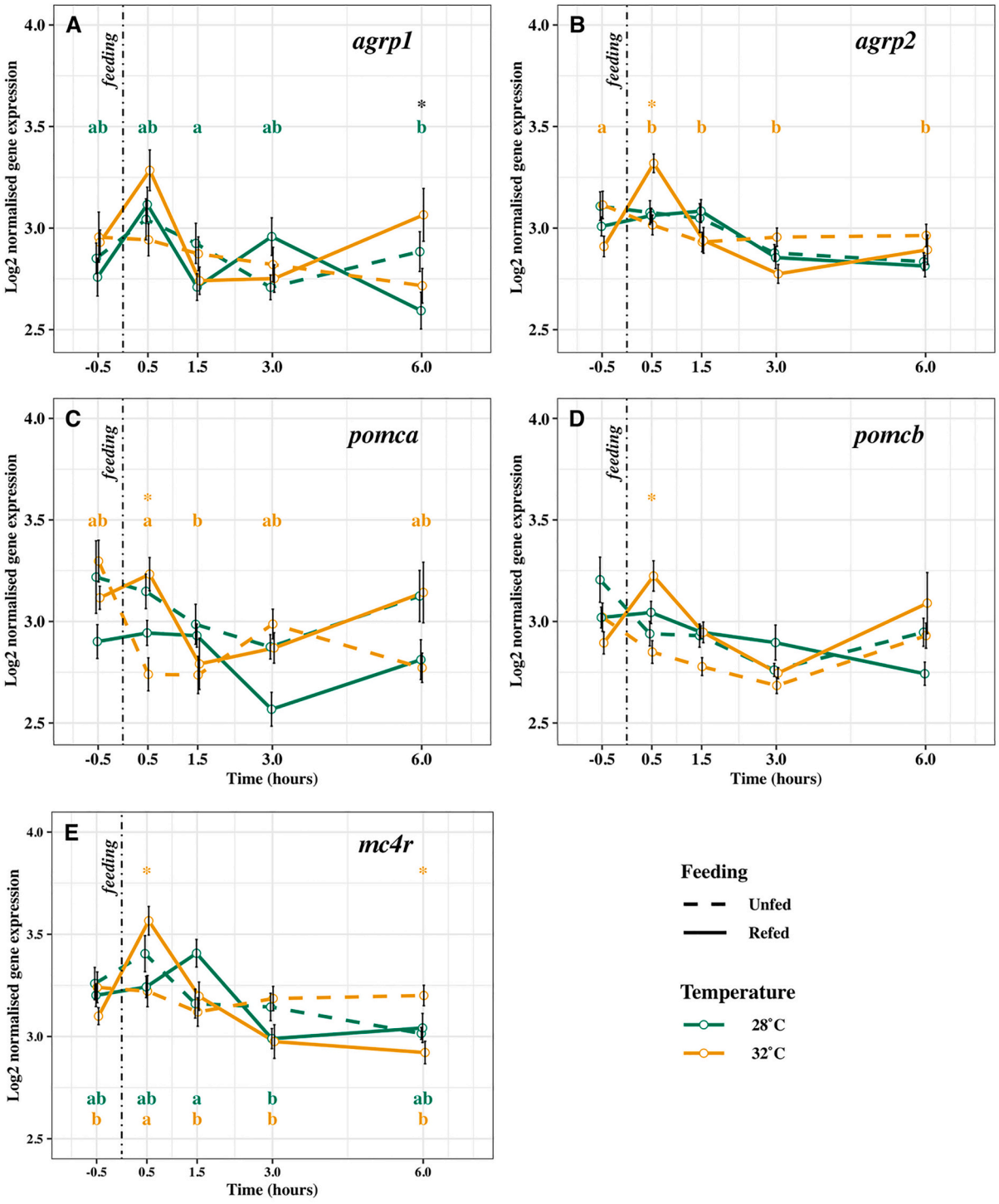

Fig. 1. Effect of temperature (28 °C vs 32 °C) combined with continuous fasting (Unfed) or refeeding of one meal (Refed) on expression of selected genes potentially involved in appetite control in clownfish: *agouti-related-protein 1&2, agrp1& 2* (A and B, respectively); *Pro-opiomelanocortin a&b, pomca*&b (C and D, respectively); and the *melanocortin-4-receptor mc4r* (E). Line color represent fish at 28 °C (green) vs 32 °C (orange), combined with continuous line style representing fasting (Unfed) (dashed lines) or refeeding one meal (Refed) (solid lines). Time represents hours after feeding the meal in the Refed group (time = 0 h; vertical black dashed line). Fish ($n = 4–5$) were sampled at intervals from 0.5 h pre-feeding to six hours post-feeding (time = −0.5; 0.5; 1.5; 3; and 6 h, respectively). Data shown as Mean ± SEM of log2 normalized relative quantity of gene expression (Y-axis). Different letters with same color (a, b) show significant differences among time points within one temperature treatment (green, 28 °C; orange, 32 °C). Asterisk (*) show significant differences between Refed and Unfed fish within each temperature treatment (green, 28 °C; orange, 32 °C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Metcalfe et al., 2016). Notably, the change in this parameter indicates fish tolerance to variable environmental factors, such as temperature and oxygen levels (Claireaux and Lagardère, 1999), and it depends on acclimation history, especially with regard to temperature and duration of acclimation (Velasco-Blanco et al., 2019). Indeed, long-term acclimation (21 days) at temperatures between 22 and 32 °C was shown not to significantly change the SMR of juvenile *A. ocellaris* (Paschke et al., 2018). In the larval stage of *A.percula*, however, SMR increased by 35%

(McLeod et al., 2013) and 55% (McLeod and Clark, 2016) when the ambient temperature increased by >3°, reaching 32.2 and 31.5 °C, respectively.

The MO_2 of the refed clownfish represents the MR of the fish fed a meal, and thus, the standard dynamic action (SDA). SDA is an obligatory metabolic cost in relation to ingesting a meal and is related to post-prandial digestion, absorption, and assimilation of ingested feed. SDA duration and peaks can vary depending on fish size, species, level of activity, water temperature, stressors, quality and quantity of the meal, and feeding ration (Behrens et al., 2012; Fitzgibbon et al., 2007; Secor, 2009). SDA can reach up to 60–80% of the maximum oxygen consumption rate (Chabot et al., 2016; Secor, 2009) and accounts for 5–20% of the gross ingested energy (Fitzgibbon et al., 2007). In the present study, both SMR and SDA were higher in clownfish at 32 °C compared to 28 °C (Table 1). However, the differences between SMR and SDA at 32 °C were smaller than those at 28 °C, indicating a lower surplus aerobic capacity and thus a lower aerobic scope at 32 °C. This suggests that the acute 4 °C increase in water temperature in our study challenged the thermal tolerance limit of the clownfish. The fact that such high temperatures are stressful for the species is also supported by previous findings in clownfish, where the induced rise in MR caused by high temperatures was actually higher than the rise in MR caused by chasing the fish (Paschke et al., 2018). This indicates that temperature is a crucial factor that affects the maximum metabolic rates and aerobic scope. At the same time, long-term acclimation, in contrast to acute exposure to tolerable higher temperatures, enables fish to adapt by adjusting their metabolic rates, reallocating their energy budget, or changing their behaviour to tolerate or even avoid thermal threats (Alfonso et al., 2021). Tolerance to temperature can be assessed as the critical thermal maximum, CT_{max}. Clownfish acclimated to 26 °C have a CT_{max} of 33.6 °C, while fish acclimated to 30 °C have a CT_{max} of 35.7 °C (Paschke et al., 2018). However, CT_{max} represents the temperature at which the fish lose their ability to maintain equilibrium and turn their abdomen up. At CT_{max}, the performance is zero, and the fish will not grow (Volkoff and Rønnestad, 2020), CT_{max}, therefore, does not represent a temperature that supports sustainable living conditions over time. At the same time, the wide distribution of *A. occelaris* from subtropical to tropical areas indicates some thermal plasticity of the species (Madeira et al., 2017; Velasco-Blanco et al., 2019); however, acutely increased water temperature resulting from heat waves poses a challenge to the species.

The differences in post-prandial energy cost (SDA) observed at the two temperatures in our study also shed new light on the reduced feed intake observed at higher temperatures (Table 1). As previously stated, SMR in fish increases as temperature increases (Wood, 2001). Elevated metabolic rates at higher temperatures also lead to a higher demand for feed to meet the increased energy requirement. In line with this, in a study over 6–8 weeks, high temperature induced increased feed intake in clownfish (Pham et al., 2021). In contrast, the present results suggest for a 4 °C rapid increase in temperature beyond the preferable temperature (30.3 °C) suggested for the species (Velasco-Blanco et al., 2019), the clownfish will not be able to fully compensate by sufficiently increasing feed intake to meet the increased metabolic demands. A study of *A. percula* showed a close correlation between SDA and the mass of prey in the gut (McLeod and Clark, 2016). Therefore, the low SDA observed at 32 °C (Table 1) correlates well with the lower feed intake at this temperature, which is in agreement with other studies on the effects of meal size on SDA (Fitzgibbon et al., 2007; Norin and Clark, 2017). This also supports other studies suggesting that the underlying mechanism for reduced appetite and feed intake at elevated temperatures over the optimum is linked to limitations in the aerobic scope for ectotherms that prevent the animal from ingesting excessive feed (Jutfelt et al., 2021; Paschke et al., 2018). The aerobic scope is the difference between the maximal metabolic rate and RMR (i.e. when the energy for all the obligatory cost of living has been allocated) (Munday et al., 2012) and thus represents the energy available for growth, activity, and the SDA. Thus, when the aerobic scope decreases steadily, there will be a smaller amount of energy available for the SDA (Volkoff and Rønnestad, 2020).

To examine the potential mechanisms underlying the observed reduced feed intake under the tested conditions, we evaluated the expression of selected key orexigenic neuropeptides *agrp1* and *agrp2*, anorexigenic neuropeptides *pomca* and *pomcb*, and the expression of *mc4r*, a receptor that integrates signals from appetite-regulating neuropeptides into a coherent physiological response in mammals (Cone, 2006). Owing to their predicted roles in central appetite regulation in fish (Rønnestad et al., 2017), we expected that there would be differences in the expression of these presumed appetite-regulating genes that correlate with temperature and feeding status. This would reflect a mechanism by which the organism would attempt to avoid hunger stress while dealing with allocating energy for survival. Notably, there were significant differences in the expression levels and dynamics of *pomca* (Fig. 1C) after feeding at both temperatures, suggesting that this gene product is correlated with hunger and satiety signals in fish. We did not observe such a clear difference in *agrp1* (Fig. 1A), a postulated orexigenic factor based on a previous study of this species (Pham et al., 2021). In addition, changes in the expression levels of the other neuropeptides involved in the regulation of appetite in response to fasting and food deprivation at elevated temperatures were inclusive. Other studies have also faced challenges in drawing firm conclusions regarding the involvement of neuropeptides based on expression data. Indeed, short-term fasting (2 days and one week) did not alter the expression levels of orexigenic (*agrp, npy)* or anorexigenic factors (*pomca2, mc4r, cart*) in Arctic char (Striberny and Jørgensen, 2017). In addition, *agrp* increased in short-term-fasted *Schizothorax prenanti*, but not after 14 days post-fasting (Wei et al., 2013b). In contrast, long-term fasted (8–29 days) sea bass (*Dicentrarchus labrax*) showed an increase in the hypothalamic expression of *agrp1*, but reduced *agrp2* expression (Agulleiro et al., 2014). Fasting Arctic charr for four months did not result in altered expression levels of *agrp* and *npy* (Jørgensen et al., 2016), while there were lower levels of *pomca1* and *pomcb* in the fish on the first day of refeeding. Expression of *mc4r* increased in Ya-fish (*Schizothorax prenanti)* after fasting for 6, 9, and 24 h and up to 14 days (Wei et al., 2013a), while fasting glass catfish (*Kryptopterus vitreolus*) for one week induced increased expression of orexin, NPY, and CART (London and Volkoff, 2019). Taken together, the literature shows that while the aforementioned genes are known to be involved in appetite control, further studies are required to understand how different neuropeptides respond and act to control feed intake under different scenarios. Additionally, although mRNA expression analysis is a highly useful approach for investigating the role of specific genes, it has inherent limitations with regard to interpreting the physiological role of translated and active protein products. As such, the development and utilisation of synergistic tools and methodologies, such as protein expression analysis via western blotting, could provide a more complete understanding of the role of the relevant genes investigated and should be considered in future studies.

Given the clear inverse relationship between appetite and stomach fullness described, for instance, in rainbow trout (Grove et al., 1978; Sam et al., 2012), mRNA data may also be considered concomitantly to explore whether there is a correlation with the content level of the various intestinal compartments. Upon refeeding the clownfish, the stomach filling shifted from completely empty to full (Table 2), and we expected that there would be a shift in mRNA expression levels, with opposite trends in orexigenic and anorexigenic signals between these points. However, there was a tendency toward a difference in the expression of only some of the genes (Fig. 1), but the data were inconsistent with regard to the observed trends and presumed functions. Still, the higher expression of the presumed anorexigenic neuroendocrine factor, *pomca*, before feeding time (−0.5 h) at 32 °C was also associated with a low feed intake, suggesting that *pomca* may be involved in reducing appetite when the ambient temperature increases acutely.

Another important variable to consider in parallel with feed intake

and stomach filling is gut passage time. Studies in some fish have shown a clear link between stomach filling (that is, gastric evacuation rate) and appetite, and the data on rainbow trout showed a close inverse correlation between stomach filling and ingested amount at the next meal (Grove et al., 1978). Therefore, stomach evacuation rate may also be correlated with the expression of appetite-regulating neuropeptides in the brain. Data from the present study suggest that the dynamic passage of food and digesta through the gut, and thus compartmental filling (Table 2), is correlated with expression over time for *pomca* and *mc4r* (**Fig. 1C** and **E**). Similar dynamic tendencies were observed at both temperatures; however, the changes were delayed at lower temperatures. Of particular note is the shift of the expression peak following the meal in refed fish from 0.5 h at 32 °C to 1.5 h at 28 °C. These two time points correlated with the same phase of food transition (Table 2) in the clownfish gastrointestinal tract (**Supplementary Material Fig. S1D**), in which the food remained in the stomach, but the gut was still empty (Phase 2) at both temperatures. After the peak, a decrease in the expression levels of the two genes was observed at 1.5 h at 32 °C and 3 h at 28°, correlating with the time at which food started to enter the intestine at both temperatures (Table 2). This shift indicates active signalling pathways in the gut-brain axis in clownfish after feeding.

Gut passage time is affected by many factors, such as the feeding regime (García-Ortega et al., 2010), feed quality (Nguyen et al., 2018), and temperature (Das et al., 2018; Volkoff and Rønnestad, 2020). Previous studies in *A. ocellaris* (Khoo et al., 2019) showed that 72-h fasting fish kept at 27 °C emptied the stomach after 36 h, and the gastric emptying rate depended on meal size. In the present study, the gut transit was faster at higher temperatures. This is also seen in several other fish, such as mahseers (*Tor tambroides*), where fish kept at 30 °C had the shortest gastric evacuation time (10*h*), whereas at 22 °C, the time was 18 h (Das et al., 2018). No differences in stomach evacuation rates were observed in Atlantic salmon at 10 and 14 °C, whereas fish kept at 6 °C showed a significant delay in stomach evacuation (Handeland et al., 2008). Several studies have found a significant correlation between stomach fullness and the mRNA expression of some brain neuropeptides, suggesting a possible link between stomach filling/distension and satiety signals (Kalananthan et al., 2020; Murashita et al., 2009). However, very few studies have explored the link between gastrointestinal tract transit rates and expression of neuropeptides signalling hunger or satiety, and there is still no clear understanding of the dynamics. In Atlantic salmon, some studies indicate a correlation, and (Valen et al., 2011) showed that elevated *pomca1* expression at 3 h post feeding correlated with a significantly reduced gastric filling, but no studies have explored how this is affected by increasing water temperature. Further studies are required for understanding how increased temperature and limited feed availability affect the complex interactions between gut transit, appetite, feed intake, and metabolic rates of clownfish.

5. Conclusion

An acute increase in temperature in fasting clownfish increased the metabolic demand but failed to fully compensate with increased appetite and feed intake when food became available. There were no clear roles for presumed orexigenic factors (such as *agrp1*) in the modulation of appetite in this condition. However, we suggest that the stress of exposure to acutely increased temperature, in combination with limited nutritional resources, affects appetite primarily via anorexigenic pathways, possibly involving *pomca*, *pomcb*, and *mc4r*. Additionally, we propose that the reduced meal size limits SDA beyond the aerobic scope to prevent impacts that reduce the performance of clownfish.

Declaration of Competing Interest

Ref.: Ms. No. CBPA-D-22-00190.
Metabolic rates, feed intake, appetite control, and gut transit of clownfish *Amphiprion ocellaris* exposed to increased temperature and limited feed availability.
Comparative Biochemistry and Physiology, Part A.
All authors declare no competing interests.

Data availability

Data will be made available on request.

Acknowledgements

We thank Dr. I. Tolås and T. Kalananthan for their comments on the manuscript. The work was funded by NORAD under the NORHED project (QZA-0485 SRV-13/0010) and supported by the University of Bergen, Norway, and Nha Trang University, Vietnam. IR also acknowledges funds from the Research Council of Norway (project #311627) and mobility grants from the Meltzer Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2022.111318.

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