

## RESEARCH ARTICLE

# Effects of temperature on survival, moulting, and expression of neuropeptide and mTOR signalling genes in juvenile Dungeness crab (*Metacarcinus magister*)

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## ABSTRACT

Mechanistic target of rapamycin (mTOR) is a highly conserved protein kinase that controls cellular protein synthesis and energy homeostasis. We hypothesize that mTOR integrates intrinsic signals (moulting hormones) and extrinsic signals (thermal stress) to regulate moulting and growth in decapod crustaceans. The effects of temperature on survival, moulting and mRNA levels of mTOR signalling genes (*Mm-Rheb*, *Mm-mTOR*, *Mm-AMPK $\alpha$* , *Mm-S6K* and *Mm-AKT*) and neuropeptides (*Mm-CHH* and *Mm-MIH*) were quantified in juvenile *Metacarcinus magister*. Crabs at different moult stages (12, 19 or 26 days postmoult) were transferred from ambient temperature (~15°C) to temperatures between 5 and 30°C for up to 14 days. Survival was 97–100% from 5 to 20°C, but none survived at 25 or 30°C. Moult stage progression accelerated from 5 to 15°C, but did not accelerate further at 20°C. In eyestalk ganglia, *Mm-Rheb*, *Mm-AMPK $\alpha$*  and *Mm-AKT* mRNA levels decreased with increasing temperatures. *Mm-MIH* and *Mm-CHH* mRNA levels were lowest in the eyestalk ganglia of mid-premoult animals at 20°C. In the Y-organ, *Mm-Rheb* mRNA levels decreased with increasing temperature and increased during premoult, and were positively correlated with haemolymph ecdysteroid titre. In the heart, moult stage had no effect on mTOR signalling gene mRNA levels; only *Mm-Rheb*, *Mm-S6K* and *Mm-mTOR* mRNA levels were higher in intermoult animals at 10°C. These data suggest that temperature compensation of neuropeptide and mTOR signalling gene expression in the eyestalk ganglia and Y-organ contributes to regulate moulting in the 10 to 20°C range. The limited warm compensation in the heart may contribute to mortality at temperatures above 20°C.

**KEY WORDS:** Temperature acclimation, Moult cycle, Marine Crustacea, Gene expression, Ecdysteroid, mTOR

## INTRODUCTION

Crustaceans, like other ecdysozoans, must shed their exoskeleton periodically to develop and grow (reviewed in Aiken and Waddy, 1992; Charmantier-Daures and Vernet, 2004; Skinner, 1985). Two endocrine glands regulate the moult cycle (reviewed in Chang and

Mykles, 2011; Webster, 2015). The X-organ/sinus gland complex in the eyestalk ganglia secretes neuropeptides of the crustacean hyperglycaemic hormone (CHH) superfamily, including moult-inhibiting hormone (MIH) and CHH (reviewed in Webster, 2015; Webster et al., 2012). The neuropeptides act on the moulting gland (Y-organ), which synthesizes ecdysteroid moulting hormones. A drop in circulating MIH activates the Y-organ and the animal enters premoult (stages D<sub>0</sub> through D<sub>4</sub>; Chang and Mykles, 2011; Skinner, 1985). Haemolymph ecdysteroid concentration increases approximately 100-fold from intermoult (C) to a peak in late premoult (D<sub>2</sub>), then drops precipitously shortly before ecdysis and is lowest in postmoult (Thomton et al., 2006; reviewed in Mykles, 2011). In the target tissues, ecdysteroids are converted to their active forms and bind to nuclear receptors. This induces, for example, cuticle formation, partial resorption of the old exoskeleton, claw muscle atrophy and limb regenerate growth (Chang and Mykles, 2011; Mykles, 2011). Moulting is suspended or delayed by adverse environmental conditions, such as crowding or temperature stress, via direct or indirect inhibition of the Y-organ by neuropeptides (Aiken and Waddy, 1976; Anger and Spindler, 1987; Chung and Webster, 2005, Pitts et al., 2017; reviewed in Aiken and Waddy, 1992). Moult suspension occurs only in intermoult and early premoult animals. In mid and late premoult, the Y-organ becomes insensitive to neuropeptide control and the animal proceeds to ecdysis without delay (Anger, 1987; Chang and Mykles, 2011).

The highly conserved mTOR (mechanistic target of rapamycin) signalling pathway regulates cell growth and metabolism by integrating signals of energy, oxygen and nutrient supply, as well as of growth factors in organisms from yeast to mammals (reviewed in Reiling and Sabatini, 2006). It has been implicated in growth and moult regulation in insects (reviewed in Arquier et al., 2010; Nijhout et al., 2014) and crustaceans (Chang and Mykles, 2011; Das et al., 2016). mTOR activity is required for Y-organ activation, as rapamycin inhibits Y-organ ecdysteroid synthesis *in vitro* and lowers haemolymph ecdysteroid titre in *Gecarcinus lateralis* induced to moult by eyestalk ablation (Abuhagr et al., 2014b, 2016). Moreover, mTOR signalling genes, such as *Gl-mTOR*, *Gl-Rheb*, *Gl-AKT* and *Gl-S6K*, are upregulated in the Y-organ of *G. lateralis* induced to moult by multiple leg autotomy or eyestalk ablation (Abuhagr et al., 2014b, 2016; Das et al., 2018; Shyamal et al., 2018). Rheb (Ras homolog enriched in brain) is a GTP-binding protein that binds to mTOR and other proteins to form the active mTOR Complex 1 (mTORC1; reviewed in Saxton and Sabatini, 2017). Rheb activity is regulated upstream by protein kinase B (PKB, also known as AKT), which prevents inactivation of Rheb by the tuberous sclerosis complex (TSC1/2). A downstream target of mTORC1 is ribosomal protein S6 kinase (S6K), which is

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**List of abbreviations**

AKT	protein kinase B
AMPK $\alpha$	AMP-dependent protein kinase $\alpha$ -subunit
C	intermoult
CHH	crustacean hyperglycaemic hormone
CW	carapace width
D <sub>0</sub>	early premoult
D <sub>1</sub>	mid premoult
D <sub>2-3</sub>	late premoult
IGF	insulin/insulin-like growth factor
MIH	moult-inhibiting hormone
mTOR	mechanistic target of rapamycin
qPCR	quantitative polymerase chain reaction
RbS3	ribosomal protein S3
Rheb	ras homolog enriched in brain
S6K	ribosomal protein S6 kinase
$t_0$	before temperature incubation
$t_{14}$	after 14 days of temperature exposure

an important activator of protein synthesis and growth. mTORC1 can interact with transcription factors and coactivators involved in mitochondrial biogenesis and lipid metabolism and synthesis (e.g. peroxisome proliferator-activated receptors, PPARs; Sengupta et al., 2010). Transcriptomic analysis shows that mTOR activity, either directly or indirectly, affects the mRNA levels of thousands of contigs in the *G. lateralis* Y-organ (Shyamal et al., 2018). A negative regulator of mTORC1 is the cellular energy sensor AMP-dependent protein kinase (AMPK), which is activated at a low ATP/ADP ratio resulting from excessive exercise, hypoxia or acute thermal stress (Hardie et al., 2006; Reiling and Sabatini, 2006). During acute heat stress, reducing cellular energy consumption and growth by inactivation of mTOR may assure survival (Aramburu et al., 2014; Bandhakavi et al., 2008; Chou et al., 2012; Gibney et al., 2013; Hansen et al., 2007; Jarolim et al., 2013). In agreement with this, AMPK activation has been observed during acute temperature stress in marine ectotherms (Anttila et al., 2013; Frederich et al., 2009; Han et al., 2013). However, there is a paucity of studies addressing chronic, sublethal effects of temperature on mTOR activity. Transcriptional changes of raptor, an adaptor protein in mTORC1 (Clark et al., 2013), PPARs (Windisch et al., 2011) and regulators of the phosphatidylinositol 3-kinase (PI3K)-AKT signalling pathway upstream of mTOR (Windisch et al., 2014) indicate the importance of mTOR signalling for the reorganisation of metabolism with repercussions on growth during long-term thermal exposures.

The purpose of this study was to determine the effects of temperature on survival, moulting, and neuropeptide and mTOR signalling gene expression in the heart, Y-organ and eyestalk ganglia of the Dungeness crab, *Metacarcinus magister*. Juveniles from Northern California and Oregon experience temperatures ranging between 10 and 25°C (Brown and Terwilliger, 1999; Tasto, 1983). They moult successfully at 21°C, but growth is reduced compared with that at 14°C (Terwilliger and Dumler, 2001). We hypothesize that this pattern is reflected in temperature- and moult cycle-dependent expression of mTOR signalling components and neuropeptides in the tissues involved in moult regulation (eyestalk ganglia and Y-organ) and the heart of juvenile decapod crustaceans. The heart has been the subject of many ecophysiological studies with respect to thermal acclimation and adaptation owing to its central function in oxygen supply (Frederich et al., 2009; Stillman and Tagmount, 2009; Tepolt and

Somero, 2014; Wittmann et al., 2012). We transferred naturally moulting juvenile *M. magister* from ambient conditions (~15°C) to six temperatures (5, 10, 15, 20, 25 and 30°C) for up to 14 days and monitored survival and moult stage progression. At the three temperatures that allowed moult progression (10, 15 and 20°C), mRNA levels were quantified by real-time RT-PCR of mTOR signalling components in the eyestalk ganglia, Y-organ and heart, and of the neuropeptides *Mm-CHH* and *Mm-MIH* in the eyestalk ganglia. There was a decrease in mRNA levels of certain mTOR signalling genes (e.g. *Mm-Rheb*) with increasing temperature, which may compensate for higher metabolic rates at higher temperature, in order to maintain consistent allocation of cellular energy to protein synthesis. As shown in previous studies of other decapod species, *Mm-Rheb* mRNA level can serve as a marker for Y-organ ecdysteroidogenic activity.

**MATERIALS AND METHODS****Animals**

Juvenile *Metacarcinus magister* (Dana 1852) were collected at the end of June 2013 from the lower intertidal of Bodega Harbor, CA, USA, using pitfall traps (Grosholz and Ruiz, 1995). Pitfall traps consisted of 20-litre buckets placed in the sediment to ground level. When crabs were collected after one tidal cycle (ca. 24 h), water temperatures in the traps ranged from 14 to 22°C. At the University of California, Davis, Bodega Marine Laboratory (BML), Bodega Bay, CA, USA, they were kept individually under a 12 h:12 h light:dark cycle in a flow-through system in trays with compartments of 7×7×11 cm with a mesh bottom and solid transparent plastic walls and lids at ambient water temperature. Ambient temperature was determined daily in one of the trays and ranged from 11 to 15°C until the experiment started in late August 2013, and usually was similar to BML ambient seawater temperature (data provided by BML; [http://boon.ucdavis.edu/data\\_seawater\\_temperature.html](http://boon.ucdavis.edu/data_seawater_temperature.html)). Salinity was 33–34 PSU ([http://boon.ucdavis.edu/data\\_seawater\\_salinity.html](http://boon.ucdavis.edu/data_seawater_salinity.html)). The crabs were cleaned and fed *ad libitum* with pieces of frozen squid three times per week. Moults were collected five days per week and carapace widths (CW) including the 10th anterolateral spines of the moults were determined to the nearest 0.01 mm using digital callipers. During maintenance at ambient temperature, most crabs moulted twice and grew in CW by approximately 30% with each moult. At the start of the experiment, CW was 42±4 mm and wet mass was 11±3 g for a total of 180 animals (means±s.d.), and moult interval ranged between 35 and 45 days owing to variation in size and ambient temperature.

**Experimental design and sample collection**

The recirculating incubation systems at 5, 10, 15, 20, 25 and 30°C each consisted of a water reservoir of 200–500 litres, individual glass jars of 470 ml (91 mm in diameter and 94 mm in height), which each housed one animal, and water tables that collected overflowing water from the jars and drained back into the reservoir tanks. The jars received water from the reservoir using a submersible aquarium pump (Eheim, Deizisau, Germany), a manifold and regular airline tubing at a flow rate of 400 ml min<sup>-1</sup> at each outlet, and were covered with pieces of plastic light diffusion panels. All systems were equipped with air pumps and air stones, a temperature regulator (Pentair Aquatic Eco-Systems, Apopka, FL, USA) and up to three 300 W heaters (Eheim Jäger). Water was changed at least once per week to maintain water quality. The crabs were introduced and taken out for dissection in a staggered fashion, so that no more than 20 crabs were kept in each recirculating system at any time of

the 14 day incubation. To sample moult stages C to D<sub>2-3</sub>, the experiment was started ( $t_0$ ) with crabs at 12, 19 or 26 days postmoult, and tissues were collected from crabs at 26, 33 or 40 days postmoult at the end of the experiment ( $t_{14}$ ). Only four sampled crabs moulted during the incubation period. Groups of 10 crabs each at 12 or 26 days postmoult at  $t_0$  were incubated at 5°C, and groups of 10 crabs each at 12, 19 or 26 days postmoult were incubated at 10, 15, 20 or 25°C. An additional group of 10 crabs at 26 days postmoult was incubated at 10°C, and an additional group of 10 crabs at 19 days postmoult was incubated at 25°C. Two groups of 10 crabs at 19 days postmoult were incubated at 30°C, but died within hours, so crabs at 12 or 26 days postmoult were not tested. To minimize thermal shock during transfer, the 5, 25 and 30°C groups were first incubated for 2 h at each of the intermediate temperature steps, i.e. at 10 or 20°C and 25°C. We controlled for the sex ratio and size distribution in each of the temperature and days postmoult groups. We checked for moults and survival five days a week, and cleaned and fed *ad libitum* with pieces of squid mantle three times per week.

At  $t_0$  and  $t_{14}$ , wet mass (g) and CW (mm) of the crabs were determined. Haemolymph samples were collected using needles and syringes (5 µl haemolymph immediately added to 50 µl dd H<sub>2</sub>O) and stored at -20°C at both time points. Haemolymph ecdysteroid concentration was quantified with an enzyme-linked immunosorbent assay (ELISA) modified from Kingan (1989) and Tamone et al. (2007) as described in Abuhagr et al. (2014a). At  $t_0$  and  $t_{14}$ , the basal endite of one of the third maxillipeds was excised and stored in crab saline and photographed under a microscope within 72 h to determine moult stage. Moult stage was determined after Drach and Tchernigofteff (1967) using illustrations of endites from Moriyasu and Mallet (1986), as well as haemolymph ecdysteroid concentrations [10–40 pg µl<sup>-1</sup> in intermoult (C), 50–160 pg µl<sup>-1</sup> in early premoult (D<sub>0</sub>), 200–600 pg µl<sup>-1</sup> in mid premoult (D<sub>1</sub>), ≥1000 pg µl<sup>-1</sup> in D<sub>2</sub>, and a drop in ecdysteroids from D<sub>3</sub> onwards]. At  $t_{14}$ , 5–18 individuals per treatment group were dissected (total  $N=93$ , see Fig. S1), and the pairs of eyestalk ganglia and Y-organs, as well as 3–4 mm cubes of heart tissue, were stored in RNAlater (Life Technologies, Carlsbad, CA, USA) at 4°C overnight and then transferred to -20°C.

### RNA isolation and cDNA synthesis

Pairs of eyestalk ganglia or Y-organs were hand-homogenized in 100 µl TRIzol reagent (Life Technologies, Carlsbad, CA, USA) using Kontes Pellet Pestle Grinders and 1.5 ml tubes (Kimble Chase, Vineland, NJ, USA). Heart tissues were homogenized with two 5 mm stainless steel balls (Abbott Ball Co., West Hartford, CT, USA) in precooled (-80°C) 2 ml tubes in a TissueLyzer II (Qiagen, Frederick, MD, USA) at 2×1 min at 30 Hz, then suspended in 1 ml of TRIzol reagent. After centrifugation for 15 min at 12,000 g at 4°C, total RNA was isolated from the supernatant. For eyestalk ganglia, the Direct-zol RNA MiniPrep kit with microcolumns (Zymo-Spin IC Column, Zymo Research, Irvine, CA, USA) was used according to the manufacturer's protocol with an in-column DNase I treatment including RNase inhibitor (RiboLock, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 37°C. Total RNA of Y-organs and heart was extracted using the phenol-chloroform method as described in Covi et al. (2010). RNA purity and concentrations were determined by measuring absorbance at 260 and 280 nm using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Then, 400 ng of eyestalk ganglia RNA or 1 µg of

Y-organ or heart RNA were reverse transcribed in 20 µl reactions using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA).

### PCR cloning

Partial cDNAs encoding *M. magister* *Mm-AKT* (GenBank accession no. KT285226), *Mm-CHH* (KY070318), *Mm-Rheb* (KT315722), *Mm-mTOR* (KT315723), *Mm-S6K* (KT367806) and *Mm-AMPKα* (KT315720) were cloned using nested specific or degenerate primers and RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE). Primers (Table S1; Integrated DNA Technologies, Coralville, IA, USA) were designed to bind to highly conserved regions, as identified by multiple sequence alignment including a suite of pancrustacean and vertebrate species. Sequences were aligned using Clustal X (<http://www.clustal.org/clustal2/>) and GeneDoc software (<http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html>). Template cDNA from eyestalk ganglia, Y-organ, heart, claw or thoracic muscle was used for initial PCR reactions, which consisted of 1 µl cDNA, 1 µl forward and 1 µl reverse primer (10 µmol l<sup>-1</sup>), 5 µl 2× PCR mastermix (Thermo Fisher Scientific) and 2 µl nuclease-free water. 3' RACE was performed with the FirstChoice RLM-RACE kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol, except PCR reactions consisted of 1 µl cDNA, 2 µl gene-specific forward primer and 2 µl reverse primer (10 µmol l<sup>-1</sup>, from RLM-RACE kit), 25 µl 2× PCR mastermix (Thermo Fisher Scientific) and 20 µl nuclease-free water. The PCR was carried out using a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation for 4 min at 96°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing at the respective melting temperature of the primers for 30 s, and elongation for 30–120 s at 72°C, depending on the expected product length (1000 bp min<sup>-1</sup>). Final elongation for 7 min occurred at 72°C, followed by a hold at 4°C.

Products were separated on a 1.0% agarose gel in TAE buffer for 90 min at 100 V, stained with ethidium bromide and extracted using the Qiaex II Gel Extraction kit (Qiagen, Valencia, CA, USA). Sequencing was carried out by Davis Sequencing (Davis, CA, USA) using the respective primers that yielded the products.

### Quantitative real-time PCR

Roche LightCycler 480 and LightCycler Fast Start DNA Master PLUS SYBR Green I reaction mix (Roche Applied Science, Penzberg, Germany) were used to quantify absolute mRNA levels of *Mm-AKT*, *Mm-Rheb*, *Mm-mTOR*, *Mm-S6K*, *Mm-AMPKα*, *Mm-CHH*, *Mm-MIH* (GenBank accession no. AF031493; Umphrey et al., 1998) and *Mm-RbS3* (ribosomal protein S3; JF276909.1; Martin et al., 2011; previously used as reference gene). Reactions were combined on 384-well plates and consisted of 1 µl cDNA template, standard, no reverse transcriptase control or negative control (nuclease-free water), 0.5 µl (10 µmol l<sup>-1</sup>) gene-specific forward and reverse primers (Table 1), 5 µl 2× SYBR Green master mix and 3 µl nuclease-free water. PCR conditions were 3 min at 95°C for initial denaturation, then 45 cycles of 30 s at 95°C, 30 s at the primer annealing temperatures (Table 1) and 20 s at 72°C for elongation, followed by 7 min at 72°C for final extension. Melting curves and crossing point ( $C_p$ ) values were determined using Roche LightCycler 480 Software version 1.5.0 SP4. Absolute mRNA levels (copies µg<sup>-1</sup> RNA) were calculated from the respective standard curves ranging from 10<sup>-10</sup> to 10<sup>-18</sup> g µl<sup>-1</sup> of purified cDNA product of each gene (see Table 1 for primers; see 'PCR cloning' above for PCR conditions and product purification).

**Table 1. Primers for quantitative real-time PCR of the respective genes, annealing temperatures (°C), product lengths (bp) and references**

Primer name	Sequence 5' → 3'	Annealing temperature (°C)	Product length (bp)	Reference
<i>Mm</i> -AKT F2	CCAGGAGGTCAGTTCGGTGG	62	252	Present study
<i>Mm</i> -AKT R2	AGCGTATGGGCCACCTCATC			
<i>Ci</i> -AMPK $\alpha$ qF1	TCCGCAAGATTAAGTCGGGTGTGT	60	117	Frederich et al., 2009
<i>Ci</i> -AMPK $\alpha$ qR1	TATCCTCAATGGTGGCTCGCTTCA			
<i>Mm</i> -CHH qF2	GAGCACGTGTGTGACGATTG	55	177	Present study
<i>Mm</i> -CHH qR2	TGTTTACTTCTTCCTGCCAACCATC			
<i>Mm</i> -MIH F1	ACGACGACTGTCCAAACCTTA	55	242	Umphrey et al., 1998
<i>Mm</i> -MIH R1	GATAACCTTGTGACTCACTC			
<i>Mm</i> -mTOR qF1	TGTTACGGATGGCACAGGAC	60	170	Present study
<i>Mm</i> -mTOR qR1	CAGTGAGCGGGAGTAGTTGG			
<i>Mm</i> -RbS3 F1	CAAGGCCAAACTCAACAGGTTCC	55	139	Martin et al., 2011
<i>Mm</i> -RbS3 R1	GTCCCTTTTACCAAGGACA			
<i>Mm</i> -Rheb qF1	CACCTGGAGCGTGTAGTG	60	207	Present study
<i>Mm</i> -Rheb qR1	GGTTGTGGCTATATCACAGAGT			
<i>Mm</i> -S6K F1	ACGCCTTCAAACGGGAGGG	62	296	Present study
<i>Mm</i> -S6K R2	CTCAATGGTGCCGCGAGAACC			

Primers are prefixed with species names: *Ci*, *Cancer irroratus*; *Mm*, *Metacarcinus magister*.

Data are presented as  $\log_2$ (fold expression) relative to the mean absolute mRNA level at stage C at 15°C (Table 2).

### Data analysis and statistics

Statistical analyses were carried out using GraphPad Prism Version 7.0b (GraphPad Software, La Jolla, CA, USA). The Mantel–Cox test was used to test effects of temperature on survival. Moulting progression is presented only for animals in D<sub>0</sub> at  $t_0$  as  $t_{14}/t_0$  ratios of ecdysteroid concentration and as progression from D<sub>0</sub> to D<sub>1</sub> or D<sub>2–3</sub>. Ecdysteroid concentrations and their ratios were  $\log_{10}$  transformed to fulfil the prerequisite of equal variances. Because moulting stage of the individuals at a given day postmoulting was variable at both  $t_0$  and  $t_{14}$  at all temperatures (Fig. S1), qPCR data were grouped by moulting stage rather than by days postmoulting. qPCR data from 5°C-incubated animals were excluded from the analysis, as no data or not enough data were available from animals in mid and late premoulting (D<sub>1</sub> and D<sub>2–3</sub>). Factorial changes in absolute mRNA levels relative to the mean absolute mRNA levels at stage C at 15°C were  $\log_2$

transformed. Outliers were detected using the ROUT test, and removed. Subsequently, one-way or two-way ANOVA and the *post hoc* Tukey multiple comparisons test were used to test for significant effects of temperature and moulting stage at the  $P < 0.05$  level. Pearson correlation coefficients ( $r$ ) and goodness of fit of linear regressions ( $R^2$ ) were determined for relationships between  $\log_{10}$ -transformed ecdysteroid concentrations and *Mm*-*Rheb* mRNA levels. An ANCOVA was used to compare the slopes and  $y$ -intercepts of the regression lines.

## RESULTS

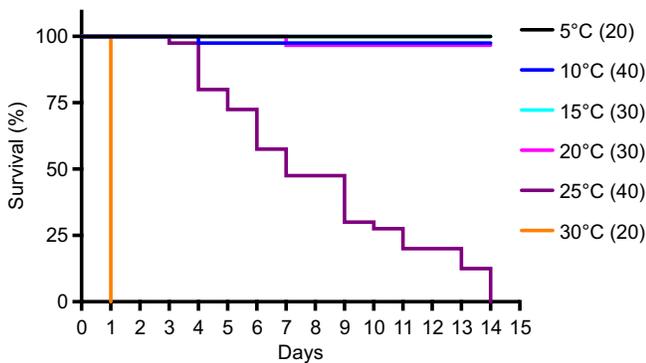
### Effects of temperature on survival and moulting progression

Temperature affected survival ( $P < 0.0001$ ). At 30°C, all animals died within 24 h (Fig. 1). At 25°C, animals started dying after 3 days, with only 48% of the animals surviving after 7 days and 0% surviving after 14 days. Survival was 100% at 5 and 15°C, and 98% and 97% at 10 and 20°C, respectively, over the 14-day incubation period.

**Table 2. Results of two-way ANOVA of  $\log_2$ -transformed  $x$ -fold gene expression relative to stage C at 15°C in eyestalk ganglia, Y-organ and heart of juvenile *Metacarcinus magister* incubated at the respective temperatures for 14 days (Figs 4–6)**

Tissue	Gene <i>Mm</i> -	Absolute expression (copies $\mu\text{g}^{-1}$ RNA) means $\pm$ s.e.m., stage C 15°C	Temperature		Moulting stage		Interaction	
			<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Eyestalk ganglia	<i>AKT</i>	1.0 $\pm$ 0.1 $\times 10^7$	54.3	<b>&lt;0.0001</b>	2.8	<b>0.0497</b>	2.2	0.0593
	<i>AMPK<math>\alpha</math></i>	2.1 $\pm$ 0.2 $\times 10^6$	35.3	<b>&lt;0.0001</b>	1.4	0.2442	1.9	0.1006
	<i>CHH</i>	2.6 $\pm$ 0.2 $\times 10^7$	13.4	<b>&lt;0.0001</b>	0.9	0.4556	2.1	0.0697
	<i>MIH</i>	1.4 $\pm$ 0.2 $\times 10^7$	30.2	<b>&lt;0.0001</b>	5.2	<b>0.0028</b>	2.7	<b>0.0208</b>
	<i>Rheb</i>	5.7 $\pm$ 0.7 $\times 10^5$	140.3	<b>&lt;0.0001</b>	0.8	0.4796	1.6	0.1622
	<i>S6K</i>	3.6 $\pm$ 0.4 $\times 10^6$	8.2	<b>0.0007</b>	3.4	<b>0.0238</b>	0.8	0.5729
Y-organ	<i>AKT</i>	2.9 $\pm$ 0.5 $\times 10^6$	10.0	<b>0.0002</b>	4.0	<b>0.0109</b>	0.6	0.7074
	<i>AMPK<math>\alpha</math></i>	7.7 $\pm$ 1.2 $\times 10^5$	0.9	0.4117	3.5	<b>0.0203</b>	1.1	0.3806
	<i>mTOR</i>	2.7 $\pm$ 0.2 $\times 10^4$	6.4	<b>0.0029</b>	4.5	<b>0.0067</b>	0.8	0.5842
	<i>RbS3</i>	2.5 $\pm$ 0.3 $\times 10^5$	4.5	<b>0.0152</b>	1.9	0.1422	0.7	0.6841
	<i>Rheb</i>	2.5 $\pm$ 0.4 $\times 10^5$	30.1	<b>&lt;0.0001</b>	17.3	<b>&lt;0.0001</b>	0.3	0.2483
	<i>S6K</i>	1.1 $\pm$ 0.2 $\times 10^6$	3.0	<b>0.0383</b>	4.8	<b>0.0047</b>	1.0	0.4558
Heart	<i>AKT</i>	2.2 $\pm$ 0.2 $\times 10^6$	8.4	<b>0.0006</b>	3.9	<b>0.0138</b>	0.7	0.6222
	<i>AMPK<math>\alpha</math></i>	5.4 $\pm$ 0.7 $\times 10^5$	6.6	<b>0.0026</b>	1.9	0.1358	1.3	0.2742
	<i>mTOR</i>	4.2 $\pm$ 0.6 $\times 10^5$	3.5	<b>0.0384</b>	0.4	0.7327	3.4	<b>0.0066</b>
	<i>RbS3</i>	4.3 $\pm$ 0.5 $\times 10^5$	1.0	0.3840	2.3	0.0824	0.5	0.8094
	<i>Rheb</i>	1.7 $\pm$ 0.1 $\times 10^5$	66.7	<b>&lt;0.0001</b>	5.6	<b>0.0020</b>	0.7	0.6546
	<i>S6K</i>	7.2 $\pm$ 0.7 $\times 10^5$	13.5	<b>&lt;0.0001</b>	2.9	<b>0.0432</b>	1.4	0.2325

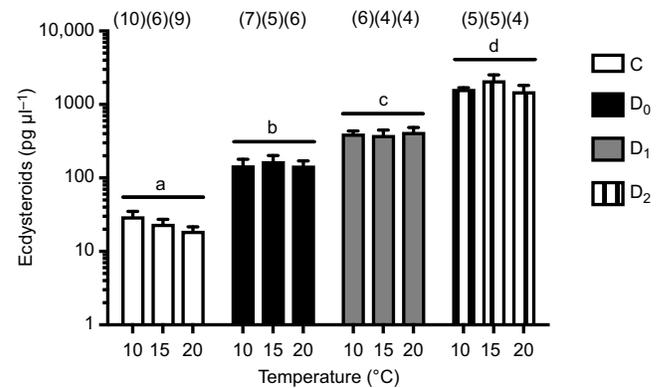
Significance at  $P < 0.05$  in bold.



**Fig. 1. Survival of *Metacarcinus magister* juveniles during 14 days of incubation at temperatures ranging from 5 to 30°C.** Survival curves were significantly different (Mantel–Cox test,  $P < 0.05$ ). Sample size ( $N$ ) is given in parentheses.

Moult progression increased with temperature. Among the group of animals in  $D_0$  at  $t_0$ , individuals tested at 5°C did not progress in the moult cycle within the observation period, whereas the crabs incubated at 10, 15 or 20°C did so (Fig. 2). Most animals at 10, 15 or 20°C exhibited  $t_{14}/t_0$  ecdysteroid ratios greater than 1, resulting in significantly higher means than the animals at 5°C ( $F = 0.54$ ,  $P < 0.0001$ ; Fig. 2A). Consequently, all the  $D_0$  animals incubated at 5°C remained in this stage (Fig. 2B), while at 10°C, 43% remained in  $D_0$  and 57% progressed to  $D_1$ . At 15°C, almost half of the animals (46%) advanced to  $D_{2-3}$ , while 23% remained in  $D_0$  and 31% progressed to  $D_1$ . At 20°C, 27% of the animals remained in  $D_0$ , while 36% progressed to  $D_1$  and 36% progressed to  $D_{2-3}$  by 14 days.

At  $t_{14}$ , haemolymph ecdysteroid levels of all the individuals incubated at 10, 15 or 20°C varied by moult stage ( $P < 0.0001$ ), but not by temperature (Fig. 3). Ecdysteroid concentrations increased significantly from moult stage to moult stage in each temperature group and were approximately 100-fold higher in stage  $D_2$  compared with stage C. In intermoult, ecdysteroid titres varied from  $19 \pm 3$   $\text{pg } \mu\text{l}^{-1}$  at 20°C to  $28 \pm 5$   $\text{pg } \mu\text{l}^{-1}$  at 10°C. In stage  $D_0$ , values increased to  $147 \pm 32$   $\text{pg } \mu\text{l}^{-1}$  at 10°C,  $169 \pm 34$   $\text{pg } \mu\text{l}^{-1}$  at 15°C and  $146 \pm 25$   $\text{pg } \mu\text{l}^{-1}$  at 20°C. In  $D_1$ , titres ranged from  $383 \pm 68$   $\text{pg } \mu\text{l}^{-1}$  at 15°C to  $420 \pm 65$   $\text{pg } \mu\text{l}^{-1}$  at 20°C. Maximal



**Fig. 3. Haemolymph ecdysteroid concentrations ( $\text{pg } \mu\text{l}^{-1}$ ) of juvenile *M. magister* in intermoult (C, white), early ( $D_0$ , black), mid ( $D_1$ , grey) or late ( $D_2$ , striped) premoult after 14 days of incubation at 10, 15 or 20°C ( $t_{14}$ ).** Results of two-way ANOVA of  $\log_{10}$ -transformed data: temperature:  $F = 0.7$ ,  $P = 0.4991$ , moult stage:  $F = 325.7$ ,  $P < 0.0001$ , interaction:  $F = 0.7$ ,  $P = 0.6253$ ; different letters denote significant differences according to the Tukey *post hoc* test at  $P < 0.05$ . Sample size ( $N$ ) is given in parentheses; animals in  $D_3$  were excluded from the graph and statistical analysis. Data are presented as means  $\pm$  s.e.m. Note the logarithmic y-axis.

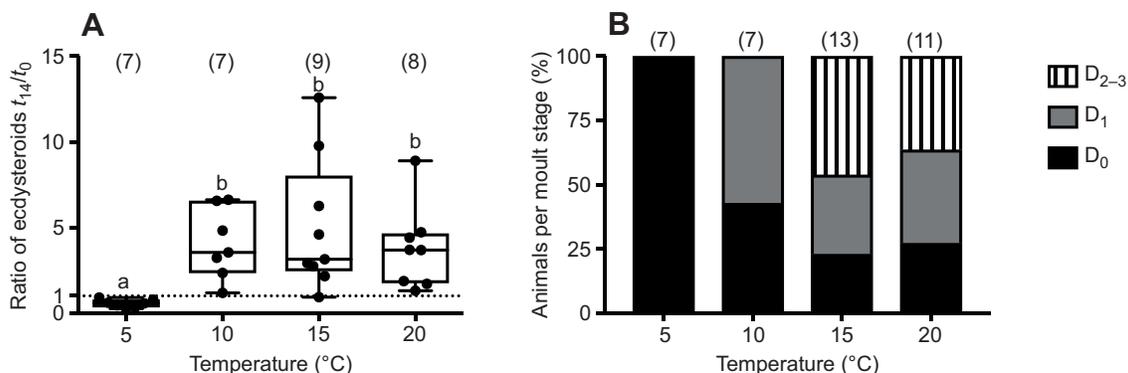
concentrations at stage  $D_2$  ranged from  $1506 \pm 318$   $\text{pg } \mu\text{l}^{-1}$  at 20°C to  $2123 \pm 403$   $\text{pg } \mu\text{l}^{-1}$  at 15°C.

### Effects of temperature and moult stage on mRNA levels

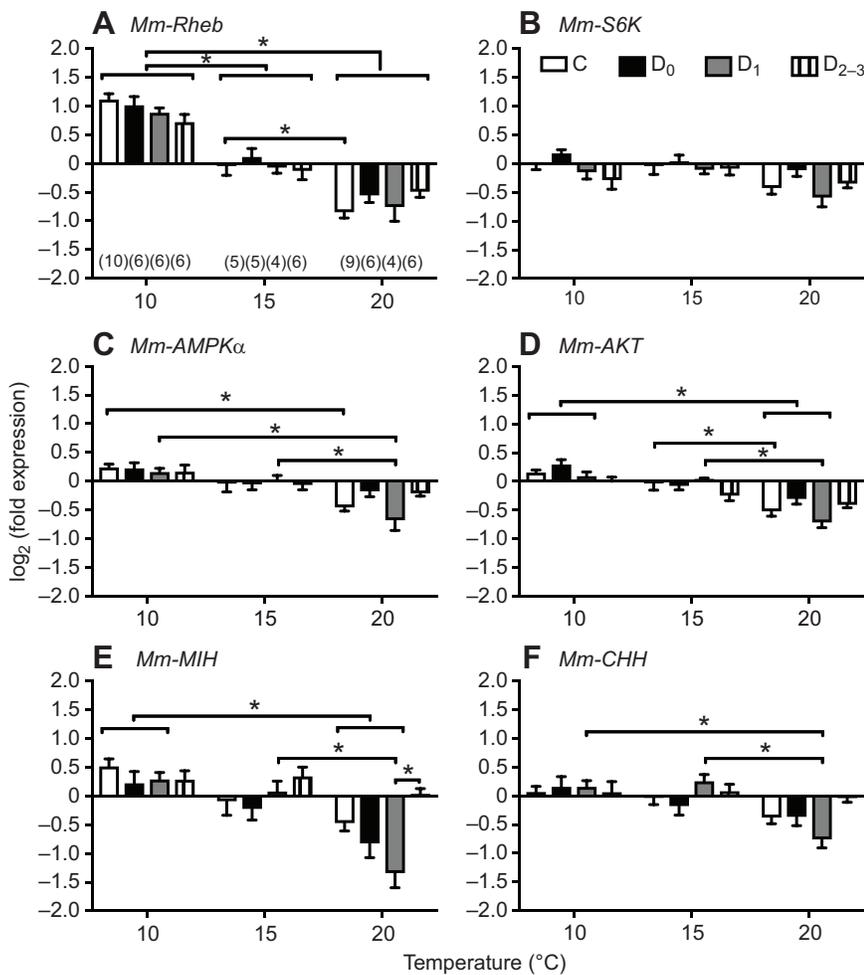
#### Eyestalk ganglia

mRNA levels in eyestalk ganglia were significantly affected by temperature, with a general trend of being higher at 10°C and/or lower at 20°C (Fig. 4, see Table 2 for results of two-way ANOVA). *Mm-MIH*, *Mm-AKT* and *Mm-S6K* were also significantly affected by moult stage. Only for *Mm-MIH* was a significant interaction observed between temperature and moult stage. *Mm-RbS3* and *Mm-mTOR* were not included in the analysis because a reliable quantification was not possible owing to low mRNA levels and residual genomic DNA.

*Mm-Rheb* was upregulated up to 2.2-fold between 15 and 10°C in all moult stages ( $P$  ranged from  $< 0.0001$  in C to 0.0033 in  $D_{2-3}$ , Tukey *post hoc* test; Fig. 4A), downregulated in stage C by 0.6-fold between 20 and 15°C ( $P = 0.0036$ ) and downregulated by 0.3-fold



**Fig. 2. Progression of the moult cycle through early premoult ( $D_0$ ) in juvenile *M. magister* is dependent on temperature.** (A) Ratio of haemolymph ecdysteroid concentrations ( $t_{14}/t_0$ ).  $t_{14}/t_0 < 1$  and  $t_{14}/t_0 = 1$  indicate no progression of the moult cycle,  $t_{14}/t_0 > 1$  indicates progression of the moult cycle. One-way ANOVA of  $\log_{10}$ -transformed data:  $F = 16.4$ ,  $P < 0.0001$ . Different letters denote significant differences according to the Tukey *post hoc* test at  $P < 0.05$ . Boxes represent medians and the interquartile range, error bars minimum to maximum values. (B) Percentage of animals that remained in early premoult (stage  $D_0$ ; black) or progressed to mid ( $D_1$ ; grey) or late premoult ( $D_{2-3}$ ; striped) at  $t_{14}$ . Sample size ( $N$ ) is given in parentheses. Only the subgroup of animals in  $D_0$  at  $t_0$  was used for this graph. See Fig. S1 for progression through other moult stages. Data pairs with unreasonably high ecdysteroid concentrations at  $D_0$  (ELISA out of range), animals in  $D_3$  (drop of ecdysteroids) and an outlier at 20°C were excluded from A but retained for B.



**Fig. 4.** mRNA levels in eyestalk ganglia after 14 days of incubation at 10, 15 or 20°C of *M. magister* juveniles in intermolt (C, white), early (D<sub>0</sub>, black), mid (D<sub>1</sub>, grey) or late (D<sub>2-3</sub>, striped) premoult. (A) *Mm-Rheb*, (B) *Mm-S6K*, (C) *Mm-AMPKα*, (D) *Mm-AKT*, (E) *Mm-MIH* and (F) *Mm-CHH*. Data are normalized to the mean absolute mRNA levels in stage C at 15°C (Table 2). For results of two-way ANOVA, see Table 2; for results of the *post hoc* Tukey test, see Results. Asterisks denote significant differences according to the Tukey *post hoc* test at *P* < 0.05. Sample size (N) is given in parentheses in A also applies to the other genes; data are presented as means ± s.e.m.

between 10 and 20°C (*P* < 0.0001). In the other moult stages, the differences between 15 and 20°C were not significant, but the differences between 10 and 20°C were all highly significant (0.3- to 0.4-fold, *P* < 0.0001). There were no significant differences in *Mm-S6K* levels in *post hoc* comparisons of biological interest (Fig. 4B). *Mm-AMPKα* levels differed in stage C and D<sub>1</sub> between 10 and 20°C (both by 0.6-fold, *P* < 0.0001, Tukey test; Fig. 4C) and in stage D<sub>1</sub> also between 15 and 20°C (0.6-fold, *P* = 0.0069). *Mm-AKT* differed between 15 and 20°C in stages C (*P* = 0.0042; Fig. 4D) and D<sub>1</sub> (*P* = 0.0002), and between 10 and 20°C in stages C to D<sub>1</sub> (*P* < 0.0001, *P* = 0.0005, *P* < 0.0001, 0.6-fold).

*Mm-MIH* levels decreased in stage C animals 0.5-fold between 10 and 20°C (*P* = 0.0007, Tukey test; Fig. 4E). In stage D<sub>1</sub>, *Mm-MIH* levels at 20°C were lower than at 10 and 15°C (*P* < 0.0001 and *P* = 0.0019, 0.3-fold). At 20°C, *Mm-MIH* was upregulated by a factor of 2.5 from stages D<sub>1</sub> to D<sub>2-3</sub> (*P* = 0.0008). Although the trends in *Mm-CHH* levels were similar to those in *Mm-MIH* levels, the differences were smaller as no upregulation occurred upon exposure to 10°C (Fig. 4F). In stage D<sub>1</sub>, *Mm-CHH* levels at 20°C were lower than at 10°C (*P* = 0.0067, Tukey test) and at 15°C (*P* = 0.0057), both by 0.5-fold.

#### Y-organs

In the Y-organ, *Mm-Rheb*, *Mm-AKT*, *Mm-AMPKα*, *Mm-mTOR* and *Mm-S6K* were significantly affected by moult stage, and *Mm-Rheb*, *Mm-AKT*, *Mm-mTOR*, *Mm-S6K* and *Mm-RbS3* were significantly affected by temperature (ANOVA; Fig. 5, Table 2). There was no significant interaction between moult stage and temperature in any

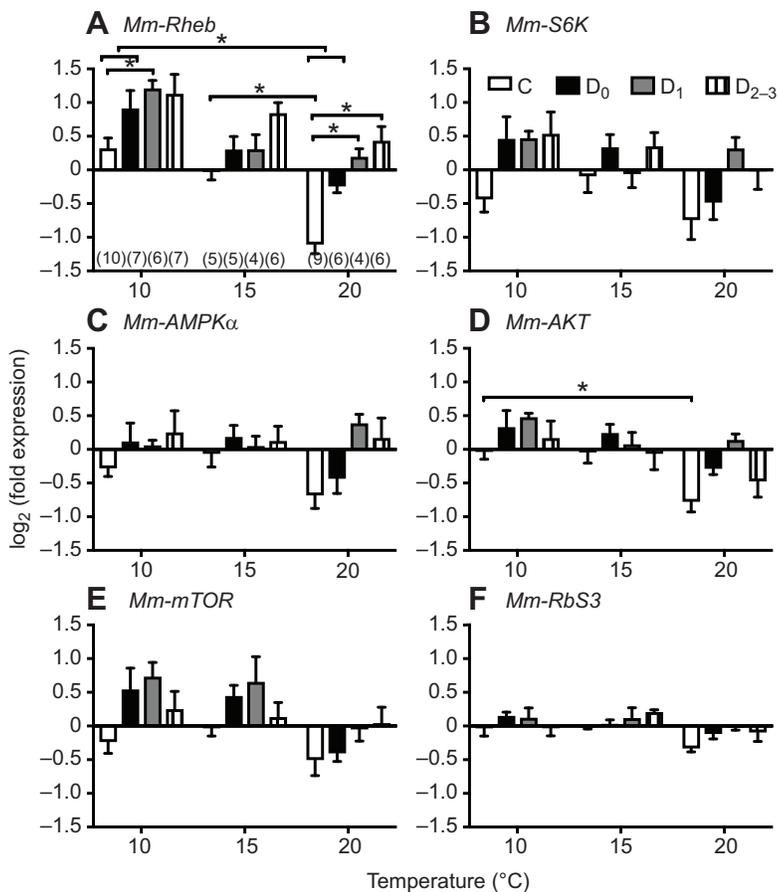
of the genes. When affected by temperature, mRNA levels decreased with increasing temperature. For *Mm-Rheb* (Fig. 5A), *Mm-S6K* (Fig. 5B) and *Mm-AMPKα* (Fig. 5C), there was a trend for an increase with progressing moult stages. For *Mm-AKT* (Fig. 5D) and *Mm-mTOR* (Fig. 5E), there was a trend for higher mRNA levels in stages D<sub>0</sub> and D<sub>1</sub> compared with stages C and D<sub>2-3</sub>.

*Mm-Rheb* mRNA levels were lower at 20°C compared with 10°C in stages C and D<sub>0</sub> by 0.4-fold (*P* < 0.0001 and *P* = 0.0050, Tukey *post hoc* test; Fig. 5A), and in stage C at 15°C compared with 20°C (*P* = 0.0111, 0.5-fold). At 10°C, mRNA levels were elevated by a factor of 1.8 in stage D<sub>1</sub> compared with stage C (*P* = 0.0389). At 20°C, *Mm-Rheb* levels increased from stage C to D<sub>1</sub> and D<sub>2-3</sub> (2.4- and 3.0-fold, *P* = 0.0028 and *P* < 0.0001). *Mm-AKT* levels were lower at 20°C compared with 10°C in stage C (0.6-fold, *P* = 0.0435, Tukey test; Fig. 5D). There were no significant differences in *Mm-S6K* (Fig. 5B), *Mm-AMPKα* (Fig. 5C), *Mm-mTOR* (Fig. 5E) or *Mm-RbS3* (Fig. 5F) mRNA levels in *post hoc* comparisons of biological interest.

#### Heart

In the heart, the mRNA levels of all genes except *Mm-RbS3* were significantly affected by temperature, and the mRNA levels of *Mm-Rheb*, *Mm-AKT* and *Mm-S6K* also varied with moult stage (ANOVA; Fig. 6, Table 2). There was a significant interaction between moult stage and temperature in *Mm-mTOR* mRNA levels.

In all moult stages, *Mm-Rheb* mRNA levels at 15 and 20°C were lower than at 10°C (*P* < 0.0001 to *P* = 0.0285, Tukey test; Fig. 6A).



**Fig. 5.** mRNA levels in Y-organ after 14 days of incubation at 10, 15 or 20°C of *M. magister* juveniles in intermoult (C, white), early (D<sub>0</sub>, black), mid (D<sub>1</sub>, grey) or late (D<sub>2-3</sub>, striped) premoult. (A) *Mm-Rheb*, (B) *Mm-S6K*, (C) *Mm-AMPKα*, (D) *Mm-AKT*, (E) *Mm-mTOR* and (F) *Mm-RbS3*. Data are normalized to the mean absolute mRNA levels in stage C at 15°C (Table 2). For results of two-way ANOVA, see Table 2; for results of the *post hoc* Tukey test, see Results. Asterisks denote significant differences according to the Tukey *post hoc* test at  $P < 0.05$ . Sample size (N) is given in parentheses in A also applies to the other genes; data are presented as means  $\pm$  s.e.m.

Factorial change ranged among the moult stages from 0.3 to 0.4 between 10 and 15°C and from 0.2 to 0.3 between 10 and 20°C. The latter were the largest fold changes found by this study; in other words, a 5.3-fold increase at a temperature decrease from 20 to 10°C in stage D<sub>1</sub>. Although not identified by the *post hoc* test, it is noteworthy that there was a trend for reduced *Mm-Rheb* mRNA levels with progression of the moult cycle by up to 0.5 at 20°C. The mRNA levels of the kinases *Mm-S6K* (Fig. 6B), *Mm-AMPKα* (Fig. 6C), *Mm-AKT* (Fig. 6D) and *Mm-mTOR* (Fig. 6E) varied little and exhibited similar patterns. There were differences only in *Mm-S6K* between 10 and 15°C in stage C ( $P = 0.0360$ , 0.6-fold, Tukey test; Fig. 6B) and in *Mm-mTOR* between 10 and 20°C in stage C ( $P = 0.0018$ , 0.6-fold; Fig. 6E).

#### Relationship between haemolymph ecdysteroid and *Mm-Rheb* levels

In eyestalk ganglia, the correlation between  $\log_{10}$  ecdysteroids and *Mm-Rheb* copies  $\mu\text{g}^{-1}$  RNA varied with temperature (Fig. 7A, see Table S2 for statistics). There was a negative correlation at 10°C ( $P = 0.0420$ ), no correlation at 15°C and a positive correlation at 20°C ( $P = 0.0159$ ). Consequently, there were differences in the slopes of the regression lines ( $F = 4.758$ ,  $P = 0.0118$ ). Given that the slopes differed greatly, it was not possible to determine whether the elevations differed significantly. Goodness of linear fits ( $R^2$ ) varied between 0.00646 (15°C) and 0.2367 (20°C).

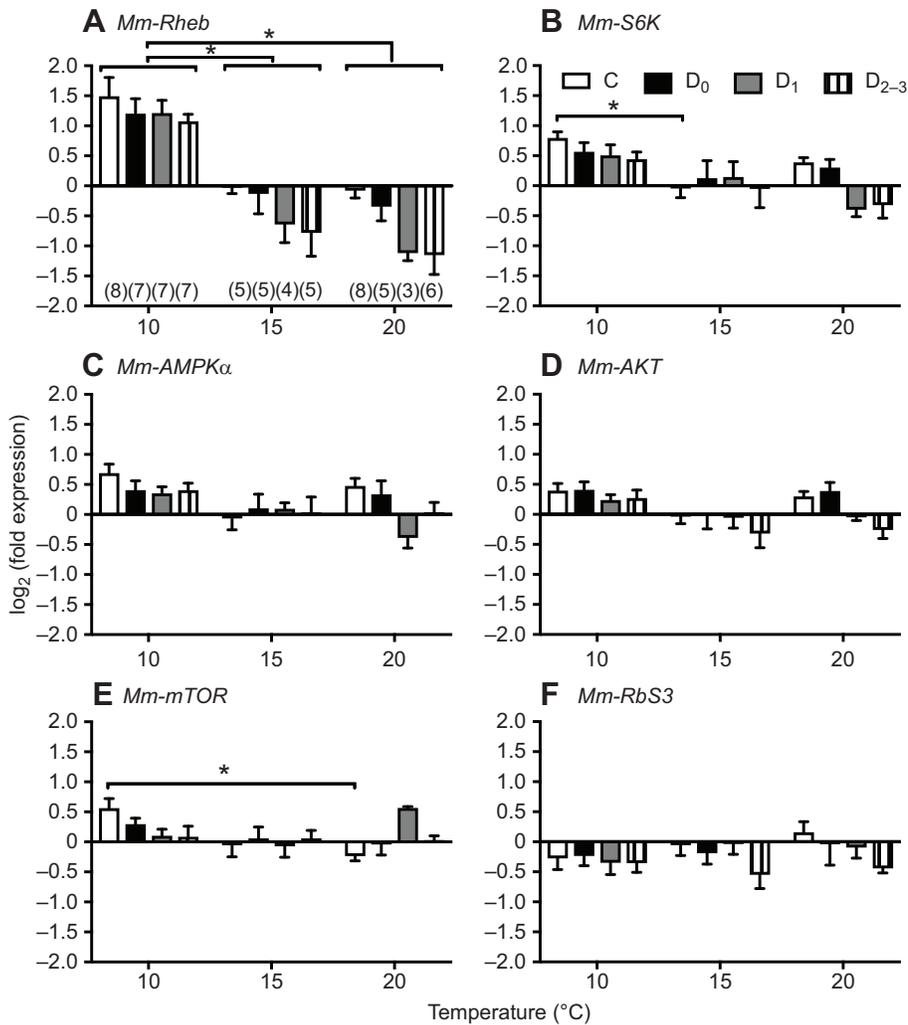
In the Y-organ, a positive correlation of the two variables was highly significant at all temperatures ( $P$  between 0.0081 and  $< 0.0001$ ; Fig. 7B, Table S2). The slopes of the lines did not differ significantly ( $F = 0.1279$ ,  $P = 0.8802$ ), but the difference between the elevations was highly significant ( $F = 23.78$ ,  $P < 0.0001$ ), which indicates an inverse relationship between *Mm-*

*Rheb* mRNA levels and temperature.  $R^2$  ranged from 0.2322 (10°C) to 0.7008 (20°C).

In the heart,  $\log_{10}$  ecdysteroids and *Mm-Rheb* levels were negatively correlated (10°C:  $P = 0.0498$ ; 20°C:  $P = 0.0004$ ) or not correlated (15°C; Fig. 7C, Table S2). There was no significant difference between the slopes of the fitted lines ( $F = 1.182$ ,  $P = 0.3136$ ), but there was a significant difference between the elevations of the lines ( $F = 51.66$ ,  $P < 0.0001$ ), indicating higher *Mm-Rheb* mRNA levels at 10°C.  $R^2$  varied between 0.0855 (15°C) and 0.491 (20°C).

#### DISCUSSION

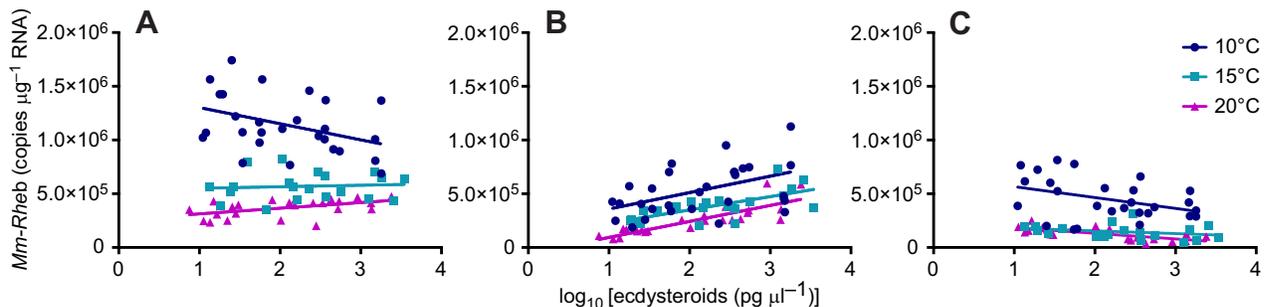
Summer water temperatures in the intertidal of embayments and estuaries of Northern California and Oregon vary between 10 and 25°C (Brown and Terwilliger, 1999; Tasto, 1983), and we observed a maximum of 22°C when collecting juveniles from Bodega Harbor during summer 2013. Larval and juvenile *M. magister* from different locations along the North Pacific coast show high survival and decreasing moult interval from 5 to 18°C, and stagnating moulting frequency and growth at higher temperatures (Brugman, 1972; Kondzela and Shirley, 1993; Sulkin and McKeen, 1989; Sulkin et al., 1996; Terwilliger and Dumler, 2001). This is consistent with our results on temperature-dependent survival and moult progression (Figs 1 and 2). Oxygen consumption rate increases up to 3-fold in *M. magister* between 10 and 20°C, resulting in extraordinarily high  $Q_{10}$  values (Brown and Terwilliger, 1999; Gutermuth and Armstrong, 1989; McLean and Todgham, 2015; Prentice and Schneider, 1979). At 20°C, metabolic maintenance costs may become so high that energy partitioned for growth is reduced (Pörtner and Knust, 2007; Pörtner, 2010;



**Fig. 6.** mRNA levels in heart after 14 days of incubation at 10, 15 or 20°C of *M. magister* juveniles in intermolt (C, white), early (D<sub>0</sub>, black), mid (D<sub>1</sub>, grey) or late (D<sub>2-3</sub>, striped) premolt. (A) *Mm-Rheb*, (B) *Mm-S6K*, (C) *Mm-AMPK $\alpha$* , (D) *Mm-AKT*, (E) *Mm-mTOR* and (F) *Mm-RbS3*. Data are normalized to the mean absolute mRNA levels in stage C at 15°C (Table 2). For results of two-way ANOVA, see Table 2; for results of the *post hoc* Tukey test, see Results. Asterisks denote significant differences according to the Tukey *post hoc* test at  $P < 0.05$ . Sample size (N) is given in parentheses in A also applies to the other genes; data are presented as means  $\pm$  s.e.m.

Sokolova et al., 2012; Terwilliger and Dumler, 2001). Thus, higher metabolic rate does not translate into faster moult progression, and the crabs seem to have reached or surpassed a maximum rate for completing premoult processes. Juvenile survival is only compromised at substantially longer exposures to this temperature than in the present study and at higher temperatures (Brugman, 1972; Kondzela and Shirley, 1993; Sulkin and McKeen, 1989; Sulkin et al., 1996; Terwilliger and Dumler, 2001).

In the temperature range 10 to 20°C, haemolymph ecdysteroid levels throughout the moult cycle of the juveniles were similar to those of adult *M. magister* (Fig. 3; Thomson et al., 2006). In the lobster *Homarus americanus*, temperature-dependent regulation of ecdysteroid titers is necessary to prevent premature (i.e. lethal) moults (Aiken and Waddy, 1975; Chang and Bruce, 1980). In the copepod *Calanus pacificus*, ecdysteroid titers decrease with lower temperatures (Johnson, 2003). It is possible that low temporal



**Fig. 7.** Relationship between log<sub>10</sub>-transformed circulating ecdysteroid levels (pg  $\mu$ l<sup>-1</sup>) and *Mm-Rheb* expression (copies  $\mu$ g<sup>-1</sup> RNA) in juvenile *M. magister* after 14 days incubation at 10°C (dark blue circles), 15°C (light blue squares) or 20°C (pink triangles) throughout the moult cycle. (A) Eyestalk ganglia, (B) Y-organ and (C) heart. *Mm-Rheb* expression as also presented in Figs 4–6 as log<sub>2</sub>-transforms of factorial change, excluding two x–y pairs of late premoult (D<sub>3</sub>) animals with ecdysteroids below the detection limit of the ELISA. For sample size (N), see Figs 4–6. For goodness of fit of linear regression ( $R^2$ ), Pearson correlation coefficients ( $r$ ) and  $P$ -values, see Table S2.

resolution precluded detection of temperature-dependent differences in ecdysteroid titre in our study. Alternatively, similar ecdysteroid concentrations at 10, 15 and 20°C in juvenile *M. magister* may result from the active regulation of both biosynthesis and degradation/elimination of moulting hormone (Mykles, 2011).

In most decapods, the neuropeptide MIH appears to be regulated post-transcriptionally over the normal moult cycle, as *MIH* mRNA levels in the eyestalk ganglia can remain elevated during premoult (Chung and Webster, 2003, 2005; Covi et al., 2012; Pitts and Mykles, 2017; Techa et al., 2015; Techa and Chung, 2015). Genetic manipulation of *Rheb* and other mTOR components in insects highlights the importance of this pathway for protein synthesis (Danielsen et al., 2016; Hall et al., 2007; Parthasarathy and Palli, 2011). In *Drosophila melanogaster*, inhibition of mTORC1 in neurosecretory brain cells that produce prothoracicotropic hormone, which stimulates ecdysteroid production in the prothoracic gland, did not alter the timing of pupariation (Layalle et al., 2008). Thus, mTORC1 does not seem to be necessary for the control of neurohormone expression, synthesis, and/or secretion in insects under normal conditions. However, environmental stressors or conditions may inhibit moulting by upregulating *MIH* expression, thus maintaining high circulating MIH levels that prevent Y-organ activation. For example, some *G. lateralis* individuals fail to moult in response to multiple leg autotomy, indicating the animals are in a blocked or chronically inhibited condition (Pitts et al., 2017). The eyestalk ganglia of blocked animals exhibit 200- to 400-fold higher mRNA levels of *Gl-MIH*, *Gl-CHH* and three of four mTOR components, including *Gl-Rheb*, than the eyestalk ganglia from control animals (Pitts et al., 2017). These data suggest that mTOR signalling enhances neuropeptide production in eyestalk ganglia of animals with an activated Y-organ to prevent or delay moulting under adverse conditions.

Our study provides important insights into the role of mTOR signalling in the eyestalk ganglia of juvenile *M. magister*. In the thermal range that allows moult progression, transcriptional regulation of *Mm-Rheb* may contribute to the control of neuropeptide synthesis in response to temperature rather than over the moult cycle. The significant interaction term in *Mm-MIH* mRNA levels indicates that moulting is regulated differently at different temperatures. At temperatures that juvenile *M. magister* tolerate over prolonged periods (10 and 15°C; Kondzela and Shirley, 1993), *Mm-MIH* was constitutively expressed over the moult cycle, as expected. A decrease of *MIH* expression in premoult was reported for *Callinectes sapidus* and in *Scylla paramamosain* (Huang et al., 2015; Lee et al., 1998) and observed in our experimental animals at 20°C. Transcription was downregulated at 20°C also in the other genes, but to a lesser extent. Unlike at lower temperatures, at 20°C, *M. magister* juveniles control MIH transcriptionally, yet with a time lag. *Mm-MIH* mRNA levels were reduced by about half from stages C to D<sub>1</sub> (Fig. 4E), which is when the committed Y-organ becomes least sensitive to MIH through the end of premoult (Chang and Mykles, 2011). In stage D<sub>2-3</sub>, *Mm-MIH* recovered by a factor of 2.5, which would allow the eyestalk ganglia to resume a greater rate of MIH synthesis in preparation for MIH release during postmoult and intermoult. Consistent with this, Techa and Chung (2015) found a time lag between transcription and peptide storage in *C. sapidus* eyestalk ganglia in premoult. Owing to the variable correlation coefficients between ecdysteroid concentration and *Mm-Rheb* expression in eyestalk ganglia, a (simple) feedback mechanism of ecdysteroid on neuropeptide production (Techa and Chung, 2015) via mTOR seems unlikely. However, transcriptional compensation of mTOR

components and neuropeptides may counteract temperature-dependent effects on neuropeptide synthesis, as hypothesized for moderate thermal stress.

mTOR-dependent protein synthesis is required for increased ecdysteroid synthesis and secretion in the activated Y-organ. Transcriptional regulation of this pathway takes place not only during moult induction in *G. lateralis* (Abuhagr et al., 2014b, 2016; Das et al., 2018; Shyamal et al., 2018), but also during naturally occurring moults in *M. magister*. However, the expression patterns differ between species (no change to up to 10-fold change), and differing degrees of post-transcriptional regulation are likely (Abuhagr et al., 2014b; 2016). In *M. magister*, *Mm-Rheb* increased up to 3-fold from intermoult to late premoult, while gene expression changes of the protein kinases mTOR, AMPK, AKT and S6K were smaller, yet significant (Fig. 5). The positive correlations between *Mm-Rheb* mRNA levels and ecdysteroid titres at all three temperatures support the role of this GTPase in the regulation of the moult cycle through the mTOR pathway (Fig. 7B). As mTORC1 is a direct target of Rheb (Long et al., 2005), *Mm-Rheb* may serve as a proxy for mTOR activity as a function of temperature and moult stage in the Y-organ of *M. magister*. Adjusting *Mm-Rheb* in the Y-organ to higher levels in the cold and lower levels in the warmth may play an important role in coping with moderate thermal stress.

AMPK was the only protein kinase in the Y-organ not significantly affected by temperature. Keeping a certain level of *Mm-AMPK $\alpha$* , especially in mid and late premoult, would maintain regulatory capacity and, at least to some extent, may help in the downregulation of metabolic cost by phospho-AMPK at 20°C. Partial inhibition of protein synthesis in the warmth may contribute to similar ecdysteroid levels across temperatures. In addition, taking into account the expression of the activating mTOR components, a 1.9-fold increase in *Mm-AMPK $\alpha$*  from intermoult to late premoult at 20°C may counterbalance the 3.0- and 1.6-fold increases of *Mm-Rheb* and *Mm-S6K*, respectively. At 10°C, by contrast, a relatively small increase in *Mm-AMPK $\alpha$*  (1.6-fold) opposes larger changes of *Mm-Rheb* (1.9-fold), *Mm-S6K* (2.1-fold) and *Mm-mTOR* (1.9-fold) throughout the moult cycle. This may promote protein synthesis and thereby ecdysteroid production at thermally reduced kinetic energy. Posttranscriptional regulation of the kinases, e.g. through phosphorylation by upstream kinases, may play an important role in the regulation of the mTOR pathway in the Y-organ, and needs to be studied in the future.

Tissue- and fibre-specific regulation of protein turnover through the mTOR pathway contributes to regulating muscle growth (Bodine et al., 2001; Goodman et al., 2011; Saxton and Sabatini, 2017), cardiac homeostasis and compensatory cardiomyocyte growth in mammals (reviewed in Sciarretta et al., 2014). In crustaceans, muscle protein synthesis and ribosomal activity are higher in premoult than in postmoult and intermoult, and are regulated by ecdysteroid and other factors (El Haj et al., 1996; reviewed in Mykles and Medler, 2015). mTOR signalling components, especially *Rheb*, are upregulated in atrophic claw muscle of *G. lateralis* during premoult after moult induction (MacLea et al., 2012) and in naturally moulting *Carcinus maenas* (Cosenza, 2016). Thus, mTOR activation may relate to increased protein turnover during claw muscle atrophy, which facilitates the passage of the tissue through the narrow joint (Covi et al., 2010; MacLea et al., 2012; reviewed in Mykles and Medler, 2015). There is a significant positive correlation between haemolymph ecdysteroid concentrations and *Rheb* expression in claw muscle, but no correlation in thoracic muscle (Cosenza, 2016; MacLea et al.,

2012). We found no correlation (at 15°C) or negative correlations (at 10 and 20°C) between *Mm-Rheb* in the heart and ecdysteroid titres of *M. magister* juveniles (Fig. 7C). Consistent with this, actin and myosin protein content decrease from intermoult to premoult at constant soluble protein and water content in abdominal muscle of juvenile shrimp (de Oliveira Cesar et al., 2006). mRNA levels of muscle proteins exhibit a similar pattern over the moult cycle in *Portunus pelagicus* (Kuballa et al., 2011). Also, heart sarcoplasmic reticulum  $Ca^{2+}$ -ATPase (*SERCA*) mRNA expression declines in late premoult crayfish (Chen et al., 2002). This points to a lower degree of heart muscle remodelling than in skeletal muscle in premoult, and an inverse relationship, if any, between ecdysteroid and heart muscle growth (Qian et al., 2014). In intermoult, low levels of ecdysteroid may contribute to cardiac muscle growth via mTOR.

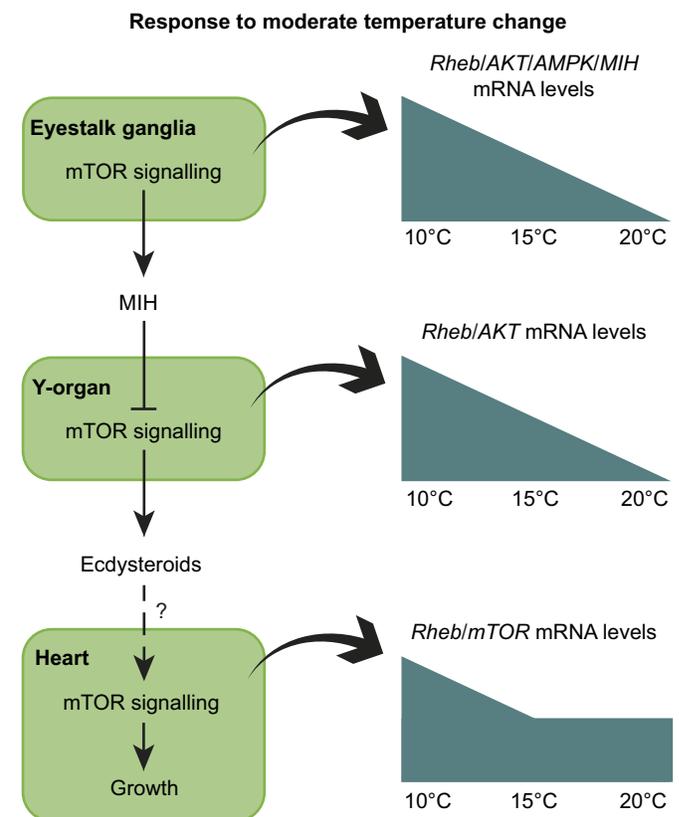
Phenotypic changes of the heart are mirrored in changes in mRNA expression of genes involved in muscle growth both during warm and cold acclimation in fish (e.g. Keen et al., 2016). Furthermore, cardiac transcriptomes vary after heat shock according to seasonal and latitudinal acclimatization in porcelain crabs (Stillman and Tagmount, 2009). In *M. magister* juveniles, cardiac expression patterns of mTOR components point to a general cold compensation of the mTOR signalling pathway, as expression of activating kinases (*Mm-AKT*, *Mm-mTOR* and *Mm-S6K*) and the inhibitory *Mm-AMPK* differ to the same extent (Fig. 6). The temperature effect was most pronounced in *Mm-Rheb* with the largest expression differences found in this study (Fig. 6A). That *Mm-Rheb* was not downregulated during warm exposure is clearly evident from overlapping 15 and 20°C regression lines when correlation to ecdysteroid concentration was tested (Fig. 7C). This indicates incomplete warm compensation of protein turnover as both protein synthesis and degradation may increase with temperature (Storch et al., 2003, 2005; Todgham et al., 2017; Whiteley et al., 1997). In agreement with this, cold acclimation of cardiac performance was observed in adult *M. magister* (Prentice and Schneider, 1979), whereas there was no evidence for seasonal (warm) acclimatization in juveniles (McLean and Todgham, 2015). Increased AMPK expression or activation may be a transient phenomenon during acute heat stress, helping to rapidly reduce energy consumption (Frederich et al., 2009; Jost et al., 2012; Han et al., 2013). Our data do not point to mTOR inhibition, and thus reduced growth, of the heart at 20°C. A sustained level of mTOR activation may aid to maintain cardiac muscle capacity (Sciarretta et al., 2014) at chronically increased heart rates expected owing to the lack of seasonal acclimatization (McLean and Todgham, 2015) at the cost of reduced body growth (Kondzela and Shirley, 1993; Terwilliger and Dumler, 2001). Body growth can only take place in the thermal range in which energy supply from aerobic metabolism exceeds maintenance costs (Pörtner and Knust, 2007; Pörtner, 2010; Sokolova et al., 2012). The thermal range that allows moulting and growth is relevant for the biogeography of a species. Knowledge of the underlying mechanisms of thermal tolerance can help to explain and predict past and future changes in distribution and biodiversity owing to climate change with impacts on fisheries (García Molinos et al., 2016; Green et al., 2014; Holsman et al., 2003; Moloney et al., 1994; Quinn, 2017; Toft et al., 2014).

## Conclusions

Consistent with previous findings, Y-organ *Rheb* mRNA levels were positively correlated with ecdysteroid titres, which indicates that Rheb is central to regulating ecdysteroidogenesis through the mTOR pathway (Chang and Mykles, 2011; Das et al., 2018; Shyamal et al., 2018). Expression patterns of the GTPase *Mm-Rheb*

may relate to temperature-dependent mTOR activation and protein synthesis during the moult cycle of juvenile *M. magister*. In all three organs, *Mm-Rheb* expression correlated most strongly with ecdysteroid titres at 20°C, which may indicate the tightest regulation of *Mm-Rheb* (and, vice versa, ecdysteroid production in Y-organs) compared with lower temperatures. This may point towards a need to control metabolic demand more stringently at higher temperatures.

Fig. 8 summarizes the effects of temperature on mRNA levels in the eyestalk ganglia, Y-organ and heart of juvenile *M. magister*. In intermoult, MIH inhibits mTOR signalling in the Y-organ, resulting in low ecdysteroid synthesis and secretion (Chang and Mykles, 2011). Low levels of ecdysteroid may foster growth of the heart. Temperature-dependent expression of mTOR signalling genes in the eyestalk ganglia and Y-organ and *Mm-MIH* in the eyestalk ganglia contributes to temperature compensation of moult control between 10 and 20°C. However, thermal compensation in the heart may be incomplete. The limited capacity for acclimation or acclimatization to 20°C in juvenile *M. magister* was apparent, as



**Fig. 8. Effects of moderate thermal stress on moulting and growth through the mTOR signalling pathway in juvenile crabs.** In intermoult, MIH keeps the Y-organ in a basal or inhibited state with low ecdysteroid secretion by inhibiting mTOR. Low levels of ecdysteroid may stimulate heart muscle growth via mTOR signalling. Moderate temperature change (10 to 20°C range) allows acclimation of the animals, at least with respect to some physiological functions. After 14 days, thermal compensation is observed in moult control, i.e. similar ecdysteroid titre across temperatures throughout the moult cycle. Mechanisms include upregulation or downregulation of *Mm-MIH* and mTOR signalling genes (*Mm-Rheb*, *Mm-AKT*, *Mm-AMPK*) during cold (10°C) or warm (20°C) exposure in the eyestalk ganglia and Y-organ. In the heart, thermal compensation of metabolism is incomplete, as oxygen demand and heart activity increase with temperature. A sustained mRNA level of *Mm-Rheb* and *Mm-mTOR* indicates a greater allocation of energy to maintaining cardiac capacity during warm exposure.

indicated in the gene expression patterns in the heart after 14 days. Lack of downregulation of cardiac *Mm-Rheb* and other mTOR components at 20°C may relate to higher protein turnover and rising maintenance costs, which may result in similar moult stage progressions at 15 and 20°C. Insufficient warm compensation of heart function may contribute to the negative effects of high temperature on long-term growth and survival. This is consistent with previous studies of juvenile *M. magister*: successful moulting occurs up to about 20°C and reduced body growth occurs at temperatures above about 15°C (Kondzela and Shirley, 1993; Terwilliger and Dumler, 2001). Future studies should quantify phosphorylation of the protein kinases (e.g. mTOR, AMPK, AKT and S6K) to determine temperature-dependent activation or inhibition of mTOR signalling throughout the moult cycle. Furthermore, studying the mechanisms involved in the crosstalk between tissues will enable us to gain new insights into how moulting and growth are regulated in response to environmental stressors.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: A.C.W., D.L.M.; Methodology: A.C.W., D.L.M.; Validation: A.C.W.; Formal analysis: A.C.W.; Investigation: A.C.W., S.A.M.B., D.A.L.-C.; Resources: A.C.W., D.L.M., E.S.C.; Writing - original draft: A.C.W.; Writing - review & editing: A.C.W., S.A.M.B., D.A.L.-C., E.S.C., D.L.M.; Visualization: A.C.W.; Supervision: A.C.W., E.S.C., D.L.M.; Project administration: A.C.W., D.L.M.; Funding acquisition: A.C.W., E.S.C., D.L.M.

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#### Data availability

New sequences identified in this study have been submitted to GenBank under the accession numbers KT285226, KT315720, KT315721, KT315722, KT315723, KT367806, KT367807 and KY070318 (<https://www.ncbi.nlm.nih.gov/genbank/>).

#### Supplementary information

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