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Research paper

Food intake, growth, and expression of neuropeptides regulating appetite in clown anemonefish (*Amphiprion ocellaris*) exposed to predicted climate changes

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ABSTRACT

The clown anemonefish (*Amphiprion ocellaris*) is a common model species in studies assessing the impact of climate changes on tropical coral fish physiology, metabolism, growth, and stress. However, the basic endocrine principles for the control of food intake and energy homeostasis, under normal and elevated sea temperatures, in this species remain unknown. In this work, we studied food intake and growth in clown anemonefish reared at different temperatures and with different food availability. We also analyzed expression of genes in the melanocortin system, which is believed to be involved in the control of appetite and feeding behavior. These were two paralogues of *pomc*: *pomca* and *pomcb*; two paralogs of *agrp*: *agrp1* and *agrp2*; and one *mc4r-like*. Groups of juvenile clown anemonefish were exposed to four experimental treatments combining (orthogonal design) two rearing temperatures: 28 °C (T28; normal) and 32 °C (T32; high) and two feeding regimes: one (1 M; 08:00) or three (3 M; 08:00, 12:00, 15:00) meals per day, fed to satiety by hand. The results showed that high temperature (T32) did not affect the average growth rate but induced a stronger asymmetrical individual body weight of the fish within the population (tank). Lower feeding frequency (1 M) resulted in lower growth rates at both rearing temperatures. Fish reared at high temperature had higher total daily food intake, which correlated with a lower expression of *pomca,* supporting an anorexigenic role of this gene. High temperature combined with restricted feeding induced higher *agrp1* levels and resulted in a higher food intake in the morning meal compared to the control. This supports an orexigenic role for *agrp1*. mRNA levels of *agrp2* responded differently from *agrp1*, supporting different roles for the paralogues. Levels of *mc4r-like* inversely correlated with fish body weight, indicating a possible size/stage dependence of gene expression. In conclusion, our results indicate that the melanocortin system is involved in adjusting appetite and food intake of clown anemonefish in response to elevated temperature and low food availability.

1. Introduction

The control of appetite and food intake is essential for maintaining metabolism and supporting growth. In fish, this control is mediated through the hypothalamus, which perceives and integrates a set of central and peripheral signals (Delgado et al., 2017; Rønnestad et al., 2017; Soengas et al., 2018). These signals, which comprise both endocrine and neuronal pathways, relay information on the status of energy and some key nutrients in the body, as well as filling and contents in the gastrointestinal tract. Appetite control is also strongly affected by environmental factors such as temperature (Nguyen et al., 2019; Volkoff and Rønnestad, 2020), stress (Conde-Sieira et al., 2018), and food availability (Striberny et al., 2015). The central control of appetite and food intake is modulated by two opposing signaling loops: (1) anorexigenic – inhibiting and (2) orexigenic stimulation (Volkoff et al., 2005). One of these regulatory pathways (1) is mediated by α-melanocyte-stimulating hormone (α-MSH), a potent anorexigenic peptide derived from pro-opiomelanocortin POMC, which acts on target cells in the paraventricular nucleus via the melanocortin 4 receptor (MC4R) (Cerdá-Reverter et al., 2011; Volkoff, 2016; Volkoff et al., 2005). The orexigenic

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pathway (2) involves the agouti-related protein (AGRP) and acts as an antagonist or inverse agonist on MC4R (Cerdá-Reverter et al., 2011; Volkoff, 2016; Volkoff et al., 2005). This basic mechanism for regulating appetite and food intake is mainly conserved among vertebrates and fish (Dores and Baron, 2011; Rønnestad et al., 2017; Soengas et al., 2018; Tao, 2010; Volkoff, 2016; Yúfera et al., 2019). However, no studies have explored these mechanisms in clown anemonefish.

In fish, Pomc is involved in food intake regulation via two or more different subtypes, whereas in mammals there is only one transcript present (reviewed by (Rønnestad et al., 2017). The *pomc* genes have been characterized in a number of species with varying numbers of transcripts. Studies focused on two transcripts of *pomcα* and *pomcβ* in Senegalese sole (*Solea senegalensis*) (Wunderink et al., 2012); pufferfish (*Tetraodon nigroviridis*) fugu *(Takifugu rubripes)* (De Souza et al., 2005); medaka (*Oryzias latipes*); and three-spined stickleback (*Gasterosteus aculeatus*). Studies involved three transcripts of *pomca1, pomca2,* and *pomcb* in rainbow trout *(Oncorhynchus mykiss)* (Leder and Silverstein, 2006) and *pomc1, pomc2,* and *pomc3* in olive flounder (*Paralichthys olivaceus*) (Kang and Kim, 2015). In Atlantic salmon *(Salmo salar)* there are four transcripts that have been investigated, *pomca1, pomca2*, *pomcb1, and pomcb2* (Kalananthan et al., 2020) Studies examining *pomc* responses to fasting in rainbow trout revealed that the expression of hypothalamic *pomca1* and *pomcb* (but not *pomca2*) increased after 4 months of starvation (Jørgensen, 2016). In olive flounder, 10 days of fasting led to the upregulation of *pomc2,* but not of *pomc1* and *pomc3* mRNA (Kang and Kim, 2015).

Conversely, Agrp plays a role in the regulation of energy homeostasis, growth of larvae and juveniles, and reproduction by stimulating food intake (Stütz et al., 2005; Zhang et al., 2012). However, *agrp1* and *agrp2* may have distinct functions (Cortés et al., 2014; Wei et al., 2013a). In zebrafish, *agrp1* stimulates appetite and food intake, whereas *agrp2* seems to be involved in the regulation of a camouflage mechanism an adaptation to the background to avoid predation (Jeong et al., 2018). Ya-fish have only one form of Agrp (Wei et al., 2013a), the observed response of Agrp in Ya-fish is similar to the response of AGRP1 in mammals, goldfish, zebrafish, and common carp (*Cyprinus carpio*), the expression increases when fasting. An orexigenic action of Agrp was observed in the GH-transgenic common carp, wherein increased food intake positively correlated with higher expression levels of *agrp1* (Zhong et al., 2013).

Mc4r belongs to a family of specific G protein-coupled receptors (Anderson et al., 2016) and has been characterized in several fish species, such as barfin flounder (*Verasper moseri*), zebrafish, goldfish, spiny dogfish (*Squalus acanthias*), fugu, rainbow trout, lamprey (*Petromyzon marinus* and *Lampetra fluviatilis*), European seabass (*Dicentrarchus labrax*), Snakeskin gourami (*Trichopodus pectoralis*), the platyfish genus (*Xiphophorus* sp.) (reviewed by (Rønnestad et al., 2017), Atlantic salmon (Kalananthan et al., 2020), and medaka (*Oryzias latipes*) (Liu et al., 2019). Mc4r participates in both anorexigenic and orexigenic regulatory pathways in the melanocortin system, and both α-Msh and Agrp have diverse signaling modalities that regulate feeding and energy homeostasis through Mc4r (Anderson et al., 2016). It has been suggested that *mc4r* may act with Agrp in the orexigenic signaling in European seabass (Sánchez et al., 2009), and *Schizothorax prenanti* (Wei et al., 2013b).

As an effect of climate change, sea temperature is projected to increase by up to 4–6 °C, which is a stress factor for tropical coral reef fish, especially clownfish (Pörtner and Farrell, 2008). The increased temperature may also lead to a reduction in zooplankton productivity that is one of the key nodes in the fish food web. This will negatively affect the survival rate, growth, and development of fish larvae and juveniles (Hoegh-Guldberg et al., 2014; Anderson and Sabado, 1995; Gale et al., 2013; Henderson, 2006; Sandersfeld et al., 2015; Striberny et al., 2015; Zeng et al., 2018) (Richardson, 2008). When temperature increases beyond the optimum organism's thermal range, rate-limiting enzymes may be affected (Schmidt-Nielsen, 1997; Yúfera et al., 2019). Temperature increases can reduce the oxygen-carrying capacity (Dowd et al., 2015) and increase the transcription of stress markers (Logan and Somero, 2011; Madeira et al., 2016; Podrabsky and Somero, 2004). Fish typically lose their appetite and eventually cease to ingest food as temperatures increase above the optimum range tolerable by the species (Shafland and Pestrak, 1982). The combination of a low availability of food and high temperatures will drive the trade-off in energy expenditure towards allocating significantly more energy to maintenance, rather than for growth (Pörtner et al., 2006) and eventually lead to metabolic suppression and poor homeostasis (Nilsson et al., 2009). Tropical fish (especially coral reef fish) are believed to be more vulnerable to global warming than their counterparts in temperate waters (Beeston, 2009; McLeod et al., 2013; Munday et al., 2012; Pörtner and Farrell, 2008). To improve our understanding of the effects of climate change, it is important to assess how sensitive the target species is to elevated water temperatures combined with low food availability.

This study explores how clown anemonefish (as a model species for tropical coral fish) responds to restricted feeding at elevated temperatures, to reflect the scenario of ongoing climate change. We also aimed to describe the basic molecular characteristics of some key players in the melanocortin system in clown anemonefish and assess the extent to which they are involved in appetite control and food intake.

2. Materials and methods

2.1. Ethics statement

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

2.2. Experimental fish and experimental design

Clown anemonefish juveniles were purchased from a commercial hatchery in Nha Trang, Vietnam. The fish were transferred into an acclimatization tank at Nha Trang University in the following conditions: temperature 28 °C ± 0.5 (representing the average summer sea temperature in Nha Trang Bay), salinity 32–33 g/L, pH 8.0–8.1, and NH_3 < 0.01 mg/L. The rearing system of the hatchery was also maintained at 28 °C ± 0.5. The fish were hand-fed to satiety three times per day with WinFAST- 600–800 μm extruded pellets containing 61% crude protein and 19% lipids (SPAROS Lda., Olhão, Portugal). After an acclimation period of two weeks, the fish were sorted and 260 similar sized juveniles (body weight 140 ± 20 mg; 19 ± 1.5 mm) were randomly distributed into 12 experimental glass tanks (0.4 × 0.5 × 0.5 m, containing 80 L water) with a density of 21–22 individuals/tank. Two sets of six experimental tanks were connected to separate recirculation units with biofilters for controlling the temperature at 28 °C (T28) for the control treatment and 32 °C (T32) for the projected temperature by the end of the century for the region, respectively. Temperature was maintained by thermal controllers (JBL ProTemp 300 W, Neuhofen, Germany). The water temperature in the T32 aquarium unit was slowly increased by 1 °C every 8 h until it reached 32 °C, while in the other aquarium unit (T28) temperature remained stable at 28 °C. The fish maintained at each temperature were divided into two groups: 3 M (the control group were provided three meals per day at 08:00, 12:00, and 15:00) and 1 M (the restricted feeding group were provided one meal per day at 08:00). The fish were fed to satiety by hand for each meal with the same diet as previously provided in the acclimation period. The diet was carefully distributed to the fish to ensure satiety and prevent waste and uneaten pellets. The amount of food consumed at each meal was recorded to calculate the average daily food intake. The experiment ceased after 52 days.

2.3. Sampling

At the end of the experiment, six fish from each tank were sampled at three time points relative to the morning meal (08:00) and the same time for all groups: 0.5 h before feeding (-0.5 h BF), one hour after feeding (1 h - FED1), and three hours after feeding (3 h – FED3). In order to minimize fish stress, only one sample was taken per day from each tank. Therefore, there were three-days of sampling for the three sampling points for each tank. In total, 210 fish were euthanatized (800 g/L MS-222, Sigma–Aldrich, St. Louis, MO, USA). Fish body weight, total length, and standard length were recorded. Fish heads were collected by a standard dissection cut behind the operculum and preserved in vials with RNAlater (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. RNA extraction

The brains of the clown anemonefish juveniles were relatively small and could not be accurately dissected from the fish head. Therefore, the whole head was used for the RNA extraction. Prior to total RNA extraction, the eyes were removed from the head segment to prevent RNA contamination. RNA was extracted with TRI-reagent (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturers protocol. Then ethanol precipitation and elimination of genomic DNA contamination was performed using Ambion TURBO DNA-free™ Kit (Life Technologies, CA, USA). The RNA quality and quantity were assessed using Nanodrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To examine RNA integrity, 25% of the total samples were analyzed using an Agilent 1000 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), resulting in RIN scores between 9 and 10.

2.5. cDNA synthesis

RNA (3 μg) was subjected to cDNA synthesis using a SuperScript™ IV First-Strand Synthesis System Kit (Invitrogen, USA) with oligo-dT primer (Oligo T $(dT)_{20}$) according to the manufacturer's protocol. No-template and no-enzyme controls were used to monitor contamination and primer-dimer formation.

2.6. Cloning, sequencing, and primer design

The cDNA produced from the pooled RNA extracted from six fish heads was subjected to PCR, cloning, and sequencing. The RNA extraction and cDNA synthesis were performed using the protocols described previously. The cloning primers were specific primers based on sequences from *Stegastes partitius*, for *agrp1* (XM_008302574), *agrp2* (XM_008283609), *pomca* (XM_008304571), *pomcb* (XM_008290702 and XM_008286942), and *mc4r* (XM_008293413). PCR were performed according to the manufacturer's protocol (GoTaq Hot Start). PCR products were successfully cloned according to the manufacturer's protocol (pGEM T Easy) and sequenced at the UoB in-house facility.

The qPCR primers were designed within the coding region of identified sequences from clown anemonefish (Table 1). Alignments and phylogenetic analyses were performed using Geneious Prime and MEGA-X Software.

2.7. qPCR

The cDNA was used as a template for qPCR reactions. A 2-fold dilution series (a pool of random samples (n = 6)) were used to evaluate the essay efficiency (Table 1) and the cDNA dilution factors (20 for *mc4r-like* and 40 for all other essays). The qPCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, CA, USA). Samples were run in triplicate using 20 ng template (40x) and iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) according to the manufacturer's instructions. Controls included an inter-run calibration (pool) as well as a negative and a no template control. The thermal cycle for each qPCR run was as follows: 50 ℃ for 2 min, 95 ℃ for 10 min, repeated 40 times. The next two steps were 95 °C for 15 s and 60 °C for 1 min, then finally 95 °C for 10 s and 60 °C for 1 s. All runs included melting curves as quality controls for specificity.

2.8. Data processing and analysis

Daily food intake, FI, was calculated by subtracting the leftover food from the food offered divided by the number of fish in each tank. Fulton's condition factor (K) was calculated as described by Froese (Froese, 2006). Data were tested for normality of distribution and homogeneity of variance using the Shapiro-Wilk test and Levene's F-test, respectively. Two-way ANOVA was performed to test the effects of temperature and feeding regime on growth by evaluating the changes in body weight and length, K, daily FI, and morning FI. When an interaction between the factors was not detected, a Student's *t*-test was applied to compare the means of those parameters between the two feeding regimes or two rearing temperatures. An F-test was applied to compare the variance in K between the two temperatures or two feeding regimes.

For mRNA expression levels, we used a relative quantification normalized to the elongation factor 1-alpha *ef1a*. The normalization procedure was based on the efficiency correction method described by Pfaffl (Pfaffl, 2004) and the applied framework described by Hellemans et al. (Hellemans et al., 2007). The variation from plate to plate was neutralized by an interrun-calibrator. The selection of the reference gene was based on the expression stability (M) that was scored using NormFinder software. The input data for the gene expression in the statistical analysis were rescaled (multiplied by 10) and then log-transformed (base = 2). A Pearson's test was applied to determine the correlation coefficients between the fish body weight (scaling effect) and

Table 1
Primers used for cloning and qPCR. Primer sequences indicated as 5->3′prime, listed as either cloning (c) or qPCR (q) and forward (F) or reverse (R) primers. A specific qPCR primer pair amplifies a designated target sequence (bp). qPCR amplification efficiency (%) was calculated based on the slope of the standard curve to monitor assay efficiency.

Gene name	primer sequence (5 → 3′)	Target sequence (bp)	Amplification efficiency (%)	
agrp1	cF: ATGTTTGGCTCAGTGCTGCT	cR: CCGACTCGGCGGCAGTA		
	qF:GCTCAGACTCTCCTCATCTCTGG	qR:TGCAGACGCATCCTCATCATA	195	104
agrp2	cF: ATGTGGAAGATCAGCGCCAA	cR: CAGAGGGTTCATCCTCCAGC		
	qF: GGAAGACTGAGAACGCCACA	qR: GAGCAGCTTTCCATCAAGCG	157	99
pomca	cF: GTGTGGCTATTGGTGGCTGT	cR: TCACTTCTGCTGCTGTCCG		
	cF: ATGTGTCCTGTGTGGCTATTGG	cR: GCTCCCTCATTTCCTCCTGTC		
	qF: GTGGCTGTGATGGTTGTGGG	qR: GGGGAGGACGAGGGAGGAA	201	98
pomcb	cF: ATGGTGTGTCTCTGTTGGCTG	cR: TATCCTCTGCGCATCCTTGA		
	qF: GAGCTCAGTGGGTTTACTGTGA	qR: TGAGCTTTAACATCTGACTCTGGT	113	104
mc4r-like	cF: CATCAGCCTGCTGGAGAACA	cR:TCATCTCCTGGCTGCGAAAG		
	qF: CATTGCGCTCATCAACGGAG	qR: CTCAGCGCGTAGAAGATGGT	162	107
ef1a	qF: AGTGCGGAGGAATCGACAAG	qR: TGCTGGTCTCGAACTTCCAC	161	99

genespecific mRNA expression. The effects of temperature, feeding regime, time of sampling, and body weight on gene expression were tested using the linear models and/or non-linear models (generalized addicted model – GAM) with the factors identified as independent variables. Model selection and number of parameters contributing to the best-fit models were based on the Akaike Information Criterion (AIC) (Rushworth et al., 2011). When an interaction was not detected, a Student's *t*-test was applied to compare the means of the fish from the two feeding regimes or two rearing temperatures. A one-way ANOVA with Tukey's post-hoc test was applied to compare the variance between the sampling points. Statistical analysis and graphs were performed using R studio 1.1.456. In all analyses a probability value<0.05 was considered statistically significant ($P < 0.05$).

3. Results

3.1. Gene sequences and phylogeny

From the PCR, we obtained partial cDNA sequences of *pomc-a, pomc-b, agrp-1, agrp2,* and *mc4r-like*. The partial clown anemonefish open reading frame (ORF) *pomc-a* nucleotide sequences (GenBank accession nos: **MK900694** and **MK900695 and MK900699**) were 591, 601 and 620 bp in length encoding sequences of 197, 200 and 206 amino acids, respectively (Fig. 1). The partial clown anemonefish ORF *pomc-b* nucleotide sequences (GenBank accession nos: **MK900696, MK900697 and MK900698**) were 787, 748, and 754 bp in length encoding sequences of 263, 250, and 252 amino acids, respectively (Fig. 1). Molecular characterizations of the clown anemonefish *pomc* sequences were performed by aligning the amino acid sequences (Fig. 1) with a phylogenetic analysis of the nucleotide sequences (Fig. 2). The core amino acid sequences for the MSH (HFRW) and β-END (YGGFM) segments are highlighted by a box in Fig. 1. The B-end sequence is only present in the POMCa-3 sequence. The clown anemonefish Pomca-3 sequence shared 63.7% of the identity with clown anemonefish Pomca1-2 sequences, and 31.3% of the identity to clown anemonefish Pomcb1-3 sequences. The three *pomcb* variants cloned using XM_008286942 specific primers clustered well within the teleost *pomcb* clade. The clown anemonefish *pomca* sequences also clustered well within the teleost *pomca* clade.

The partial clown anemonefish *agrp-1* ORF nucleotide sequence (GenBank accession no: **MK900693**) was 237 bp long and encoded a sequence of 77 amino acids (Fig. 3). The partial clown anemonefish *agrp-2* ORF nucleotide sequence (GenBank accession no: **MK900692**) was 330 bp long and encoded a sequence of 110 amino acids (Fig. 3). The molecular characterization of clown anemonefish *agrp* is provided in Fig. 3 (the alignment of the amino acid sequences) and Fig. 4 (the phylogenetic analysis of the nucleotide sequences). The signal peptide is highlighted by a box in the partial Agrp1 sequence. Solid lines demonstrate the suggested disulfide bonds following the guidelines of Wei et al. (2013b) in clown anemonefish Agrp2 (Fig. 3). The clown anemonefish *agrp1* and *agrp2* clustered well within the teleost *agrp1* and *agrp2* clades, respectively (Fig. 4). The partial clown anemonefish ORF *mc4r-like* nucleotide sequence (GenBank accession no: **MK900700**) was 764 bp in length. The clown anemonefish *mc4r-like* sequence clustered well within the teleost *mc4r-like* clade (Fig. 5).

3.2. Effects of temperature and feeding regimes on fish growth and food intake

The number of meals per day affected growth at both temperatures (Table 2). Restricted feeding (1 M) decreased the growth, body weight, and length of the fish. However, the average growth was similar at both temperatures for the two feeding regimes (1 M and 3 M). There were no significant differences in the mean condition factor (K) between the two temperatures, but there was a higher variance in K in the fish kept at an elevated temperature of 32 °C (T32) compared to the control (T28). Both

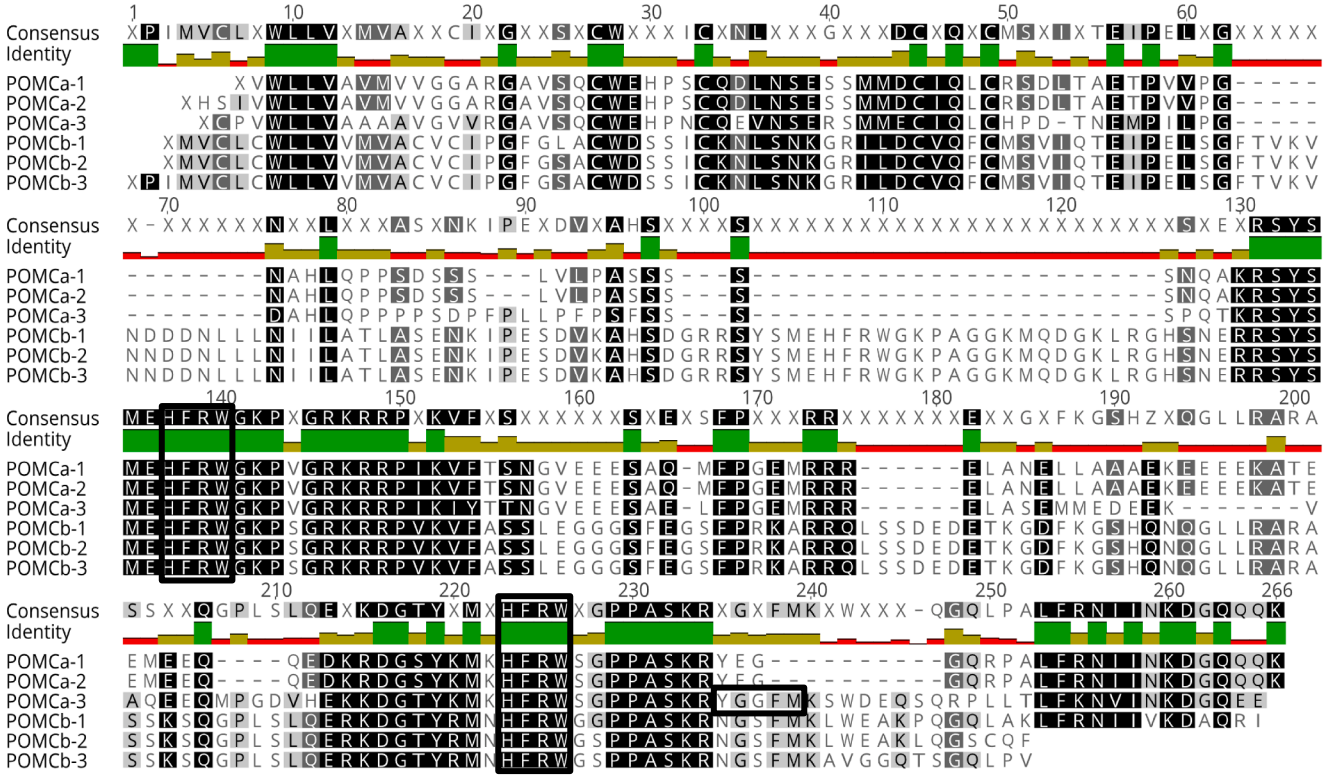

Fig. 1. Alignment of the clown anemonefish Pomc amino acid sequences. Alignments were made using the submissed clown anemonefish GenBank amino acid sequences in Geneious software. GenBank Accession Nos. are as follows: Pomca-1 (**MK900694**), Pomca-2 (**MK900695**), Pomca-3 (**MK900699**), Pomcb-1 (**MK900696**), Pomcb-2 (**MK900697**), Pomcb-3 (**MK900698**) The core amino acid sequences for the MSH (HFRW) and β-End (YGGFM) segments are boxed.

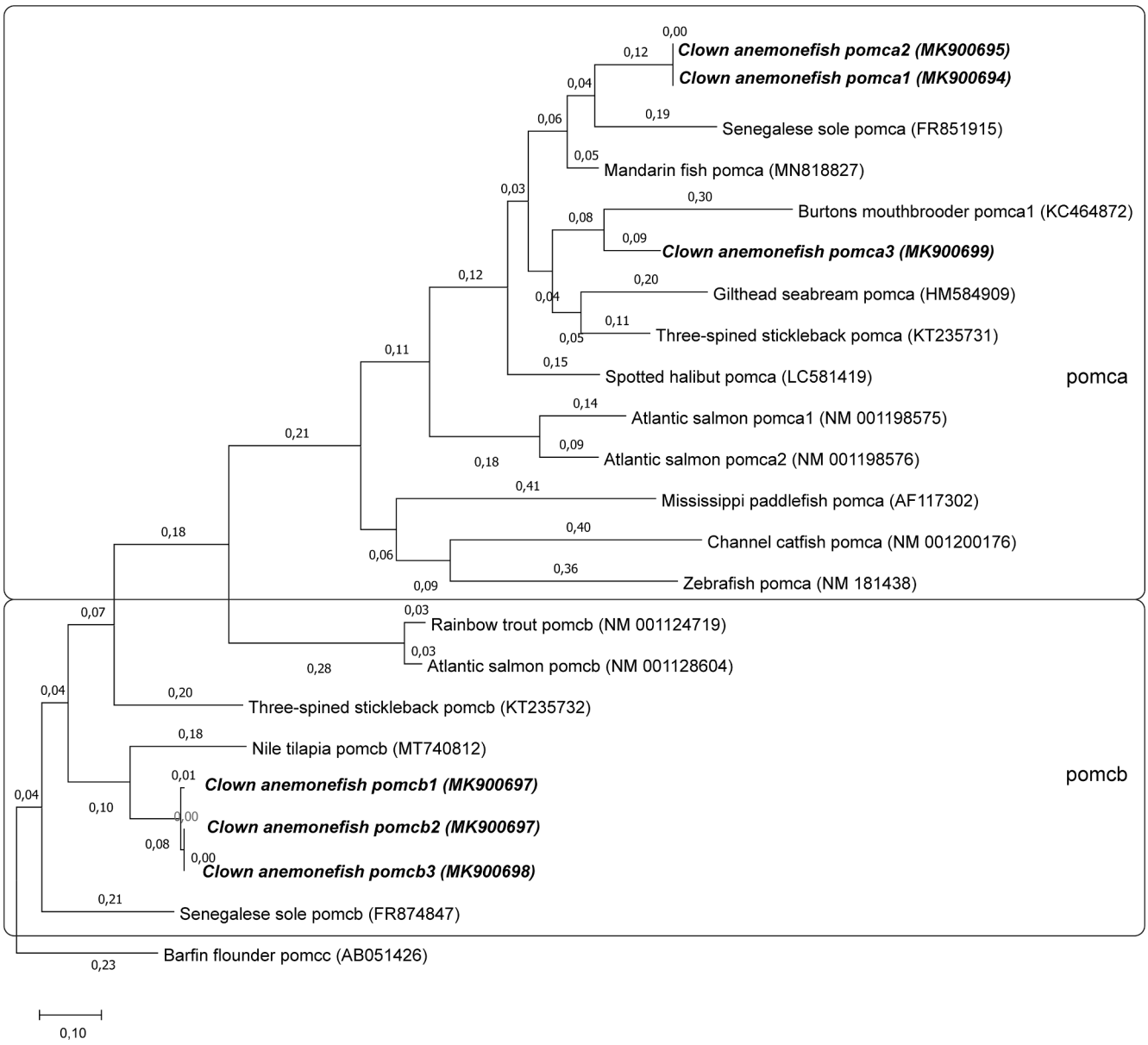

Fig. 2. Phylogenetic analysis of the clown anemonefish nucleotide sequences of *pomcs*. Alignments were made using the submissed GenBank nucleotidesequences in MEGA-X software. GenBank Accession Nos are as follows: Clown anemonefish (*pomca-1*, **MK900694**), Clown anemonefish (*pomca-2*, **MK900695**), Clown anemonefish (*pomca-3*, **MK900699**), zebrafish (*pomca*, **NM_181438**), Senegalese sole (*pomca*, **FR851915**), three-spined stickleback (*pomca*, **KT235731**), Gilthead seabream (*pomca*, **HM584909**), Atlantic salmon (*pomca*1, **NM_001198575**), Atlantic salmon (*pomca*2, **NM_001198576**), Mandarin fish (*pomca*, **MN818827**), Burton's mouthbrooder (*pomca1*, **KC464872**), Spotted halibut (*pomca*, **LC581419**), Mississippi paddlefish (*pomca*, **AF117302**), Channel catfish (*pomca*, **NM01200176**), three-spined stickleback (*pomcb*, **KT235732**), Senegalese sole (*pomcb*, **FR874847**), Rainbow trout (*pomcb*, **NM_001124719**), Atlantic salmon (*pomcb*, **NM_001128604**), Clown anemonefish (*pomcb-1*, **MK900696**), Clown anemonefish (*pomcb-2*, **MK900697**), Clown anemonefish (*pomcb-3*, **MK900698**), Nile tilapia (*pomcb*, **MT740812**), and outgroup Barfin flounder (*pomcc*, **AB051426**). Evolutionary relationships among the fish sequences were inferred from ML analysis of nucleotide sequences using a global alignment with free end and gaps.

temperature and the number of meals significantly affected the fish daily food intake and morning food consumption (08:00). In regard to the morning food consumption, on average, for both groups (T28 and T32), fish subjected to restricted feeding (1 M) consumed 44% more ($p < 0.001$) than fish fed three times per day (3 M), but their total daily food consumption (FI) had reduced by 52% ($p < 0.001$). T32 showed higher food consumption, both in the morning meal (20%, $p < 0.001$) and in the total daily FI (19.6%, $p < 0.001$). There were no interactions between feeding regime and temperature that affected fish growth and daily FI ($p > 0.05$). However, analysis of the morning food intake revealed a significant interaction between temperature and the number of meals ($p < 0.05$).

3.3. Effects of temperature and feeding regime on the mRNA expression of *agrp1, agrp2, pomca, pomcb, and mc4r-like*

The T32 fish had higher *agrp1* levels ($p = 0.001$) and lower *pomca* levels ($p < 0.001$) compared to the T28 group (Fig. 6A and 6C, respectively). Fish fed restricted volume of food (1 M) had an elevated *agrp1* expression ($p = 0.001$) (Fig. 6A). Regardless of the temperature differences and feeding regime variations, *agrp2* expression increased 1 h after feeding (FED1) compared to BF ($p = 0.03$, Fig. 6B). Neither the temperature and feeding regime, nor the time relative to feeding effected *pomcb* expression ($p > 0.05$, Fig. 6D). The effects of the experimental factors (T and M) on *mc4r-like* expression were highly influenced by the

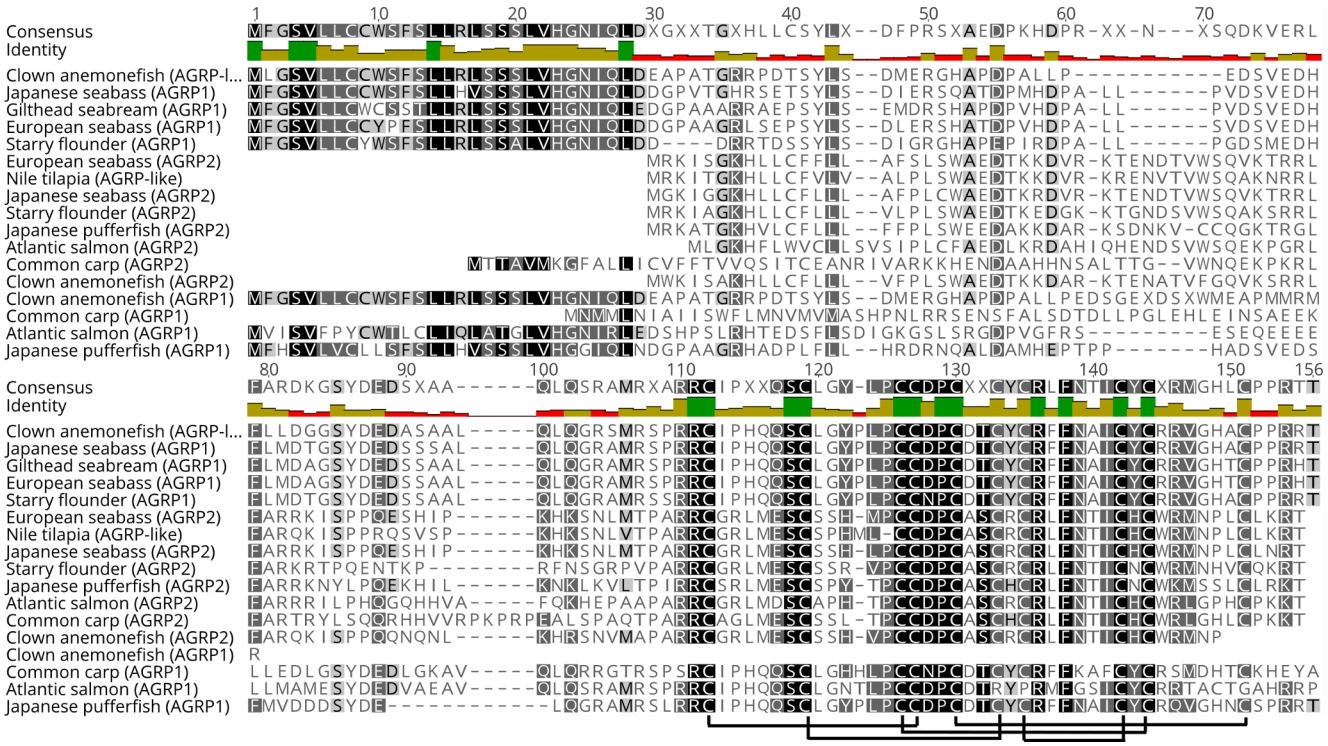

Fig. 3. Alignment of the clown anemonefish Agrp amino acid sequences. Alignments were made using submissed GenBank amino acid sequences in Geneious software. GenBank Accession Nos are as follows: clown anemonefish (Agrp-like, **XP_023152276**), Nile tilapia (Agrp-like, **NP_001266508**), Japanese seabass (Agrp1, **AIJ03132**), gilthead seabream (Agrp1, **AMZ00814**), European seabass (Agrp1, **CCF78543**), starry flounder (Agrp1, **APY24031**), clown anemonefish (Agrp1, **MK900693**), common carp (Agrp1, **CBX89934**), Atlantic salmon (Agrp1, **NP_001140149**), Japanese pufferfish (Agrp1, **NP_001092125**), Japanese seabass (Agrp2, **AIJ03133**), starry flounder (Agrp2, **APY24032**), Japanese pufferfish (Agrp2, **NP_001092126**), Atlantic salmon (Agrp2, **NP_001140150**), common carp (Agrp2, **CBX89935**), clown anemonefish (Agrp2, **MK900692**), and European seabass (Agrp2, **CCF78544**). Solid lines indicate suggested disulfide bonds in the clown anemonefish Agrp2. The box represents the signal peptide in the partial Agrp1 sequence, as suggested by UniProtBlast (A0A3QIBLN9).

fish population hierarchy, which induced variation in body weights. This indicated a negative correlation between *mc4r-like* and body weight ($R = -0.42$, $p < 0.01$; see Supplementary data Fig. S.). The deviance explained 43.4% of the variation, and the nonlinear models explained the *mc4r-like* expression dependence on body weight. Hierarchical growth was present in all groups, and the covariation between body weight (BW) and *mc4r like* expression was significant within treatments 1 M and T28 ($p = 0.02$), 1 M and T32 ($p < 0.001$), and 3 M and T32 ($p = 0.045$) (Fig. 6E). A covariation between BW and *mc4r like* expression was also significant between fish before the 08:00 morning feeding (BF) ($p < 0.001$, Fig. 6F).

4. Discussion

4.1. Food intake and growth

Changes in temperature may lead to poor maintenance in physiological homeostasis (Long et al., 2012) and result in the redirection of energy from somatic growth to cytoprotective pathways (Madeira et al., 2016). Our results support previous suggestions that clown anemonefish are thermally sensitive (Nilsson et al., 2010). At elevated sea temperatures, the species will require a higher abundance of food to sustain growth. Unfortunately, food availability may become a limiting factor as the ocean continues to get warmer, resulting in adverse growth effects in both individuals and the population hierarchy.

The thermal tolerance range varies extensively among fish species (Pörtner et al., 2017; Whitney et al., 2016) and also varies with the trait or the physiological function under investigation. In general, performance is maximal within a certain temperature range, and declines when it is outside the optimal range, reaching zero at the upper and lower critical temperatures (Volkoff and Rønnestad, 2020). Fish typically lose their appetite and stop feeding at critical temperatures prior to mortality, at least in the short term. In the present study, clown anemone fish retained their appetite and were able to increase their food intake and maintain growth when sufficient food was provided (T32; 3 M). Thus, for clownfish, 32 °C is still below the upper critical temperature at which the fish growth ceases. However, the high temperature coupled with a limited food supply challenged the energy balance of the fish. Due to their ectothermic nature, increased temperature leads to increased metabolic rate, resulting in higher energy demands. Increased temperature combined with low food availability, forces a trade-off in the energy expenditure for basal physiological processes (mainly standard metabolic rate and activity), and leaves a low or even a negative budget for growth. In our study, there was a significant decrease in body weight and length in the clown anemonefish reared at 32 °C with restricted feeding. This result suggests that warmer ocean scenarios may have a negative impact on fish growth. However, food availability, rather than temperature, was the major factor that affected the growth of clown anemonefish juveniles. Interestingly, similar results were shown in orange clownfish (*Amphiprion percula*) at the larval stage (McLeod et al., 2013). In our study, fish exposed to the higher temperature (T32) showed a 20% higher food consumption (in the combined feeding regimes) than the T28 fish, without contributing to higher growth. Therefore, when subjected to restrictive feeding (although each meal provided were fed to satiety) the clown anemonefish juveniles were not able to compensate the high temperature by eating more in a single meal (fed to satiety) for the increased energy requirements, resulting in lower growth rate. However, Juvenile Snook managed to increase the FI in a single meal up 52% if it was fed only one meal per day, resulting in similar FI and growth rates in fish fed one, two or three meals per day (García-galano, 2003). Our data demonstrates that when anemone clownfish were trained to anticipate three meals per day, the

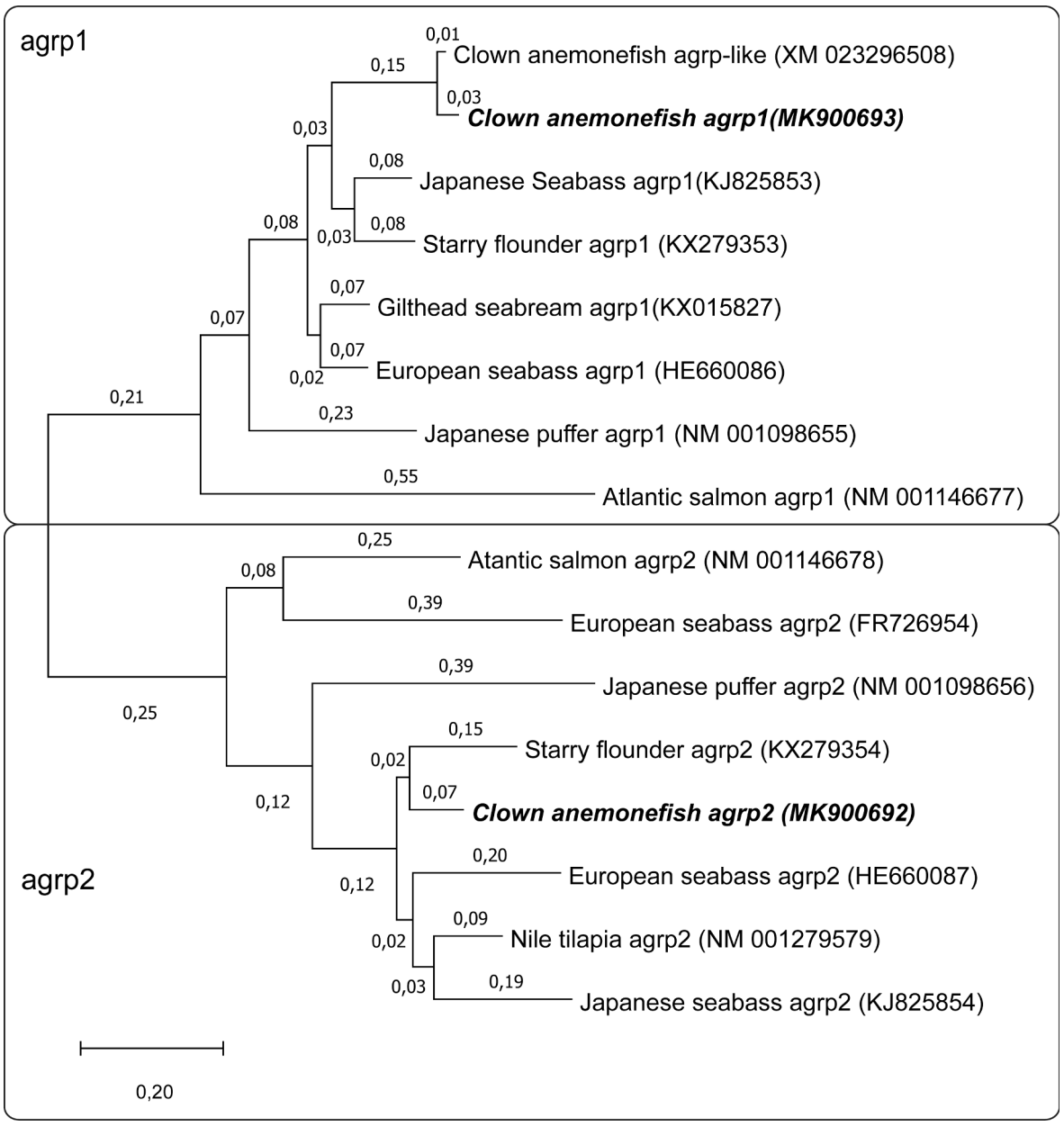

Fig. 4. Phylogenetic analysis of clown anemonefish nucleotide sequences of *agrp*. Alignments were made using submissed GenBank sequences in MEGA-X software. GenBank Accession Nos are as follows: Clown anemonefish (*agrp*-like, **XM_023296508**), Clown anemonefish (*agrp1*, **MK900693**), common carp (*agrp1*, **FR726953**), Atlantic salmon (*agrp1*, **NM_001146677**), Japanese pufferfish (*agrp1*, **NM_001098655**), Japanese seabass (*agrp1*, **KJ825853**), Gilthead seabream (*agrp1*, **KX015827**), European seabass (*agrp1*, **HE660086**), Starry flounder (*agrp1*, **KX279353)**, European seabass (*agrp2*, **HE660087**), Nile tilapia (*agrp2*, **NM_001279579**), Japanese seabass (*agrp2*, **KJ825854**), Starry flounder (*agrp2*, **KX279354**), Japanese pufferfish (*agrp2*, **NM_001098656**), Atlantic salmon (*agrp2*, **NM_001146678**), and Clown anemonefish (*agrp2*, **MK900692**). Evolutionary relationships among the fish sequences were inferred from ML analysis of nucleotide sequences using a global alignment with free end and gaps. .

satiation that led to cessation of feeding at the end of the first meal occurred at a lower Gastro-intestinal tract (GIT)-fullness than when the fish consumed only one meal per day. It is likely that the latter represents the maximum size of a single meal and may be limited by the maximal filling capacity or distension of the GIT (most likely stomach). If satiation occurs below the maximal filling capacity, other factors become involved. This could include hormonal or neuronal signaling pathways stimulated by the presence of nutrients in the GI tract and/or in the tissues or plasma post absorption. In addition, the 1 M fish may also possess higher "hunger" signals at the onset of feeding that may stimulate higher ingestion rates, but this was not systematically evaluated in the present study.

In the present study, there were no significant differences in the condition factor (K) between the different groups. However, there were large differences in the variance in K among the fish in the different treatments. The group in an elevated temperature (T32) with a low level of food availability (1 M) had a high variance in K. This was likely to be due to competition and the variable food access between individuals in this treatment. The larger fish were more aggressive, had better food access, and grew faster, thus resulting in a higher K value. The smaller specimens received less food, showed retarded growth, and became leaner and thus had a lower K value. At the end of the experiment, the fish from T32 and 1 M showed a stronger asymmetric growth profile (and therefore, variance in K) compared to the other groups. Similar results have also been reported in other clown anemonefish species from the temperate zone, including wide-band anemonefish *(Amphiprion*

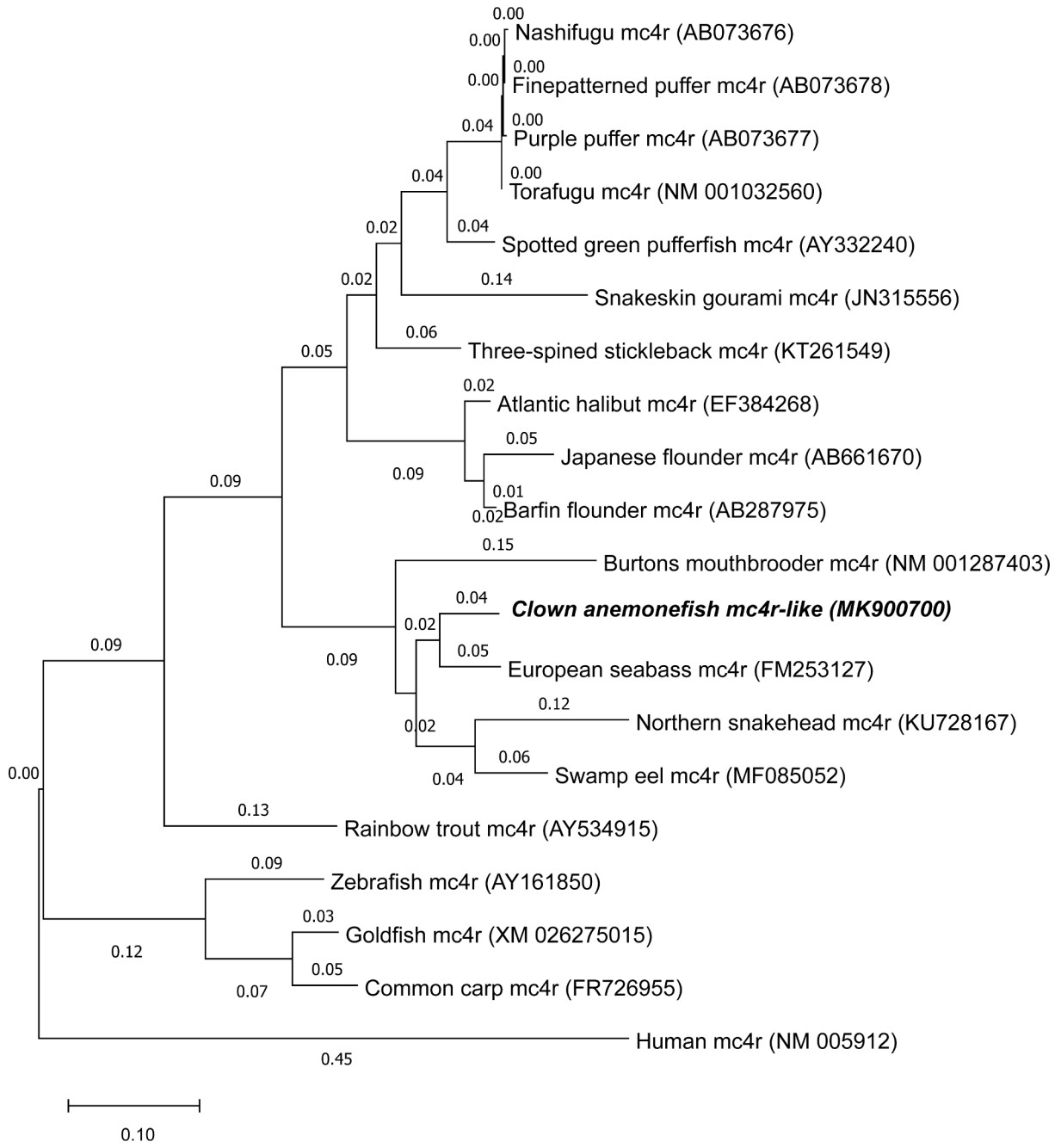

Fig. 5. Phylogenetic analysis of clown anemonefish nucleotide sequence *mc4r-like*. Alignments were made using submissed GenBank sequences in MEGA-X software. GenBank Accession Nos are as follows: torafugu (*mc4r*, **NM 001032560**), snakeskin gourami (*mc4r*, **JN315556**), common carp (*mc4r*, **FR726955**) zebrafish (*mc4r*, **AY161850**), clown anemonefish (*mc4r-like*, **MK900700**), Atlantic halibut (*mc4r*, **EF384268**), Japanese flounder (*mc4r*, **HQ230046**), barfin flounder (*mc4r*, **AB287975**), Burton's mouthbrooder (*mc4r*, **NM_001287403**), European seabass (*mc4r*, **FM253127**), purple puffer (*mc4r*, **AB073677**), nashifugu (*mc4r*, **AB073676**), finepatterned puffer (*mc4r*, **AB073678**), Northern snakehead (*mc4r*, **KU728167**), spotted green pufferfish (*mc4r*, **AY332240**), three-spined stickleback (*mc4r*, **KT261549**), Asian swamp eel (*mc4r*, **MF085052**), rainbow trout (*mc4r*, **AY534915**), goldfish (*mc4r*, **XM_026275015**), and human (*mc4r*, **NM005912**). Evolutionary relationships among the fish sequences were inferred from ML analysis of nucleotide sequences using a global alignment with free end and gaps.

latezonatus) (Rushworth et al., 2011). Anemone fish are hierarchical, and the hierarchical growth profile of an anemonefish *Amphiprion* population depends on food intake and individual growth, which is closely associated with rank in the fish school (Buston, 2003a, 2003b; (Iwata et al., 2012). Under unfavorable conditions such as high temperature and low food availability (as provided in the current experiment), competition among the fish in the tank is likely to result in a more pronounced hierarchy, and thereby asymmetry, within the group. The high morning FI suggests that clown anemonefish may possibly have "learned" to "predict/anticipate" how much and when they were fed each day. This awareness may eventually improve their motivation for feeding and food acquisition and utilization (Montoya et al., 2010; Sánchez-Vázquez and Madrid, 2001). Therefore, in addition to the physiological regulation of food intake by hunger and satiety signals, there may be other cognitive hedonic cues. It is possible a reward system may be affecting the appetite, which may contribute to explaining why the morning FI was higher in fish fed one meal per day. One could expect that the neuropeptides (the central factors regulating appetite) should reflect this long-term regulation of feeding behavior.

Table 2
Comparison in fish growth performance (body weight and length; Fulton's condition factor (K)) and daily food intake (Daily FI) and food intake consumed in the morning at two temperatures and two feeding regimes. Data shown as Mean ± SEM (except (*) which shows variances of K). Lowercase (a,b) or uppercase (A,B) letters in the same row show significant differences in effects of either number of meals or temperature (respectively) according to Student *t*-test. Different letters u,v,x,y in the same row show significant interactions effects of both temperature and meals according to Barlett's test and F-test.

Temperature	T28		T32		p-value		
Number of meals	1 M	3 M	1 M	3 M	Temperature	Meals	Interaction
Final body weight (mg)	$494^{a} \pm 27$	$714^{b} \pm 28$	$506^{a} \pm 30$	$710^{b} \pm 30$	*ns*	$p < 0.001$	*ns*
Final total length (cm)	$2.89^{a} \pm 0.05$	$3.28^{b} \pm 0.04$	$2.92^{a} \pm 0.06$	$3.28^{b} \pm 0.05$	*ns*	$p < 0.001$	*ns*
Final standard length (cm)	$2.29^{a} \pm 0.04$	$2.60^{b} \pm 0.04$	$2.30^{a} \pm 0.05$	$2.57^{b} \pm 0.04$	*ns*	$p < 0.001$	*ns*
K (mean ± SEM)	3.96 ± 0.09	3.97 ± 0.06	4.04 ± 0.12	4.09 ± 0.05	*ns*	*ns*	*ns*
K variance(*)	0.38^{u}	0.18^{v}	0.81^{x}	0.14^{v}	$p < 0.01$	$p < 0.001$	$p < 0.001$
Daily FI (mg $ind^{-1}day^{-1}$)	$6.67^{Aa} \pm 0.24$	$13.21^{Ab} \pm 0.31$	$8.35^{Ba} \pm 0.30$	$15.71^{Bb} \pm 0.34$	$p < 0.001$	$p < 0.001$	*ns*
Morning FI (mg $ind^{-1}day^{-1}$)	$6.67^{u} \pm 0.24$	$3.82^{v} \pm 0.13$	$8.35^{x} \pm 0.30$	$4.54^{y} \pm 0.16$	$p < 0.001$	$p < 0.001$	$p < 0.01$

4.2. Expression of neuropeptide genes

This is the first study characterizing some of the key appetite regulators in the brain of clown anemonefish. This includes partial coding of the cDNA sequences for the neuropeptides *pomc, agrp*, and the receptor *mc4r-like*. Clown anemonefish *pomcs* are well conserved, including the Pomc sequences for the translation products MSH and β-END. The *agrp*s sequences feature conserved cysteine bridges and signal peptides. The *mc4r-like* sequence clustered well with the teleost *mc4r-like* and *mc4r* sequences.

4.3. Transcript quantities of agrp1 and agrp2 support different functions of the two paralogs

Previous studies have investigated the roles of *agrp1* and *agrp2* genes during fasting in carp (Wan et al., 2012), Atlantic salmon (Murashita et al., 2009), zebrafish (Jørgensen et al., 2016; Drew et al., 2008), and seabass (Agulleiro et al., 2014) and *agrp* in goldfish and Ya-fish (reviewed by (Volkoff, 2016). Together these studies indicate that the different responses of *agrp* and its paralogs to feeding and fasting are species-specific and affected differently by environmental factors. Further, it was suggested that the relationship between *agrp1* and food intake depends on prior experience with food availability (anticipation) and temperature (Delgado et al., 2017). In this study, the two *agrp* paralogs in clown anemonefish responded differently. The *agrp2* expression varied as a response to feeding. The *agrp1* expression was generally higher at the 08.00 meal in the food restriction groups (1 M) compared to the control group (3 M). This was most likely an adjustment to stimulate hunger and ensure a higher food intake in the morning meal. Thus, *agrp1* seems to act as a long-term orexigenic signal that stimulates a higher food intake to support energy requirements when food accessibility is limited.

4.4. mc4r-like abundance links to growth and hunger suppression

Although *mc4r-like* results did not show any clear correlation with food intake in the clown anemonefish, the data indicated a role in controlling growth and energy expenditure, as reported for other species (Anderson et al., 2016; Boonanuntanasarn et al., 2012; Delgado et al., 2017; Josep Agulleiro et al., 2013; Sánchez et al., 2009; Striberny et al., 2015). In our study, *mc4r-like* was negatively correlated with body weight ($R = -0.42$) (Supplementary data), with a higher expression of *mc4r-like* in smaller fish. Although this correlation is evident ($R = -0.42$), the variation in *mc4r-like* cannot only be explained by body weight. The nonlinear regression models showed the effects of experimental factors (feeding regime and temperature) and hunger between body weight and the transcript levels of *mc4r-like*. This correlation was also observed in the control group, which displayed growth suppression without any food restrictions. When the clown anemonefish were subjected to high temperature with restricted feeding, it caused increased pressure on the fish physiology and resulted in both higher variations in condition factor and lower growth (see the previous discussion on food intake and growth). Thus, *mc4r-like* expression may be linked to growth patterns and possibly future maturation of individuals within a population with high social hierarchal pressures (Buston, 2003a, 2003b; (Iwata et al., 2012; Pörtner et al., 2006). Similar roles for the *mc4r* genes were previously suggested in other species. In medaka, Mc4r is involved in controlling very different traits, including hatching time, development, growth, and puberty (Liu et al., 2019). In swordtail fish (*Xiphophorus multilineatus*), Mc4r has been implicated in the modulation of food intake and consequently, the onset of puberty and maturation (Morris et al., 2018). These authors suggested that the individual reproduction strategy of the adult was determined by early-stage growth patterns and was correlated with *mc4r* expression.

Our results also propose a link between the level of *mc4r-like* and hunger since the gene was expressed significantly higher in smaller fish prior to feeding. This indicates that the receptor may be involved in suppressing hunger in small clown anemonefish. In small clown anemonefish, the motivation to eat is also affected by stress and the aggressive behavior of dominant individuals in the tank. It is known that MC4R can bind to both α-MSH and AGRP (Tao, 2010). The ratio of Agrp and α-Msh will likely determine the activation level of the Mc4r and thus whether higher-order orexigenic or anorexigenic pathways are stimulated (Valen et al., 2011). However, the direct role and regulation of Mc4r in the control of appetite in clownfish remains unclear.

5. The anorexigenic action of pomca was influenced by feeding regime and temperature

Irrespective of feeding regime, the clown anemonefish exposed to high temperatures had an increase in their food intake and showed reduced levels of *pomca*. This suggests that lowering the anorexigenic stimuli through a pathway (where Pomc is involved) will lead to the fish consuming larger meals. *pomca* may also be involved in long-term appetite regulation (that is possibly dependent on temperature) and is clearly inversely related to growth. Previous studies support an anorexigenic role of *pomca* in fasting rainbow trout (Jørgensen et al., 2016) and *pomca1* in Atlantic salmon (Murashita et al., 2011). In larval zebrafish, *pomca* transcript levels were lower in fish fed *ad lib.* compared to that in fish with restricted feeding (Löhr et al., 2018). These results support our hypothesis that *pomca* transcript levels play a role in the regulation of short-term feeding. Similar to rainbow trout (Leder and Silverstein, 2006) and Atlantic salmon (Murashita et al., 2011), clown anemonefish *pomcb* does not seem to play a role in the long-term regulation of feeding. However, the decline in *pomcb* levels in rainbow trout refed after four months of fasting (Jørgensen et al., 2016) suggests the role of *pomcb* in adjusting the allocation of energy and appetite after extended periods without food, in this species. The increased expression of anorexigenic genes (Jørgensen et al., 2016) and reduced expression of orexigenic genes (Murashita et al., 2009; Wan et al., 2012) during fasting may suggest a strategy to minimize the stress caused by long-term hunger/starvation (Conde-Sieira et al., 2018; Folkedal et al.,

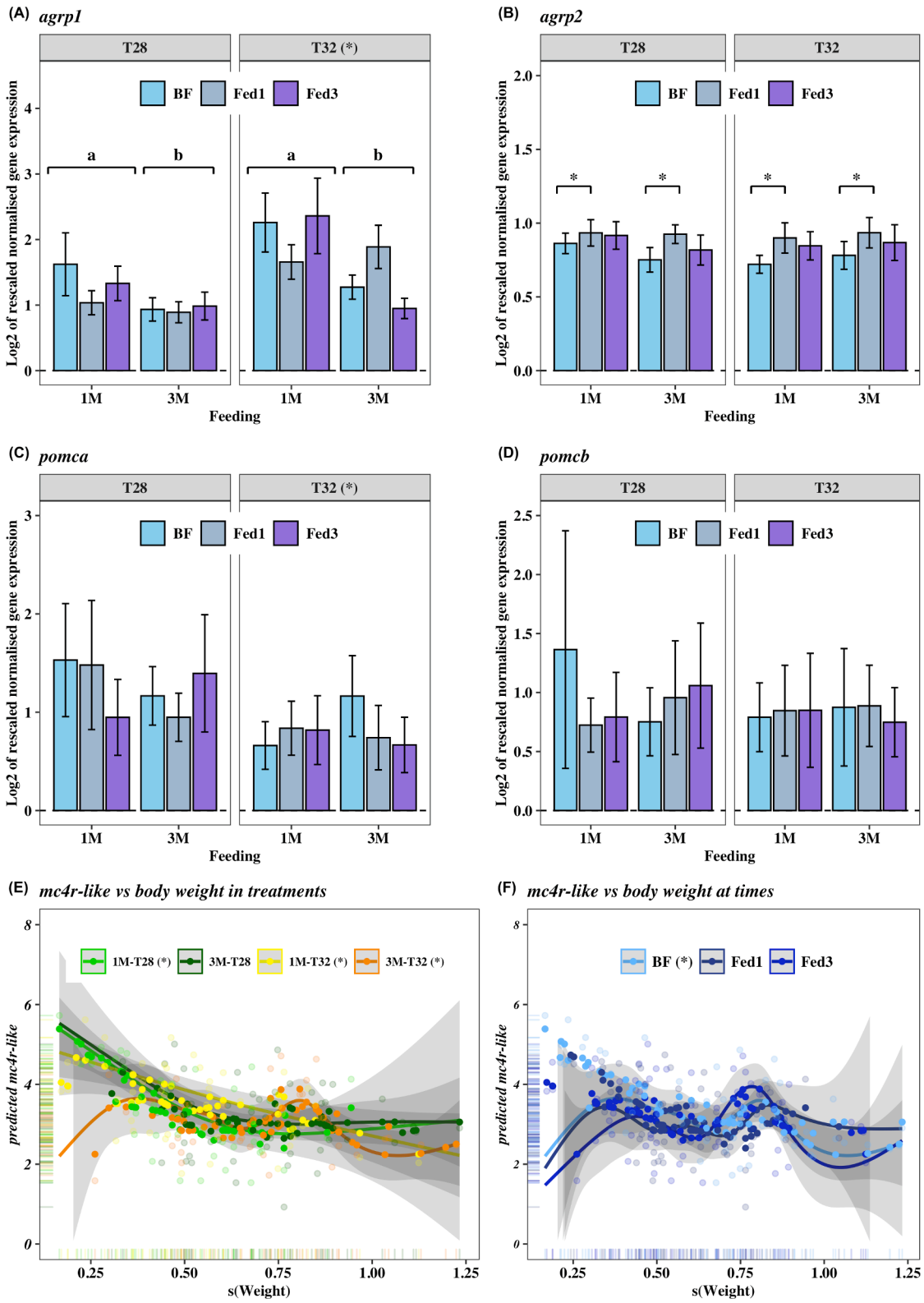

Fig. 6. Expression of *agrp1, agrp2, pomca, pomcb* (bar charts; A, B, C, and D), and *mc4r-like* (non-linear regressions; E and F) as effects of feeding regimes (1 M and 3 M) and temperatures (T28 and T32) at different sampling time points (BF, Fed1 and Fed3). Details on the treatments, are provided in the materials and methods section. The bar charts represent the log2 conversion of the normalized gene expression with Mean ± SEM. Different letters (a and b) above the bars in (A) show the significant differences between the feeding regimes (1 M and 3 M); the asterisks in (B) denotes any significant difference between sampling time points. Non-linear regression models (formula: y = s(x)) show the effects of different treatments (combined between feeding regimes and temperatures) (E): 1 M - T28; 3 M - T28; 1 M - T32 and 3 M - T32; with sampling time points (F): BF, Fed1, Fed3 on the relationship between the expression of *mc4r-like* and bodyweight (gr) (X-axis). The (*) on the legends in E and F shows significant effects of the categorical factors provided on the smooth lines.

2012). This result also implicates the hypothalamic network of appetite neuroendocrine controllers as regulators of energy expenditure.

6. Conclusion

Juvenile clown anemonefish, normally residing in tropical waters of temperatures around 28℃, seem to be able to acclimate to a temperature of 32 °C with sufficient food availability. A high temperature of 32 °C may be suboptimal (not beneficial) for juvenile clown anemonefish development. The high temperature combined with restricted food resources may result in asymmetric growth in the local population, which may reduce the effective reproductive population size. Restricted feeding mimics a reduction of the available natural trophic resources as suggested by climate change. Therefore, climate change may act as a limiting factor for clown anemonefish growth. However, the effect of the interaction of the two factors, temperature and food availability, was not significant in the present study.

The data suggests that *agrp1* is involved in stimulating the food intake of the clown anemonefish. Conversely, *agrp2* may be an anorexic short-term regulator of appetite. Both *pomca* and *mc4r-like* levels respond like anorexic factors but also show correlations with size (growth) and temperature. Further studies on the interactions of Mc4r with both Pomc and Agrp are needed to detail the complex interactions involved in neuroendocrine regulation in clown anemonefish, especially to explain the interactions between individual growth and gene expression. The mRNA expression levels of these brain neuropeptides responded to both food availability and temperature to regulate food intake and growth. The hierarchical behavioral pattern of clown anemonefish clearly influences growth and food intake. Future studies should aim to elaborate the neuroendocrine control on appetite and food intake in regulating growth and reproduction of individuals and populations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113719.

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