



UNIVERSITY^{of}
TASMANIA

Integrating physiology, behaviour and molecular
mechanisms to understand impacts of ocean
warming on southern calamari (*Sepioteuthis
australis*)

by

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STATEMENTS AND DECLARATIONS

DECLARATION OF ORIGINALITY

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person, except where due acknowledgment is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the ruling of the Safety, Ethics, and Institutional Biosafety Committees of the University. All research was conducted with approval from the University of Tasmania Animal Ethics Committee A0017463 and the Department of Primary Industries, Parks, Water and Environment (18125).

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ABSTRACT

Accelerating climate change will strongly influence marine species across the world, altering the composition, stability and function of ecosystems, and ultimately affecting the wellbeing of the human communities dependent on them. Ectotherms could be particularly vulnerable to temperature changes, as temperature is a major factor influencing their internal regulation (e.g. cellular or physiological mechanisms). Not surprisingly, increases in water temperatures have already been documented shown to have an impact on marine ectotherms with changes in body size, reproduction and geographical distribution. Understanding species responses to ocean warming is a key challenge for contemporary ecology, evolution and conservation.

Ocean temperatures are expected to increase by 2 °C to 4 °C by the end of the century, yet the rate and magnitude of change is not the same across the world. In south-eastern Australia, ocean warming is increasing almost four times faster than the global average; this region can therefore function as a ‘natural laboratory’ where temperature effects might be accelerated. Research to date has focused on how species within this region may shift geographically as waters warm; however, little is known about how species might perform and interact in response to thermal challenges within their existing ranges. Southern calamari (*Sepioteuthis australis*) are an ecologically and economically important endemic species of southern Australia and northern New Zealand. To date, studies examining the effect of ocean warming on cephalopods have been under-represented in the literature in comparison with other group (e.g., fish or marine invertebrates), despite their important role in marine ecosystems as both predator and prey. They are highly plastic and fast-growing species with short generation times, which may allow them to adapt faster than other species to changing environmental conditions, improving their chances of survival. Consequently, by studying rapidly responding species such as squid, in fast-changing regions of the world, we can examine the mechanistic links between ocean warming and the biological responses in advance of the broader scale impacts predicted for the future.

The main aim of this thesis is to understand how temperature affects the physiological (oxygen consumption and upper thermal limits) and behavioural performance (thermal preferences and predatory behaviour) of adult southern calamari, as well as the molecular pathways (transcriptomics) underpinning this performance.

Physiological and behavioural responses to changes in water temperature can provide important insights regarding species sensitivity and vulnerability to ocean warming. Currently, it is still not clear if changes in temperature will induce shifts in the aerobic metabolism and thermal preferences of squid. The first component of this study aimed to examine the effects of different acclimation temperature on the metabolic activity and behavioural thermal preferences of southern calamari in adult stages (Chapter 2). To do this, intermittent-flow respirometry was used to measure standard, routine, maximum and excess post-exercise oxygen consumption rates, as well as aerobic scope. In addition, a custom-made shuttle box system was utilised to evaluate the thermal preferences of individuals. The results suggested that acclimation temperatures influence squid metabolism and preferences, and that individuals seek out thermal environments that are more favourable for their metabolic capacities. Overall, metabolic rates (standard, routine, and maximum) increased at higher acclimation temperatures, with a simultaneous reduction in the aerobic scope at 22 °C and 25 °C. These results suggest that metabolism in southern calamari could be limited by water temperatures above 22 °C. Coincidentally, squid actively seek temperatures between 15 to 22 °C, which seem to be temperatures more beneficial for squid physiological capacities.

In addition to altering physiological responses, warming may also impact predator–prey dynamics in various ways. To further determine an individual’s performance under changing environmental conditions, predatory behaviour of southern calamari across temperature treatments (13, 16, 19, 22 and 25 °C) (Chapter 3) was also examined by way of the interaction

of individual squid with a common prey species, Australian salmon (*Arripis trutta*). Here, squid behaviour differed between treatments, with the ability and decision-making processes (e.g. motivation, capture success) to capture the prey influenced by temperature. At elevated temperatures, southern calamari showed an increase in predation rate, capture success and number of strikes, meanwhile the latency time was greatly reduced. However, handling time increased with temperature.

In addition to behavioural thermoregulation, species have another major mechanism to cope with changes in environmental conditions – acclimation. The ‘live fast, die young’ life history strategy of squid, with fast-paced life histories and multiple generations within short time windows, might facilitate or favour a fast acclimation or adaptation in squid. To examine this, phenotypic plasticity (upper thermal limits) and genome-wide gene expression (RNA-seq) were used (Chapter 4). Squid were exposed to rapid increases in temperature to establish the effect of acclimation on their upper thermal limit (CT_{max}), and RNA-seq was used to examine the potential mechanisms involved in individual adaptation to temperature. Southern calamari demonstrated a plastic response to temperature, increasing their CT_{max} significantly. The transcriptomic study suggested that squid used post-transcriptional RNA modifications such as splicing activity, as well as apoptotic and immune responses, to adjust to temperature changes.

This thesis demonstrates for the first time, how acclimation temperature affects southern calamari performances, by integrating physiological (metabolic activity and critical thermal limits), behavioural (thermal preferences and predatory performance), as well as molecular (RNA-seq analysis) responses to understand mechanisms of temperature adaptation. Overall, the results indicate that this species could maintain phenotypic plasticity in some traits (CT_{max}) and behavioural performance measures (predatory strategies) under ocean warming conditions, possibly associated with the highly plastic transcriptional response found in this study.

However, other important traits such as aerobic scope, survivorship and the ability to capture prey could be negatively impacted by an increase in ocean temperatures. As squid are key components of trophic webs, transferring energy across trophic levels, the findings of this study might have implications at the community and ecosystem levels, by way of cascading effects. Consequently, a more holistic approach integrating different measures of performance, as well as exploration of the mechanisms involved in adaptation, is needed to better predict future responses. Greater understanding of species climate change responses will be critical for underpinning development of more appropriate management and climate adaptation strategies to better support healthier marine ecosystems, fisheries and aquaculture now and into the future.

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Chapter 1: General introduction

1.1 Cephalopods

Cephalopods are unique in many physiological and ecological ways (for example, inking, jet propulsion and learning capacity) and are broadly considered the most cognitively complex of the invertebrates. They evolved in the marine ecosystem during the Cambrian period (~530 Ma) from a monoplacophoran-like mollusc (Hildenbrand et al., 2021), and are now a diverse group with 800 species of living cephalopods (Hanlon and Messenger, 2018a) differentiated into two groups – Nautiloidea and Coleoidea (Boyle and Rodhouse, 2007). The latter, which include species of octopus, squid, and cuttlefish (herein referred to as cephalopods), show significant divergences from other molluscs, with a major modification of the body plan organisation. They have a greatly modified internal regressed shell, with specific characteristics of the neuromuscular complex, such as arms, funnel, and muscular mantle (Bonnaud-Ponticelli and Boletzky, 2016).

One of the most significant adaptations in this group is their life history strategy of ‘live fast and die young’ or ‘life in the fast lane’ (Boyle and Rodhouse, 2007). Most cephalopods are fast-growing animals (e.g., *Loligo reynaudii* show a increment on mantel length between 0.75 to 1 mm/day (Mwanangombe et al., 2021)) with short life cycles, for example most of species of squid live for a year or less (Jackson and O'Dor, 2001), exhibiting wide plasticity in life history traits, influenced primarily by the interactive effects of food availability (quality and quantity), light cycle and temperature (Pecl et al., 2004b). The later might be the most influential, allowing them to substantially vary growth trajectories (Forsythe et al., 2001). Not

surprisingly, recent work has indeed shown that cephalopod populations are increasing globally (Doubleday et al., 2016).

Cephalopods have also developed other effective evolutionary adaptations which make them a distinctive and extremely successful group by many measures. These include complex neural organisation supporting flexible behaviours and advanced cognition, including learning and memory abilities, mimicking/camouflage, and social interactions (Mather and Dickel, 2017; O'Brien et al., 2018; Schnell and Clayton, 2019). For instance, their large brain allows the processing of a significant amount of information (Mather and Dickel, 2017). Moreover, their advanced vision and their unique locomotion mode of jet propulsion allow for high-speed responses, which support predatory behaviours and predator avoidances (Bartol et al., 2001; Boyle and Rodhouse, 2007). They also have mechanisms to eject ink, confusing any predators and giving them a better chance to escape (Caldwell, 2005; Derby et al., 2013). Furthermore, they have cells and organs (chromatophores, iridophores, leucophores and papillae) capable of rapidly changing their body colour and pattern which can be used for camouflage as well as communication for social interactions (Hanlon and Messenger, 2018a).

Cephalopods are a major component of many marine ecosystems and their physiology, behaviour and sensory strategies have allowed them to effectively inhabit most regions from shallow to deep water (Boyle and Rodhouse, 2007; Villanueva et al., 2017). They can be found in almost all marine habitats, including coral reefs, seagrass beds, rocky reefs, sandy plains, open ocean and intertidal zones (Hanlon and Messenger, 2018a), serving as both important predators and prey (Wood and O'Dor, 2000; Pimentel et al., 2012). They, especially squid, have a role in the structuring of marine ecosystems, linking different trophic levels due to their voracious prey consumption and high production rates (de la Chesnais et al., 2019).

Furthermore, fisheries for this group represent a major target worldwide, being one of the four most highly valuable groups, representing around 5% of the total captures with annual landings ranging from 2 million tonnes in 1980 with a peak of 4.9 million tonnes in 2014, then, catches declined to about 3.6 million tonnes in 2017 and 2018 (Arkhipkin et al., 2015; FAO, 2020; Schickele et al., 2021). Despite their major economic and ecological role, information on cephalopod biology, ecology, and physiology exists for only approximately 8% of the known species, limiting our understanding of this important group across ecosystems (O'Brien et al., 2018).

1.2 Cephalopod performance under ocean warming

Given the current rates of change in marine species and their ecosystems in response to climate change, understanding the complex relationship between species performance and environmental stressors is essential to identify future populations and ecosystem dynamics. The present chapter aims to examine the current status of our knowledge in relation to the impact that ocean warming has on cephalopod species. Ocean warming is introduced, followed by a discussion of the effects on life history, and physiological and behavioural performance, as well as the potential mechanisms this group might use to handle changing conditions.

1.2.1 Ocean warming

The earth is facing global and unprecedented anthropogenic changes where the magnitude and rapid nature of these changes greatly exceed historical rates (Blois et al., 2013; Smith et al., 2015; Millar et al., 2017). The ocean absorbs approximately 93% of the additional anthropogenic heat entering the climate system (Johnson and Lyman, 2020). Over recent decades an increase in water temperatures has been observed across the world's oceans (Figure 1.1), with projections indicating that temperature could rise between 1 °C (SSP1-2.6) to 4 °C

in the worst scenario (SSP5-8.5) by the end of the century given current trajectories of greenhouse emissions (IPCC, 2021). In addition to longer-term climatic trends, short extreme events such as heatwaves have been recorded as more frequent and intense and are likely to increase in magnitude in the future, which could have devastating consequences at population and ecosystem levels for many species including cephalopods (Oliver et al., 2019). The impact of rising temperatures has already been observed around the world (Parmesan, 2006; Hoegh-Guldberg and Bruno, 2010; Poloczanska et al., 2013; Scheffers et al., 2016), and further disruption is predicted as global temperatures continue to increase, potentially leading to more major and extensive changes in ecosystem functions as well as higher extinction rates (Parmesan, 2006; Scheffers et al., 2016).

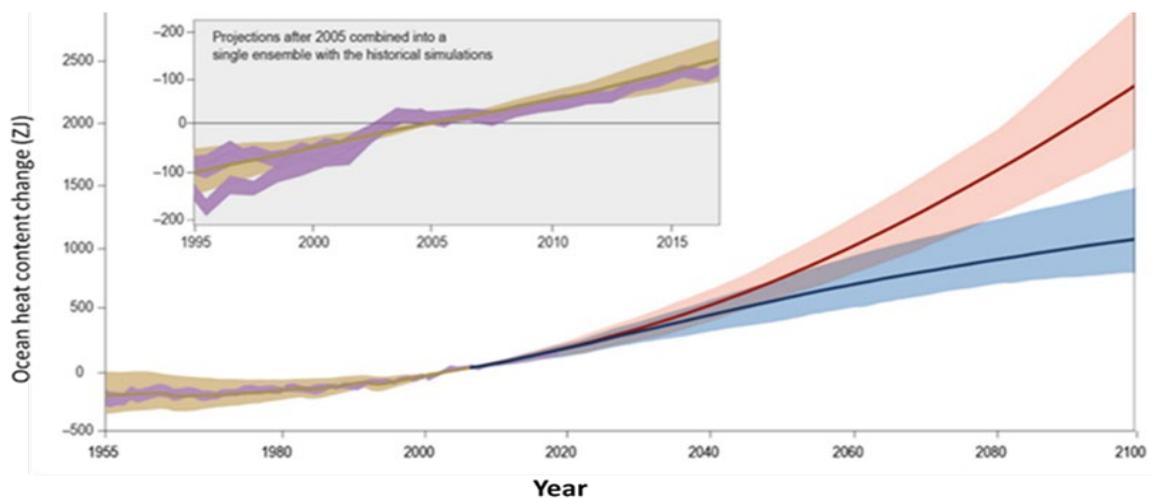


Figure 1.1. Time series of globally integrated upper 2000 m ocean heat content changes (ZJ), relative to the period 2000–2100, as inferred from observation (magenta) and simulated for historical periods (tan) (insert figure). The solid blue and red lines are the model mean of the representative concentration pathways (RCP) 2.6 (blue) and 8.5 (red), while the shading indicates each ensemble’s 5th to 95th percentile range. The 2.6 and 8.5 scenarios are respectively the very stringent and extreme pathways, where RCP requires that carbon emissions started to decline by 2020 and will go to zero by 2100, meanwhile RCP 8.5 is the worst possible scenario (IPCC special report, 2019, Chapter 5: Changing ocean, Marine ecosystems, and Dependent communities (Bindoff et al., 2019).

1.2.2 Direct effects of ocean warming on the life history, physiology and behaviour of cephalopods

Ectotherms are highly dependent on external temperature as it plays a major role in their internal regulation and changes can significantly impact their whole performance, ultimately

affecting their overall fitness and survival (Pörtner and Knust, 2007; Pörtner and Peck, 2010; Bozinovic and Pörtner, 2015). Currently, studies investigating the effect of ocean warming on cephalopods are under-represented in the literature, limiting our knowledge of how this critical group will respond to future challenges (Higgins et al., 2012; Xavier et al., 2015). So far, temperature has been observed to affect almost every aspect of life history in cephalopods (Pimentel et al., 2012; Vijai et al., 2015). For instance, higher temperatures reduce the length of embryonic developmental time, increasing premature hatching, and malformations such as mantle detachment, body deformities and eye dimorphism (Pimentel et al., 2012; Uriarte et al., 2012; Zúñiga et al., 2013; Repolho et al., 2014; Caamal-Monsreal et al., 2016; Uriarte et al., 2018). Increases in mortality rates have also been linked to elevated temperatures in early life stages, for example in the embryonic development of *Loligo vulgaris* (Figure 1.2) and *Octopus vulgaris* (Pimentel et al., 2012; Rosa et al., 2012; Uriarte et al., 2012; Repolho et al., 2014). Furthermore, faster growth rates have been observed as a function of warmer temperatures (Pecl, 2004), as have reductions in body size (Figure 1.2) (Boletzky, 1994; André et al., 2009; Repolho et al., 2014). A major concern regarding the impact that ocean warming may have on active species is the energetic costs of living at higher temperatures. All organisms live within a defined range of temperatures (thermal window) and outside of this range, important processes such as growth and reproduction are compromised due to the reduction in aerobic scope, protein denaturation and enzyme inhibition (Pörtner and Knust, 2007; McDonnell and Chapman, 2015). Due to the current warming rates however, it is predicted that in many species thermal tolerances will be exceeded, especially for those already living close to their thermal limits (T_{pejus}). To cope with these stressful conditions, individuals will be forced to adjust their physiological and behavioural performances (Killen et al., 2013). Therefore, understanding the physiology and behaviour of species under changing conditions could help us to identify species thermal sensitivity and vulnerability to climate change.

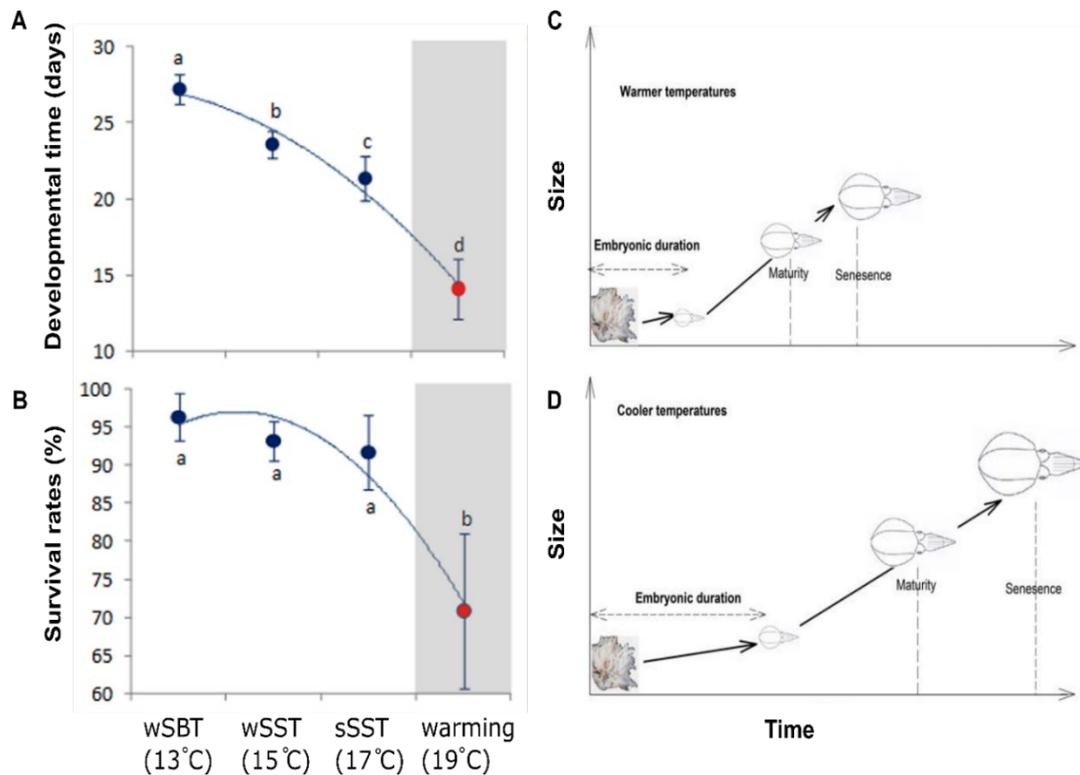


Figure 1.2. Example of the effect of temperature on life history traits in squid. A) developmental time (days); B) survival rates in early stages of ontogeny in *Loligo vulgaris* (Rosa et al., 2012); C) schematic diagram showing the potential effect of warmer water on squid ontogeny in comparison with D) showing the effect of cooler temperatures (Pecl and Jackson, 2008).

Cephalopods are known for having one of the highest metabolic rates in the whole animal kingdom (O'Dor and Webber, 1986; O'Dor et al., 2002), and for an energetically inefficient mode of locomotion – jet propulsion (O'Dor and Webber, 1986), which could also further limit their aerobic budget under ocean warming scenarios. Consequently, understanding the possible impact that temperature changes will have – on energetic requirements of daily activities such as growth and digestion, on reproductive success, and ultimately species survivorship – could be valuable in predicting species performance under future conditions. Oxygen limitation at higher temperatures is believed to be an important constraint on the response of marine organism to climate change (Pörtner and Peck, 2010). To date, cephalopod oxygen consumption rates, both standard SMR (minimum metabolic cost of maintaining biological functions) and routine RMR (metabolic cost of biological functions of maintenance, including

spontaneous activity) metabolic rates have been shown to increase with temperature in both early life stages (e.g. in early ontogeny of *Loligo vulgaris*, *Sepia officinalis* hatchling ((Pimentel et al., 2012; Rosa et al., 2012) and *Octopus mimus* (Uriarte et al., 2012; Zúñiga et al., 2013) and adults (*Dosidicus gigas* (Rosa and Seibel, 2010)). However, to the best of my knowledge, no studies have reported the effect of elevated acclimation temperature on the aerobic scope and recovery period after exhaustive exercise in cephalopods, creating an important knowledge gap in the understanding of how their aerobic budget and ultimately their daily activities could be impacted by ocean warming. Measurements of aerobic scope have often been used as a proxy of metabolic thermal optimum, which is the total oxygen available after basal cost of maintenance, for the organism to perform activities such as reproduction, or foraging (Figure 1.3) (Pörtner and Knust, 2007; Pörtner and Peck, 2010). However, to the best of my knowledge, no studies have reported the effect of elevated acclimation temperature on the aerobic scope and recovery period after exhaustive exercise in cephalopods, creating an important knowledge gap in the understanding of how their aerobic budget and ultimately their daily activities could be impacted by ocean warming.

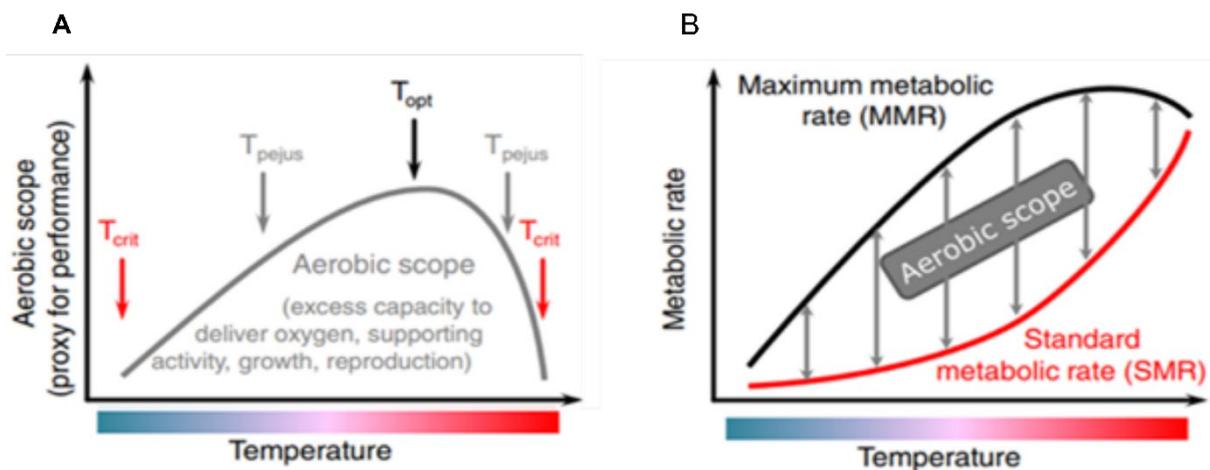


Figure 1.3 Aerobic scope, A) aerobic scope as a proxy of organismal performance for measuring the impact of temperature (T_{opt} = optimal temperature, T_{pejus} = temperature pejus, T_{crit} = critical); B) definition of aerobic scope as the amount of energy available between the standard and maximum metabolic rates which is defined as highest rate of aerobic energy conversions (Verberk et al., 2016).

While the primary concern about the effects of elevated temperature on marine species has been on physiological performances, the behaviour of species could also be significantly affected by changes in environmental conditions (Amarasekare, 2015). In marine species, studies have shown that temperature increases modified predatory and anti-predatory behaviours such as motivational response, attack speed and escape speed (Kidawa et al., 2010; Grigaltchik et al., 2012; Sentis et al., 2012; Horwitz et al., 2020). These behavioural changes could potentially impact communities by altering the stability and strength of trophic interactions (Rall et al., 2010; Grigaltchik et al., 2012). Surprisingly, studies of cephalopod behavioural responses to temperature changes are few and far between.

Cephalopods have a major role linking trophic levels and food webs from different habitats, and their fast responses to environmental conditions might increase their importance in terms of ecosystem function and their provisioning role in global fisheries (de la Chesnais et al., 2019). Consequently, understanding the response of their predatory and avoidance behaviours to ocean warming is key to predicting future community and ecosystem dynamics. To date, only one study has examined how temperature affects escape responses in cephalopods, showing that *Doryteuthis* (formerly *Loligo*) *opalescens* was able to maintain escape performance when temperature was reduced (Neumeister et al., 2000). However, the effect of elevated temperatures on predatory responses in this group has not been examined, although some studies have examined such behavioural responses in relation to ocean acidification (Spady et al., 2014; Spady et al., 2018; Zakroff et al., 2018). For example, in squid paralarvae (*Doryteuthis pealeii*), the capacity to swim appears to be resilient against to acidification (Zakroff et al., 2018), while in adults of *Idiosepis pygmaeus* antipredator and predatory behaviours were altered by exposure to elevated CO₂, increasing activity or decreasing predation rates (Spady et al., 2014; Spady et al., 2018). Due to their important position in many ecosystems, understanding the future behavioural responses of cephalopods to ocean warming

is essential as their responses could potentially lead to cascading effects at community levels and ultimately alter the stability and functioning of ecosystems.

1.2.3 Coping with a warming world

Cephalopods, due to their inherent phenotypic plasticity and fast-paced life history strategies might respond to future changing environmental conditions faster than other species, yet the mechanisms associated with this are still poorly understood. To cope with the rise in temperatures, species could respond to the new conditions by behavioural thermoregulation (movement), acclimation through phenotypic plasticity, or evolutionary adaptation (across generations) (Parmesan, 2006; Hoffmann and Sgró, 2011), noting that these responses are not mutually exclusive (Donelson et al., 2019).

Behavioural thermoregulation could act as a mechanism of thermal refuge for species, moving their distribution to alleviate the effects of stressful conditions. To date, studies have examined thermal preferences during the early life stages of cephalopods via a horizontal gradient (Higgins et al., 2012; Zúñiga et al., 2013). Both species of octopus examined, *Octopus mimus* and *huttoni*, seem to have the capacity to select specific environmental conditions, avoiding lethal temperatures. However, the effect of ocean warming on the preference of adults has not previously been examined in any cephalopod species, creating an important gap in our understanding of how key species could move and distribute in the future. Additionally, thermal preferences, in combination with thermal tolerance limits, are determinants of where species live and so studies on these processes and responses could be beneficial in identifying possible climate-driven changes in distribution in the future (Ern et al., 2016).

Acclimation plays a main role in species' survival under changing conditions. In cephalopods, for instance, acclimation temperature has been shown to have a positive effect on their critical thermal limits, increasing their thermal tolerance window in early life stages, (Rosa et al., 2012;

Speers-Roesch et al., 2018; Uriarte et al., 2018). Summer embryos adapt better to warming conditions than do winter progeny, suggesting that parental effects as well as individual thermal history might play crucial roles in the thermal adaptation of cephalopods (Rosa et al., 2012). The ability of cephalopods to increase their thermal limits via acclimation could confer significant advantages in coping with rapidly changing conditions. However, studies investigating these limits in adults, as well as across generations, are currently absent, creating a gap in our knowledge of their thermal biology. Early stages are considered more susceptible to environmental changes than adults, however, if adults fail to adapt, reproduction and ultimately recruitment will not succeed, resulting in negative consequences for populations.

For species to acclimate and adapt, modulation of gene expression is key (Place et al., 2012). Recently, in cephalopods species, an extensive RNA editing (Adenosine to inosine) process has been observed, which might confer advantages in this group to rapidly respond and acclimate to changes in environmental conditions via phenotypic plasticity (Garrett and Rosenthal, 2012b; Liscovitch-Brauer et al., 2017). For example, this mechanism is used by polar octopus promoting adaptation of their K⁺ channels to cold temperatures (Garrett and Rosenthal, 2012a). RNA editing quickly changes and diversifies genome expression (Liscovitch-Brauer et al., 2017; O'Brien et al., 2018). However, this process is still poorly understood as observational studies are lacking. Additionally, to prevent thermal stress and keep cellular balance, an antioxidative defence system involving non-enzymatic antioxidant molecules (e.g. glutathione, carotenoids), and an enzymatic cascade (e.g. superoxide dismutase, catalase), has been observed in cephalopods at early life stages (Repolho et al., 2014; Sanchez-García et al., 2017). In *Loligo vulgaris* heat shock proteins (HSPs) have been found in the paralarvae stages but not in embryos (Rosa et al., 2012). HSPs are known to be key in the regulation of thermal stress, assisting with different cellular processes such as protein translocation or apoptosis regulation (Garrido et al., 2001). However, the role of this protein

family in cephalopods is not clear and previous results have suggested that the HSPs family might not have a central role in protection against thermal stress in this group, or may be life stage-specific (Rosa et al., 2012). To date, only three species of the Cephalopoda family have an assembled and annotated genome (Albertin et al., 2015; Kim et al., 2018; Belcaid et al., 2019). Still the development of molecular techniques like *de novo* assembly (RNA-seq) offer the unique opportunity to examine the genetic expression in non-model species, identifying pathways in response to stressors, which could highlight mechanisms of phenotypic plasticity and evolutionary adaptation to better understanding of likely future responses in a changing world.

1.2.4 Highlights

The effects of ocean warming are complex, and probably life stage-specific, yet cephalopods seem to be able to adapt faster to these challenges due to their inherent high phenotypic plasticity, and resourceful ways of diversifying their genetic expression, like RNA editing. The little information available for most species makes it difficult to predict what is likely to happen with cephalopods as a group in the near future. Nevertheless, environmental temperature clearly plays an important role in driving the life history traits and physiology (including, growth, embryogenesis, metabolism) of cephalopods, which means warming waters are likely to impact these processes at each life stage (Repolho et al., 2014; Caamal-Monsreal et al., 2016). Through the likely effects on each stage, changing environmental conditions will also alter the recruitment, abundance and distribution of cephalopod species, in turn influencing their sustainable exploitation and economic importance (Rodhouse et al., 2014; Schickele et al., 2021). Consequently, studies examining the thermal capacity of cephalopods to respond to ocean warming are essential to predicting their performance, distribution, and the impact of these processes on their associated ecosystems.

1.3 Thesis aims and structure

1.3.1 Aims

Effects of ocean warming on species and ecosystems have already been observed around the world. While there have been several studies documenting how increases in temperature can affect particular processes in a small number of cephalopod species, information regarding these effects in cephalopods more broadly is still limited. Furthermore, most of the studies focus on one trait in isolation (for example, upper thermal limits; behavioural thermoregulation; metabolism), and studies integrating different performances under different thermal conditions are lacking.

The main aim of this thesis is to understand the role of acclimation to temperature in squid performances in order to understand the most likely responses of southern calamari (*Sepioteuthis australis*) to climate change. This is key to predicting how changing environmental conditions will ultimately impact the fitness, geographic distribution and abundance of species. Given the critical role that cephalopods play in many ecosystems, it is also important to project the future responses of their associated communities and ecosystem dynamics. This thesis provides, for the first time, an integrative approach to how squid (in this case, southern calamari) might perform under different climate scenarios. To accomplish this, physiological and behavioural performances are measured under different thermal conditions (13°C, 16 °C, 19 °C, 22 °C and 25 °C), representing likely temperatures of current and future Tasmanian waters. Specifically, the responses of metabolic rates and thermal preferences (Chapter 2), predatory responses (Chapter 3) and upper thermal limits (Chapter 4) were measured. In addition, omics techniques were used to examine molecular pathways involved in squid thermal regulation and the mechanisms for potential adaptation to higher and lower temperatures (Chapter 4).

These chapters advance our knowledge of how species, in particular squid, could respond to ocean warming, improving our understanding of their physiological thermal tolerances, behavioural performances and the molecular mechanisms driving their responses to change in temperature conditions. This information is essential to better predicting the future composition and function of ecosystems, and to develop more appropriate adaptation strategies for the conservation and protection of marine resources, particularly given the huge potential squid have for influencing ecosystems as both predator and prey.

1.3.2 Thesis structure

The thesis consists of five chapters: a general introduction, three experimental data chapters, and a final discussion synthesising the results from the previous chapters. Chapters here are presented as individual manuscripts for publication, so some repetition occurs in their introductions.

The main questions for each chapter were:

- Chapter 1: Identified current knowledge and main gaps in the literature regarding the effects of ocean warming on cephalopods.
- Chapter 2: Will acclimation temperature affect the metabolism and temperature preferences of southern calamari in the adult stages. If so, will squid prefer to actively move to temperatures more favourable for their metabolic capacities or, will they prefer to stay close to their acclimation temperature.
- Chapter 3: How will predatory behaviour of southern calamari respond to modification of acclimation temperature?

- Chapter 4: Will squid be able to modify their thermal limits in response to changes in temperature conditions by phenotypic plasticity? And what molecular mechanisms are involved in thermal acclimation of squid?

1.4 Study species: Southern calamari (*Sepioteuthis australis*)

Southern calamari (*Sepioteuthis australis*) (Figure 1.4) are an inshore species belonging to the Loliginidae family. This species is endemic to the southern Australian and northern New Zealand coasts (Lu and Tait, 1983), and is commonly found in shallow waters (< 20 m deep) over seagrass meadows or in sandy habitats (Moltschaniwskyj and Steer, 2004). They are critical components of coastal ecosystems, not only as a primary consumer of crustaceans and fish, but also as a food source for numerous marine species. They are ecologically, economically and socially important species, targeted by both recreational and commercial fisheries. In the last two decades this species had been increasing in commercial significance across southern Australia, particularly in South Australia and Tasmania (Hunt et al., 2011).

Southern calamari live at least 280 days (Triantafillos and Adams, 2001) with most adults caught in the fisheries between 6 and 10 months of age (Pecl, 2004). They are a relatively large species, weighing up to 4 kg and measuring up to 54 cm in mantle length, and are sexually dimorphic (males being large than females) (Pecl, 2004). There are two genetically distinct groups and a hybrid of these is found across their distribution (Triantafillos and Adams, 2001).

Mature southern calamari usually aggregate in coastal waters to mate and spawn, with low levels of spawning taking place throughout the year (Moltschaniwskyj and Pecl, 2002). For example, in Tasmanian waters a peak in spawning occurs between October and January each year (Moltschaniwskyj and Pecl, 2002; Pecl et al., 2004a). Although southern calamari spawn multiple times within a season, the deposition of egg batches takes place within a one-year life

span with population resilience and annual recruitment still dependent upon successful reproduction in each generation, and the survival of eggs and hatchlings (Moltschaniwskyj and Pecl, 2002). Egg masses containing between 10 and 600 egg strands, are deposited and attached to the seagrass *Amphibolis antarctica* (Pecl, 2001; Moltschaniwskyj and Pecl, 2002; Moltschaniwskyj and Pecl, 2006). Each strand typically contains between 3 and 9 eggs (Moltschaniwskyj and Pecl, 2002) and the embryonic phase lasts 1–2 months (Steer et al., 2003; Pecl and Moltschaniwskyj, 2006).

In southern calamari, information regarding the effect of temperature on individual performance is minimal. A few studies have examined the embryogenesis, showing that developmental time and hatchling success is reduced at higher temperatures of acclimation (Steer et al., 2003). Highly plastic responses in growth rates have also been linked with seasonal environmental conditions in Tasmanian waters, where individuals that develop in periods of warming water grow faster than those that develop in colder periods (Pecl, 2004). The performance of southern calamari has not been previously studied in terms of the physiological or behavioural responses to changes in acclimation temperature, creating an important gap in our knowledge of how this ecologically, economically and socially important species may respond to environmental changes in southern Australia.



Figure 1.4. photo of southern calamari (*Sepioteuthis australis*) (credit to Jason Caruso)

Chapter 2: Effects of acclimation temperature on metabolic rate and temperature preferences in southern calamari

Abstract

Ocean warming effects on marine species are already being observed around the world. For species to survive in now fast-changing environments, they need to adapt or move, so understanding how organisms will respond to these changes is of vital importance. Studying rapidly responding species to changes in conditions, such as squid, allows us to examine the mechanistic links between ocean warming and biological responses in advance of wider scale impacts predicted for the future. Squid are fast-growing, short-lived, voracious predators that play an integral role in the structure and function of many marine ecosystems and are a basis of economically important fisheries around the world. While most previous studies have investigated individual physiological or behavioural performances of species in isolation, for us to more accurately understand species responses to climate change, a more holistic approach considering multiple measures of performance is needed. Here, I aim to understand how temperature affects metabolic physiology and behaviour preference in southern calamari (*Sepioteuthis australis*). Standard, routine and maximum metabolic rates, and excess post-exercise oxygen consumption were examined via intermittent-flow respirometry to identify the energetic requirements of squid at different thermal acclimation scenarios (13 °C, 16 °C, 19 °C, 22 °C and 25 °C). Our results indicate that temperature of acclimation influences squid metabolism with standard, routine and maximum metabolic rates increasing by 30–40% from 13 °C to 25 °C. A concurrent reduction of the aerobic scope at the higher temperature

treatments (22 °C and 25 °C) suggests that in southern calamari, physiological performance could be limited by increasing ocean temperatures above 22 °C. Furthermore, results here show that squid actively seek out physiologically favourable temperatures. Individuals at 25 °C always preferred temperatures cooler than their acclimation temperature ($T_{pref} = 22.5 \pm 1.7$ °C), opposite to that of individuals acclimated at cooler temperatures (13 °C) which preferred temperatures around two degrees warmer ($T_{pref} = 15.5 \pm 3$ °C). Therefore, southern calamari might be using behavioural thermoregulation as a mechanism of thermal refugia to alleviate the thermal stress produced by future ocean warming, moving to more beneficial environmental conditions – those which favour physiological capacity, and ultimately improve fitness and overall performance.

Key words: aerobic scope, cephalopods, climate change, metabolism, temperature, temperature preferences.

2.1 Introduction

Climate change has occurred repeatedly throughout the earth's history; however, recent rates of warming greatly exceed historical change (Blois et al., 2013). Currently, greenhouse gas emissions are on track for a rise in ocean temperatures between 2 °C and 4 °C by the end of the century (IPCC, 2021) as well as an increase in the frequency and intensity of short extreme events like marine heatwaves (Oliver et al., 2019). Environmental temperature is one of the most important factors influencing internal processes in ectotherms (Pörtner and Peck, 2010; Lienart et al., 2014), including on the physiological mechanisms that support different behavioural, phenological, demographic and distributional responses (Pörtner and Farrell, 2008; Duncan et al., 2019). Long-term effects of increases in water temperature on marine species include changes in body size (Daufresne et al., 2009; Audzijonyte et al., 2020), reproduction (López-Galindo et al., 2019) and redistribution (Pinsky et al., 2013; Poloczanska et al., 2013),

altering ecosystem functioning, composition and stability – ultimately affecting the wellbeing of human communities dependent on marine resources (Vergés et al., 2014; Pecl et al., 2017). Therefore, understanding how species might respond to future warming conditions is a crucial challenge of our time.

Physiological and behavioural responses to temperature can provide important insights into species sensitivity or vulnerability to climate change. As temperature changes, individuals are forced to adjust their physiological and/or behavioural traits (for example, metabolism, feeding intake and movement) to handle the changing conditions, either enhancing performance or limiting the expression of it (Killen et al., 2013). While increasing temperature can have some positive effects, for example, increased growth rate, this only occurs within a species optimal range, beyond which important processes are compromised due to the reduction of aerobic capacity (Pörtner and Knust, 2007), protein denaturation and enzyme inhibition, which could potentially gradually reduce long-term fitness (McDonnell and Chapman, 2015). However, mobile ectotherms often have the behavioural capacity to avoid unfavourable temperatures by seeking out and moving into environments with less challenging temperatures where they can more optimally regulate their physiological functions and maximise fitness (Careau et al., 2008; Breau et al., 2011; Khan and Herbert, 2012; Habary et al., 2016). Previous studies of aquatic organisms have mostly explored the effect of temperature in physiological (Nilsson et al., 2009; Rummer et al., 2014) or behavioural traits in isolation (Freitas et al., 2015; Nay et al., 2015) of species. However, individuals' physiology and behaviour, as well as life history, are often correlated, and organismal responses to stressors may lead to coordinated changes (Debecker and Stoks, 2018). The complex relationships between physiological and behavioural performances and environmental stressors such as temperature remain unclear, as does the question of whether environmental changes will lead to uniform or different responses to the

same challenge. For instance, the pace-of-life syndrome (POLS) hypothesis is being developed as a framework for understanding the correlation between traits – integrating life history with behavioural and physiological traits along a fast–slow continuum (Careau et al., 2008; Debecker and Stoks, 2018). However, despite the demonstrated strong effect temperature has on most of the performances associated with the POLS and the importance of global warming, the influence of temperature has only recently been incorporated into the framework (Goulet et al., 2017; Debecker and Stoks, 2018; Goulet et al., 2018) underlining the necessity of a more integrative approach. Consequently, to fully understand how organisms will respond to ocean warming and possible adaptations, the integration of physiological and behavioural measures is essential.

Physiological processes such as metabolism play an important role in daily activities, reproductive success and ultimately species survivorship (O'Brien et al., 2018). Consequently, understanding the energetic requirements under stressful conditions could be important in predicting performances under future warming conditions. Aerobic scope (AS) has been commonly used as a trait to measure animal performance and tolerance of temperature change, examining the aerobic capacity to use the energy available to support physiological processes above the basal cost for maintenance (Clark et al., 2013). The oxygen- and capacity-limited thermal tolerance hypothesis (OCLTT) suggests that the capacity to supply oxygen to the tissues is optimal between specific thermal limits (pejus temperatures), after which oxygen transport is limited, leading to a decrease in aerobic scope and animal performance (Pörtner and Peck, 2010; Pörtner et al., 2017). The hypothesis assumes that a species' thermal optimum (*Topt*) will coincide with the temperature at which aerobic scope is maximised (Holt and Jørgensen, 2015). However, recent studies on several marine species have conversely reported that aerobic scope does not necessarily predict organism responses or processes at high temperatures, (Clark et al., 2013; Ern et al., 2014; Norin et al., 2014) and that individual

processes and overall fitness respond differently to temperature (Holt and Jørgensen, 2015; Fitzgibbon et al., 2017). Even if aerobic metabolism is important in daily activities and overall fitness, organisms are complex, and they employ different mechanisms to respond and adapt to ocean warming and these are often interdependent, for example through behavioural thermoregulation or acclimation via phenotypic plasticity.

Physiological capacities and behavioural thermoregulation seem to be directly correlated (Habary et al., 2016), where species potentially prefer to move to temperatures (T_{pref}) closer to their metabolic thermal optima (T_{opt}) to maximise overall performance. The distribution limits of organisms, from local to global scales, is related to their physiological temperature tolerance and/or behavioural thermal preferences (Khan and Herbert, 2012) (Killen, 2014). The preferred temperature (T_{pref}) is defined as the temperature range most commonly selected by ectotherms, with temperatures outside of this range potentially disadvantageous for individuals and possibly decreasing overall fitness (Khan and Herbert, 2012). Their capacity to adjust to changing temperatures by behavioural modifications may provide a safety mechanism for ectotherms under environmental stress (Breau et al., 2011). Additionally, individual preferences for a particular temperature range could vary depending on age, weight, food availability, season, water quality or light, and further influenced by biotic interactions such as competition or predation risk (Noyola et al., 2013; Killen, 2014). Thus, behavioural thermoregulation could offer important insights into species distribution limits and variation in habitat uses, as well as in identifying species traits that are informative for understanding species responses (such as migration patterns and range shifts) in a changing world.

The waters of south-eastern Australia are among the most rapidly warming regions in the world and may be considered as a natural laboratory for climate change impacts and adaptation measures in marine ecosystems (Hobday and Pecl, 2014). The waters of the Tasman Sea

between New Zealand and south-eastern Australia have been warming 3–4 times the global average as a result of the intensification and poleward extension of the East Australian Current (EAC) (Ridgway, 2007; Oliver et al., 2015), with a warming rate of 0.2 °C decade⁻¹ in eastern Tasmanian waters (Shears and Bowen, 2017). In addition to the long-term trends, marine heatwaves have been recorded more frequently and intensely in this part of the world (Oliver et al., 2017). These extreme climatic events can have severe impacts, including effects on biodiversity, fisheries and aquaculture, bringing potentially significant ecological changes and socioeconomic consequences. In Tasmania, modification of coastal ecosystems associated with longer-term climate trends have been identified – for example, kelp deforestation or changes in biodiversity as many species have been recorded shifting their distribution range further south (Johnson et al., 2011; Last et al., 2011; Pecl et al., 2019). Nevertheless, we have little understanding of how species, within their existing range, are responding to warming waters now and into the future.

Sepioteuthis australis (southern calamari) are a large loliginid squid, endemic to southern Australia and northern New Zealand. Throughout these waters, this species is targeted by both commercial and recreational fisheries. Along Tasmanian waters, southern calamari experience average summer temperatures of 19 °C with a maximum of 21 °C. Commonly, this species is found in shallow waters (< 20 m deep) over seagrass meadows or sandy habitats (Moltschaniwskyj and Steer, 2004) with a lifespan of approximately one year. Cephalopod studies examining the effect of global warming have been under-represented in the literature (Higgins et al., 2012), resulting in limited information on how temperature is likely to influence the mechanisms underpinning their physiological performance, thermal tolerances and behavioural preferences. For instance, neither the metabolism nor the behavioural preferences of southern calamari relative to temperature has been previously investigated. Their extremely fast growth rates and rapid turnover at population level means that they can respond quickly to

environmental changes and are likely to play an important role in the response of local marine ecosystems to climate change (Pecl and Jackson, 2008). Consequently, understanding how cephalopods respond to temperature is essential, as this group plays an important role in the stability of ecosystems and are economically important as high value fisheries around the world (Wood and O'Dor, 2000; Pecl, 2004). Squid are active, muscular, and negatively buoyant species with high oxygen consumption rates and low blood oxygen carrying capacity (O'Dor and Webber, 1986; Oellermann et al., 2012). In accordance with the OCLTT, previous research on squid and cuttlefish suggests that oxygen availability to tissues under ocean warming would limit performance (Pörtner and Zielinski, 1998; Pörtner, 2002; Melzner et al., 2006). A direct relationship between preferred and acclimated temperature was observed in early life history stages of *Octopus maya* (Noyola et al., 2013). However, the effect of temperature on the behavioural thermal preferences in adult stages of cephalopods has not been previously investigated, creating an important gap in our understanding of how a highly mobile and responsive key species of coastal ecosystems around the world like squid may respond to thermal challenges. Neither the metabolism nor the behavioural preferences of southern calamari relative to temperature has been previously investigated.

To fill this gap, I have three specific research aims: 1) to examine the effect of temperatures on southern calamari metabolism by measuring oxygen consumption rates to identify the squid thermal optimum (T_{opt}); 2) to determine if acclimation temperature influences squid behavioural thermoregulation and their thermal preferences; and 3) finally, to examine if there is a link between metabolism and thermal preferences in southern calamari, exploring whether the acclimation temperature induced shifts in the T_{pref} corresponding with changes in aerobic metabolism. Understanding the relative roles of acclimation, energetic requirements and behavioural thermoregulation is key to predicting how warming waters will ultimately impact fitness and distribution of individual species (Habary et al., 2016). Those could be used to

develop more appropriate management and climate adaptation strategies, leading us to healthier marine ecosystems, fisheries and aquaculture, now and into the future.

2.2 Materials and methods

2.2.1 Squid collection and holding conditions

Southern calamari were collected in the nearshore environment off the south-east coast of Tasmania (43°00'27.0"S 147°19'32.5"E) using rod and line with a squid jig. Collection of animals occurred between December 2018 and May 2019, when the seawater temperature ranged between 14 °C and 19 °C. Ten squid were captured per trip with 100 individuals caught in total. Mantle length ranged between 110 mm and 247 mm with a mean weight of 244 g ± 91.67 g. The squid were then transferred to the Institute for Marine and Antarctic Studies (IMAS) research facilities at the University of Tasmania for temperature acclimation treatment and the experimental phase. Two circular tanks (2.2 m diameter and 0.8 m high) connected to a recirculated water system were used to hold 20 individuals per treatment (10 individuals per tank). The holding system contained multiples stages of filtration (drum filter, foam fractionator, biofilter, and UV treatment) to keep the quality of water suitable for squid. Water flow into the holding tanks was maintained at 30 ± 2 L/h and conditions were kept stable at appropriate levels of: pH 7.7–8.2; salinity 34–36‰; NO₃<10 mg L⁻¹; NO₂<0.1 mg L⁻¹; NH₄<0.25 mg L⁻¹; and oxygen saturation <110%. The system was connected to the heat unit (Aqua hort heat and chill heat pump LWH030SC) to keep the temperature treatment stable during the acclimation phase. A photoperiod of 13 hr/11 hr light–dark cycle was established, corresponding to conditions of natural light during the summer months, and included a programmed 30 minutes of sunset and 30 minutes of sunrise. Squid are strict carnivores

requiring live prey (Forsythe et al., 2001), so were fed a diet of a live Australian salmon (*Arripis trutta*) or garfish (*Hyporhamphus melanochir*) (body size > 150 mm) per individual and day.

During the first 12 hours the squid were kept at the same temperature as at collection, avoiding thermal stress to the animals. The temperature was then increased or decreased by 1 °C every 12 hours until the treatment temperature was reached. Five different temperature treatments were conducted (13 °C, 16 °C, 19 °C, 22 °C and 25 °C), representing current and possible future Tasmanian water conditions. For each treatment, 20 squid were acclimated for 7 days at the required temperature treatment before any experimental procedures were undertaken.

2.2.2 Experimental trial

On the days of the experiments, three squid were randomly removed from their holding tank at the same time (1:30 pm). Two were transferred to the respirometry chambers where the metabolic activity experiment was undertaken. The other squid was placed in the shuttle box tank for the behavioural thermoregulation experiments. The metabolic activity and thermal preferences experiments ran simultaneously (Figure 2.1). Individuals were also fasted for a 24-hour period prior to introduction into the chambers, to reduce any possible effects of specific dynamic action (SDA) (Clark et al., 2013).

2.2.2.1 Metabolic rates measurements

Intermittent-flow respirometry, similar to that described by Jensen et al. (2013) was used to measure oxygen consumption rates ($\dot{M}O_2$). Specifically, standard (SMR), routine (RMR), maximum (MMR) rates, and aerobic scope (AS) were measured. SMR represents the minimum metabolic cost of maintaining biological functions; RMR is defined as the cost of biological functions of maintenance, including the cost of spontaneous activity and maintenance of posture and equilibrium; and MMR is the highest rate of aerobic energy conversion. Excess

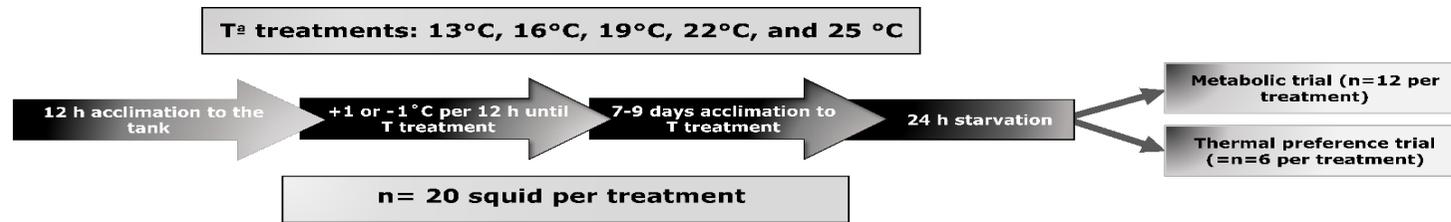
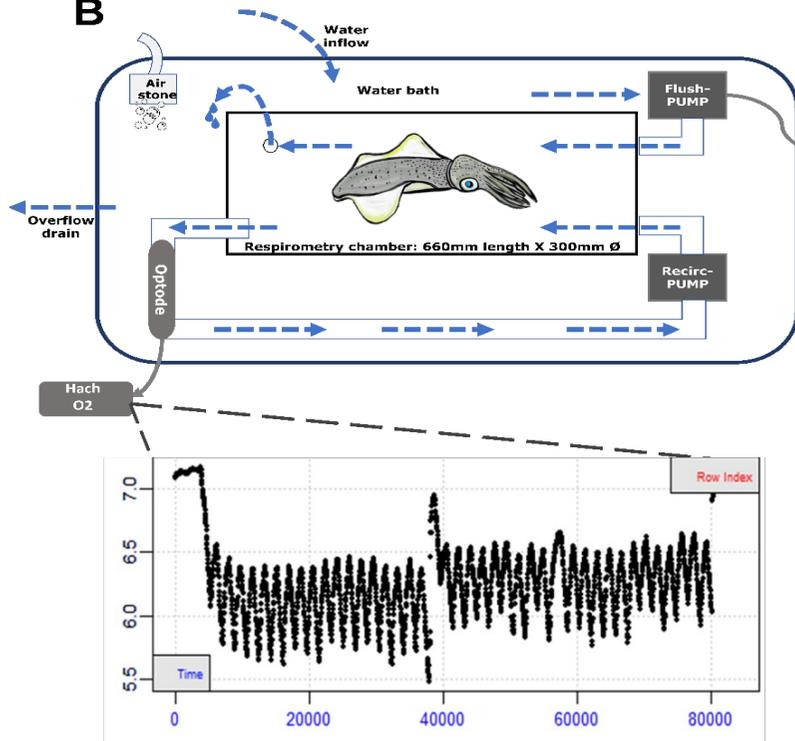
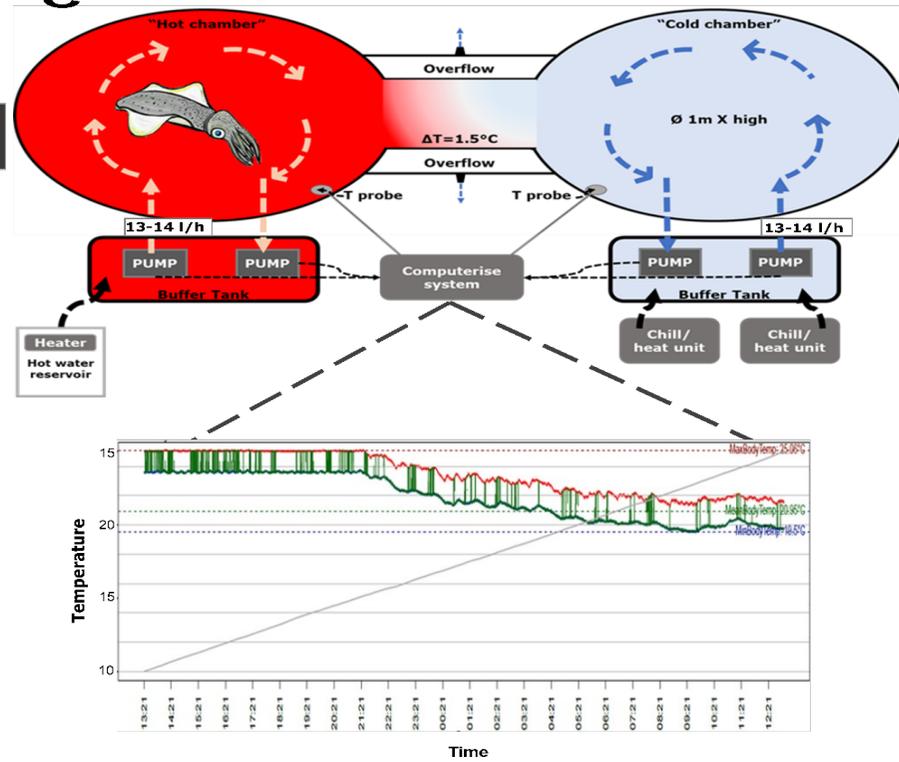
post-exercise oxygen consumption (EPOC), which represent the amount of oxygen required to restore tissue and cellular homeostasis after exhaustive exercise and can be used as an indicator of anaerobic capacity (Fitzgibbon et al., 2014b; Bouyoucos et al., 2020), was also calculated as well as the time that the squid would need to recovery from anaerobic exercise and return to routine metabolism after exhaustive exercise.

The intermittent-flow respirometry system (Figure 2.1) consisted of a 46.6 litre horizontal circular perspex acrylic chamber (0.66 m length X 0.3 m diameter) submersed in a temperature maintenance sump, receiving constant water supply at the flow rate of 15–20 l/h. The temperature of the bath was controlled (the same as the treatment temperature) by a heat/chill unit (Aquahort heat pump VL130R) and dissolved oxygen maintained at 100% saturation by an air stone. Two identical respirometry chambers were used to run metabolic activity experiments. Each chamber was placed in a separate temperature maintenance sump and isolated from external visual disturbance with curtains that surrounded the chamber, with experiments run under complete darkness to minimise any activity or stress to the squid.

Two submersible aquarium pumps (Quiet One 1200, Aquasonic, NSW, Australia) were connected to each chamber, ensuring ensured water circulation at a rate of 1.0 exchange min^{-1} within the chambers and passed the O_2 probe (recirculation pump) and the other (flush pump) to intermittently flush the chambers with new water at a rate of 1.0 exchange min^{-1} at 15 min intervals. Luminescent dissolved oxygen optodes (Hach LDO, HQ40D, Hach Company, USA) were sealed within the recirculation pump line and recorded oxygen level of the chambers every 30 sec. The respirometry chambers were considered functionally closed when the flushing pump was off, creating a 15 min closed (measurement) and a 15 min flush cycle. The O_2 content in the chamber was never less than 80% as per (Svendsen et al., 2016). Both the

recirculating and flush system were connected with the chambers by non-permeable tygon tubing.

Once in the chambers, individual squid were allowed a one-hour acclimation period, then SMR and RMR were recorded for eight hours. Squid were then removed from the respirometry chamber at the beginning of a flushing cycle and placed into a circular tank (1.0 m diameter and 0.6 m high), where the exhaustion protocol was conducted. The exhaustion protocol involved encouraging individual squid to swim by gently prodding it with a net until it could not maintain equilibrium, defined as the point where squid failed to attempt to escape, resting on the bottom of the tank for a minimum of five seconds (Norin et al., 2014), upon which the individual was returned to the respirometry chamber and O₂ was measured again for the next 12 hours to obtain MMR and EPOC parameters. Time between the end of the exhaustion protocol and closure of the chambers was always less than 20 s, including approximately 3 seconds of air exposure. Following removal of the squid from the chamber at the end of the respiratory trial, a measure of background respiration of the chamber was recorded for one hour. Once the respirometry trial was completed, squid volume, weight and mantle length were recorded.

A**B****C**

Oxygen consumption rates ($\dot{M}O_2$) were expressed in $\text{mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$ and were calculated as $\dot{M}O_2 = V_R W \frac{dCO_2}{dt}$, where V_R is the effective respirometry volume (l) of water (calculated as total respirometry volume minus the squid volume); W is the squid body mass (kg), and $\frac{dCO_2}{dt}$ is the slope of the linear decrease in O_2 content during the closed cycle. To calculate $\frac{dCO_2}{dt}$, linear regressions were applied to the change in oxygen concentration during the 15 min closed cycle and measures with a $R^2 < 0.95$ were discarded from the analysis. $\dot{M}O_2$ rates were calculated using the respR package (Harianto et al., 2019). Squid metabolic rates (SMR, RMR, MMR, aerobic scope and EPOC) were calculated similarly to that described by Fitzgibbon et al. (2014) SMR was calculated as the mean of the lowest 12.5% $\dot{M}O_2$ (2 measures) and RMR as the overall mean of the 8-hour periods. MMR was determined as the maximum $\dot{M}O_2$ during the 12-hour periods after the exhaustion protocol and aerobic scope (AS) was estimated as the difference between MMR and SMR. Recovery time was calculated when an individual $\dot{M}O_2$ after exhaustive exercise came back to within one standard error of the RMR, and EPOC was calculated as the area under the curve resulting between the MMR and the time that need to recovery. Each temperature treatment was designed to have 12 squid, but due to technical problems and some mortalities, numbers ranged between 6 and 12 per treatment (specific numbers can be found in Table 2.1).

2.2.2.2 Thermal preference trial

A custom-made shuttle box system was used to examine temperature preferences (T_{pref}) in squid randomly allocated to five temperature treatments: 13 °C, 16 °C, 19 °C, 22 °C and 25 °C ($n = 5, 5, 6, 6$ and 6 individuals). The experimental shuttle box (Figure 2.1) included two circular chambers (1.0 m diameter and 0.5 m high) with a passage (0.2 m long and 0.15 m wide) between, which allowed the animals to move freely between the two compartments. One of the

chambers was considered 'warm' and the other was considered 'cold' with an average temperature difference of 1.5 °C between them. A custom-made computerised system was used to track the individual as well as control the temperature. This system controlled the temperature within and between chambers pumping (with a submersible water pump GRECH Ctp-5800) either cold or hot water at a flow rate of 13–14 l/h into the colder or warmer compartment depending on the position of the individual. A buffer tank connected with the two heater/chiller units (Teco TK2000 chiller) supplied water to the colder compartment, and a buffer tank attached to a water reservoir with two immersion heaters (Instra Elements and Engineering, Caringbah, Australia) supplied water to the warmer chamber. A camera on top of the shuttle box always allowed tracking of the individual. In each compartment a probe (temperature probe for TEMP-4 and TMP-Reg, Loligo Systems), with a measurement range of –40 °C to 180 °C and accuracy of ± 0.3 °C, was continuously monitoring the temperature and red LED light strips were distributed uniformly under the whole shuttle box to facilitate the squid tracking. The box was completely isolated by curtains to avoid any external stimulus to the individuals.

Two experimental modes were used – static and dynamic. In static mode the temperature in the chambers was constant allowing the individuals to familiarise themselves with the set-up. In dynamic mode, the temperature in the chambers was changed depending on the position of the individual, maintaining the 1.5 °C of difference between chambers. Maximum and minimum temperature limits (28 °C and 7 °C) were set to ensure the survival of the squid. For each trial, a randomly selected single squid was placed in one of the chambers in static mode for 8 hours, followed by 12 hours in dynamic mode. The temperature in one of the compartments was set to be the same as the treatment at the beginning of the experiment.

T_{pref} was defined as the average temperature at which individuals spent most time during the 12-hour dynamic period. The upper and lower temperature avoidances, which is defined as maximum or minimum temperatures that squid faced before moving to the other compartment were calculated as the maximum/minimum temperature experienced by the squid in the chamber during the dynamic phase. The temperature range selected was estimated as the difference between the minimum and maximum that the squid achieved (Gervais et al., 2018).

2.2.3 Data analysis

Thermal curves were built to examine the metabolic performance and behavioural preferences of southern calamari. Generalised linear models (GLMs) with a gaussian distribution were used to explore the effect of temperature treatments (13 °C, 16 °C , 19 °C , 22 °C and 25 °C) on SMR, RMR, MMR and AS. Generalised linear mixed models (GLMMs) with a gaussian distribution were applied to test the effect of the temperature in EPOC and recovery time performance with the capture/holding squid group as a random effect. Preliminary analysis of the metabolism parameter included temperature treatment, weight, and the respirometry chambers as factors, as well as the capture/holding group as a random effect. The respirometry chamber was eliminated from the analysis as there was not a significant effect ($P > 0.05$) on any of the performance traits examined. In the case of SMR, RMR, MMR and AS, the random effect also showed no influence, so was dropped from the model, allowing for simpler GLMs to be fitted. GLMMs were also used to examine the acclimation temperature effect on squid T_{pref} , maximum/minimum avoidances and temperature range, where the capture/holding group was used as a random effect.

In all the cases, normality and homogeneity of the residuals were verified by inspection of the residual-fit plots, and log transformation of the variable measure was used where needed (for SMR). Akaike information criterion (AIC) values were used to establish the best fit of the

model for each measure of performance (see supplementary material, Appendix 1). Statistical analyses were performed with the statistical software R version 3.6.1 (2019) and GLMM models were fitted using the lme4 package (Bates et al., 2015).

Table 2.1. Result of the performances measures in southern calamari acclimated at different temperatures (13, 16, 19, 22 and 25 °C), values (\pm SD) of the metabolic rates (SMR, RMR, MMR, AS, EPOC and recovery time), and behavioural performance (preferred temperatures, maximum/minimum avoidances, and range) with number of individuals (n) per temperature treatments as indicated.

Measure	13°C		16 °C		19 °C		22 °C		25 °C	
	n	mean \pm sd	n	mean \pm sd	n	mean \pm sd	n	mean \pm sd	n	mean \pm sd
SMR (mg O ₂ /h/kg)	5	270.2 \pm 101.4	12	265.1 \pm 43.3	10	330.2 \pm 84.2	10	364.5 \pm 64.7	12	395.9 \pm 90.8
RMR (mg O ₂ /h/kg)	5	312.2 \pm 112.8	12	324.3 \pm 37.1	10	421 \pm 103.2	10	432.3 \pm 56.1	12	471.1 \pm 91.7
MMR (mg O ₂ /h/kg)	4	342.4 \pm 130.7	11	437.5 \pm 44.4	10	523.3 \pm 120.2	8	547.5 \pm 50.6	7	565.5 \pm 48.2
AS (mg O ₂ /h/kg)	4	82.2 \pm 32.2	11	182.3 \pm 62.5	10	193.2 \pm 53.4	8	189.6 \pm 65.6	7	187 \pm 57.2
EPOC (mg O ₂ /kg)	4	44.2 \pm 43.02	11	402.2 \pm 549.4	7	250.8 \pm 431.5	7	412.6 \pm 508.9	6	120.6 \pm 126.
Recovery time (h)	4	0.9 \pm 0.5	11	1.6 \pm 1.2	7	1.1 \pm 1.1	7	1.6 \pm 1.5	6	0.7 \pm 0.5
T _{pref} (°C)	5	15.5 \pm 3	5	16.9 \pm 2	6	19.4 \pm 2	6	21.7 \pm 0.6	6	22.5 \pm 1.7
T _{max} (°C)	5	18.5 \pm 3	5	18.7 \pm 2	6	20.8 \pm 1.5	6	23 \pm 0.6	6	25.1 \pm 0.4
T _{min} (°C)	5	13.5 \pm 1.3	5	16 \pm 1.4	6	17.6 \pm 1.1	6	20.2 \pm 0.3	6	21.2 \pm 1.6
Range (°C)	5	5 \pm 1.7	5	2.8 \pm 0.7	6	3.1 \pm 1.5	6	2.6 \pm 0.5	6	3.9 \pm 1.3

2.3 Results

2.3.1 Metabolic activity

The model with the best fit for the relationship between temperature and SMR and RMR was the exponential (log-log model), whereas a quadratic regression with log-transformed variables was applied to examine MMR, AS, EPOC and recovery time (Table 2). Temperature had a significant effect on the SMR ($\chi^2 = 25.02$; $df = 1$; $P < 0.001$), RMR ($\chi^2 = 27.52$; $df = 1$; $P < 0.001$), MMR ($\chi^2 = 30.51$; $df = 2$; $P < 0.001$) and AS ($\chi^2 = 11.55$; $df = 2$; $P < 0.001$). However, EPOC ($\chi^2 = 2.36$; $df = 2$; $P = 0.31$) and recovery times ($\chi^2 = 2.57$; $df = 2$; $P = 0.27$) did not show significant differences between different treatments. SMR and RMR increased exponentially with temperature with an increment in oxygen consumption of 31% and 33% respectively

between the 13 °C and 25 °C treatments (Figure 2.2). MMR also increased by around 37% to 40% with temperatures at 22 °C and 25 °C compared with lower treatments (Figure 2.2). Aerobic scope showed an increase until reaching the maximum oxygen consumption of $193.17 \pm 53.54 \text{ mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$ at treatment 19 °C, following a decrease at 22 °C and 25 °C (Figure 2.2). These results suggested that the thermal optimum for aerobic scope in southern calamari would be between 19 °C and 22 °C. Recovery time in most of the individuals across temperature treatments was less than 2 hours, with only 11% of squid taking more than 2 hours to recover to routine metabolic rate levels correspondingly with larger values (Figure 2.3). EPOC results show a high individual variation in the same acclimation treatments (Figure 2.3), yet the higher values found were in temperatures 16 °C, 19 °C and 22 °C (Table 2.1). Mortality varied among temperature treatments, reaching the maximum of 36.6% (4 individuals) at the highest thermal scenario (25 °C), with 20% recorded at 22 °C and no individuals died at 19 °C. At low temperatures (13 °C and 16 °C), the mortality was less than 10% with only one squid death per treatment. All the squid mortality was recorded during the metabolic trial after the exhaustion protocol.

2.2.2 Behavioural thermal preferences

The relationship between temperature and T_{pref} , and maximum/minimum avoidances were fit by exponential curves. Acclimation temperature had a significant effect on the squid T_{pref} ($\chi^2 = 38.6$; $df = 1$; $P < 0.001$). Individuals acclimated to 16 °C, 19 °C and 22 °C preferred similar temperatures to their acclimation temperature ($16.9 \pm 2 \text{ °C}$, $19.4 \pm 2 \text{ °C}$ and $21.7 \pm 0.6 \text{ °C}$ respectively), whereas squid acclimated at 13 °C preferred warmer waters ($T_{pref} = 15 \pm 3.1 \text{ °C}$) and the opposite occurred with individuals at 25 °C choosing temperatures between 2 °C and 3 °C cooler ($T_{pref} = 22.5 \pm 1.7 \text{ °C}$) (Figure 2.4 and Table 2.1). As temperature in the shuttle box in the dynamic phase was adjusted relative to the squid's position, results showed a significant

difference ($\chi^2 = 6.85$; $df = 1$; $P < 0.001$) in squid temperature preferences between chamber modes (static vs dynamic) (appendix 1, Figure 1.1.). Acclimation temperature also had a significant effect in the temperature maxima and minimum avoidance for the different treatments ($\chi^2 = 25.7$; $df = 1$; $P < 0.001$ and $\chi^2 = 131.76$ $df = 1$; $P < 0.001$ respectively). However, there was no differences between the selected temperature range ($\chi^2 = 4.17$; $df = 2$; $P = 0.12$). Despite the non-significant effect, it appeared that the squid that acclimated to the 13 °C or 25 °C treatments experienced a wider range of temperatures compared with individuals acclimated at 16 °C, 19 °C or 22 °C (Figure 2.5).

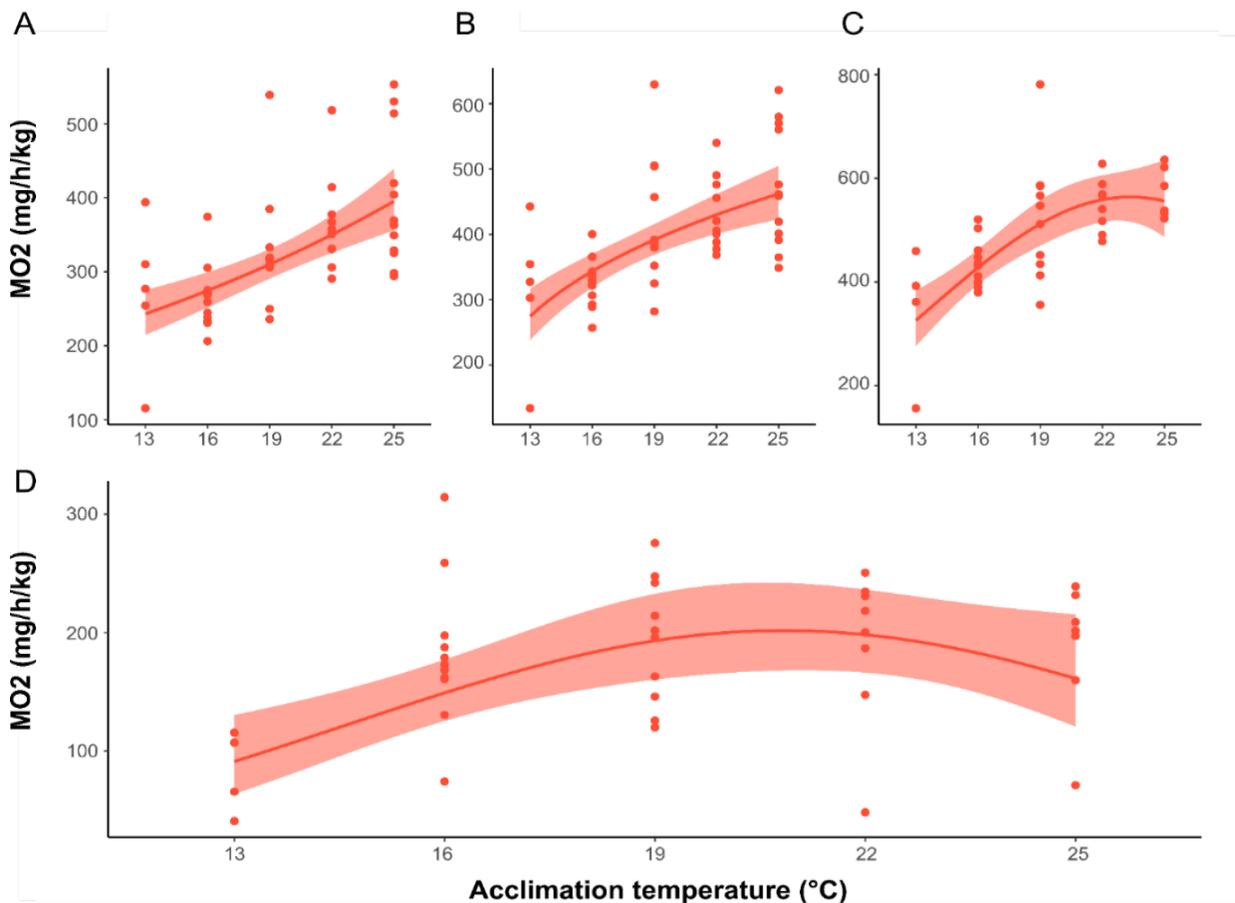


Figure 2.2. Metabolic rates (mg O₂/h/kg) of southern calamari measured at different temperatures of acclimation (13, 16, 19, 22 and 25 °C). (A) Standard metabolic rate (SMR). (B) Routine metabolic rate (RMR). (C) Maximum metabolic rate (MMR). (D) Aerobic scope (AS). Thick line represents the expected value of the GLM model for the variable measured. The band corresponds to the 95% confidence interval. Individual values are represented by red points.

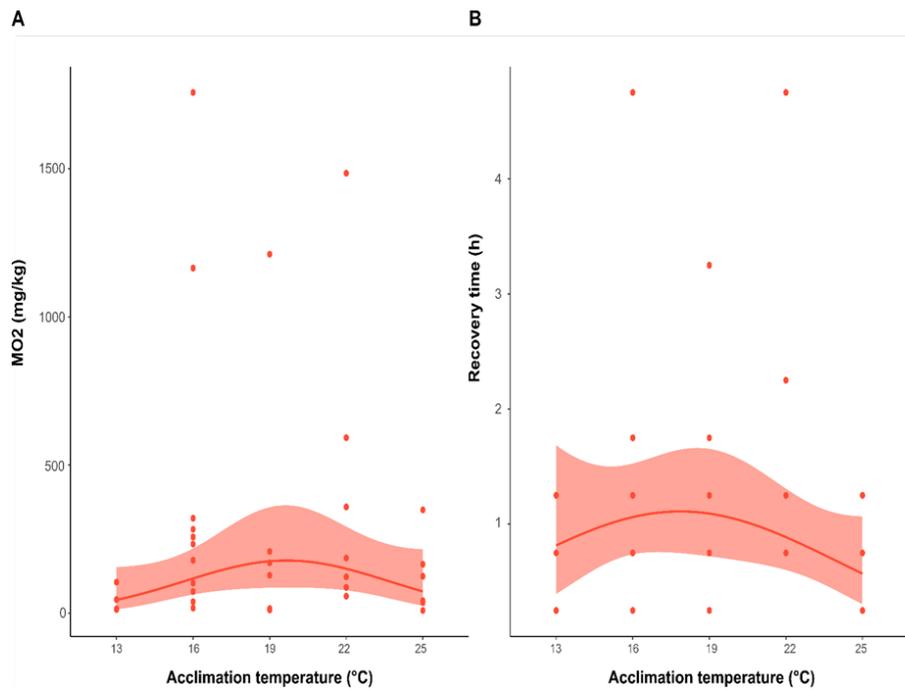


Figure 2.3. GLMMs model of the EPOC and recovery times of southern calamari at different temperatures of acclimation (13, 16, 19, 22 and 25 °C). (A) Excess post-exercise oxygen consumption (EPOC) (mg O₂/kg). (B) Squid recovery time (hours) after exhaustion protocol. Individual's measure indicated by the red points, where the value of the variable responses is the red line, and the confident interval is represented by the band.

2.4 Discussion

An ectotherm's capacity to survive is directly related to environmental temperature, so understanding species thermal performance is of vital importance to predict population responses to future climate change (Sinclair et al., 2016). This study examines, for the first time, the relationship between metabolism (T_{opt}) and behavioural performance (T_{pref}) in adult squid acclimated to different thermal scenarios. Results indicate that southern calamari display physiological and behavioural responses depending on the acclimation temperature, where their metabolism seems to lack the capacity to acclimate to temperature, yet individuals chose to actively move, regulating their environmental temperature. This movement could help to alleviate the metabolic constraints produced by thermal stress, and could be potentially used as a faster strategy until adaptation might occur (Habary et al., 2016).

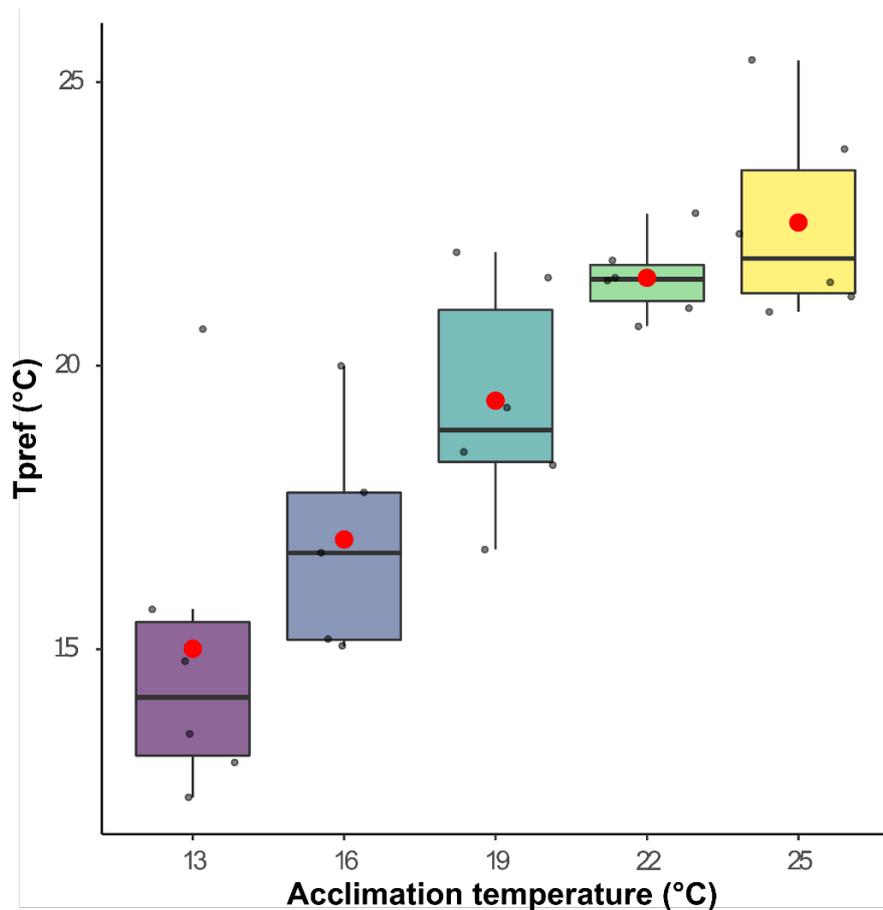


Figure 2.4. The behavioural thermal preference (T_{pref}) of southern calamari acclimated to 13, 16, 19, 22 and 25 °C, boxplots representing T_{pref} where the points indicate individual values and boxes represent first and third quartiles. Within each box, median is represented by the solid line and mean by red point.

Oxygen consumption rates (SMR, RMR and MMR) in southern calamari increased exponentially with rising acclimation temperature, reaching the highest point at 25 °C. This suggested that squid will need a higher energetic requirement (~ 30%) to support basal and maintenance processes with the increasing ocean temperatures projected for south-eastern Australia. In contrast, AS decreased above 22 °C, implying the amount of aerobic energy available for other biological processes such as growth or reproduction would decrease at increasing water temperatures, which may have detrimental effects on different performances and survival (Pörtner and Peck, 2010). Similar results were found for the Humboldt squid (*Dosidicus gigas*) where routine metabolic rates increased with temperature from 8.9 to 49.85

$\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ between 10 °C and 25 °C (Rosa and Seibel, 2010). At early life stages of cephalopods, for example in *Loligo vulgaris*, *Sepia officinalis* (Pimentel et al., 2012), or *Octopus vulgaris* (Repolho et al., 2014), oxygen consumption rates (RMR) were also found to increase with water temperature, meanwhile survival and growth decreased.

Metabolic results in the current study are in accordance with the hypothesis that at high temperatures, performance of ectotherms would be limited as oxygen delivery to the tissues would not be enough to meet the increasing oxygen demands associated with a higher metabolism (Pörtner and Knust, 2007; Pörtner and Farrell, 2008). Recorded measurements of aerobic scope of southern calamari here indicate that the physiological thermal optimum (T_{opt}) for the species will be found between 19 °C and 22 °C. The OCLTT assumes that this thermal optimum coincided with the ideal temperature where physiological responses are maximised, and that performance and long-term fitness would be reduced at temperatures beyond this limit (Pörtner and Knust, 2007). Coincidentally, the squid that acclimated to high temperatures (22 °C and 25 °C) in the present study also suffered an increase in mortality (20% and 36% respectively) compared with lower treatments which were less than 10% at both 13 °C and 16 °C. Meanwhile no mortality was recorded at treatment 19 °C. Not surprisingly, all the mortalities occurred during or after the exhaustion protocol, where squid used jet propulsion. Squid use this locomotion mode for high-speed responses for escape. However, jet propulsion is well known to be an inefficient locomotory system requiring high levels of energy from the squid's mantle muscle (O'Dor and Webber, 1986; O'dor, 1988). These results show that in southern calamari, energy requirements are likely to increase as water temperatures rise and it seems that the high energy cost of jet propulsion at those temperatures could be link with the increase in squid mortality. This could have potentially significant consequences for squid survivorship as individuals rely on jet propulsion as defence and predator responses (Bartol et al., 2001).

EPOC is used to measure recovery periods after anaerobic activity and previous studies of marine species have reported EPOC to increase with temperature (Fitzgibbon et al., 2014a; Briceño et al., 2020). However, in this study EPOC, along with recovery time, did not show a relationship with temperature ($p > 0.05$). Most of the squid among treatments took less than 2 hours to recover from anaerobic activity, with only 4 squid taking longer than 2 hours to recover, corresponding with individuals at 16 °C, 19 °C and 22 °C. Higher aerobic scope is linked with faster recovery after exhaustive exercise in fish (Killen, 2014), as individuals use a fraction of their aerobic scope until they recover completely from anaerobic exercise (Killen et al., 2015). In this study, however, it seems that longer recovery periods are associated with higher aerobic scope, as found in individuals at 19 °C and 22 °C. Those individuals potentially may have a higher anaerobic capacity for sustain swimming and then took longer to recovery from exhaustive exercise due to the use of more glycogen or production of lactate. This could potentially be associated with the high inter-individual variation (phenotypic plasticity) found in this species. Also, the increase in mortality at higher temperatures suggested that anaerobic capacity of squid would be likely to decrease, subsequently reducing squid performance after exhaustive exercise. The overall decrease in performance at higher temperatures may have important implications for southern calamari populations in Tasmanian waters, as current summer temperatures already reach 21 °C, suggesting that with further warming southern calamari will be exposed to ambient temperatures beyond their metabolic thermal tolerance.

Here, high inter-individual variation was found in metabolic rates across treatments. This variation is commonly found in a wide range of taxa and it could be linked to the animal's behaviour and personality, but the ecological relevance of these relationships is not fully understood (Killen et al., 2012). A portion of individual variation in metabolism could be explained by some individuals being more reactive or bold than others and as a result, their energy expenditure might be different, which could be associated with different metabolic

performances. RMR has been linked with fitness and behavioural fitness-related traits (Burton et al., 2011). Southern calamari have been identified as an extremely plastic species, with high levels of individual variations across different life traits (such as size or condition) and population level (Pecl et al., 2004a). However, our understanding of the relationship between energetic traits and personality in cephalopods is still limited and further studies linking physiological inter-individual variation to personality and behavioural traits would be important to an understanding of the variation in this highly plastic species.

Behavioural thermoregulation has been identified in other marine ectotherms, such as fish and invertebrates, as a safety mechanism used to alleviate the impact of temperature on individuals by their moving to more favourable conditions (Breau et al., 2011; Zúñiga et al., 2013; Nay et al., 2015). Here, southern calamari reveal separate responses to preferred temperatures across treatments, suggesting their ability to select their thermal environments. Individuals at 25 °C preferred temperatures 2–4 °C lower than their acclimation temperature, corresponding with temperatures more beneficial for their physiological performance as indicated by AS (19–22 °C). The lowest AS ($82.25 \pm 35.2 \text{ mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$) exhibited by southern calamari was found in individuals at 13° C which also moved to waters 2 °C above their temperature treatment. Individuals that acclimated to 16 °C, 19 °C and 22 °C showed a preference for temperatures closer to their acclimation temperature, corresponding with the temperature ranges at their natural habitats in Tasmanian waters during the summer months. This could indicate that southern calamari have potentially developed a preference for this temperature range, likely linked to their thermal tolerance, to improve performance. At early life stages, cephalopod individuals also choose to actively seek more favourable water conditions – for example the paralarvae of *Octopus huttoni* (Higgins et al., 2012) or *Octopus mimus* (Zúñiga et al., 2013). Results of this study suggest that squid may behaviourally avoid environments near their

thermal limits to provide a safety margin, offering to the individual an alternative to alleviate the metabolic cost associated with a future increase in temperatures.

Table 2.2. Results of the regressions of the southern calamari metabolic and behavioural thermoregulatory performances in response of temperature, including standard metabolic rates (SMR), routine metabolic rates (RMR), maximum metabolic rates (MMR), aerobic scope (AS), excess post- exercise oxygen consumption (EPOC), recovery time, thermal preferences (T_{pref}), maximum temperature avoidance (T_{max}), minimum temperature avoidance (T_{min}) and range.

Measure	Regression	a	b	c	p-value
SMR (mg O ₂ /h/kg)	Exponential	5.4	0.1		< 0.001
RMR (mg O ₂ /h/kg)	Exponential	5.6	0.3		< 0.001
MMR (mg O ₂ /h/kg)	Quadratic	6.2	1.1	-0.5	< 0.001
AS (mg O ₂ /h/kg)	Quadratic	5.1	0.9	-1.26	< 0.001
EPOC (mg O ₂ /kg)	Quadratic	4.7	0.03	-3.3	0.4
Recovery time (h)	Quadratic	-0.1	-1.3	-1.49	0.4
T_{pref} (°C)	Exponential	1.1	0.6		< 0.001
T_{max} (°C)	Exponential	1.5	0.53		< 0.001
T_{min} (°C)	Exponential	0.8	0.71		< 0.001
Range (°C)	Quadratic	1.1	-0.28	1.06	0.1

Once the species-specific thermal optimum is exceeded, species will be inclined to shift to cooler conditions to maximise their physiological capacities and fitness (Perry et al., 2005; Habary et al., 2016). However, southern calamari will be constrained from moving further south to alleviate the pressure of increasing temperatures as the suitable shallow habitat for this species is limited poleward of Tasmania waters. Thus, species might potentially need to make concessions between preferred water temperature and other factors like suitable habitat, resources or predation risk (Nay et al., 2015). Further studies should investigate the combination of different factors such as food and other habitat resources and environmental conditions, to identify possible species trade-offs in the future.

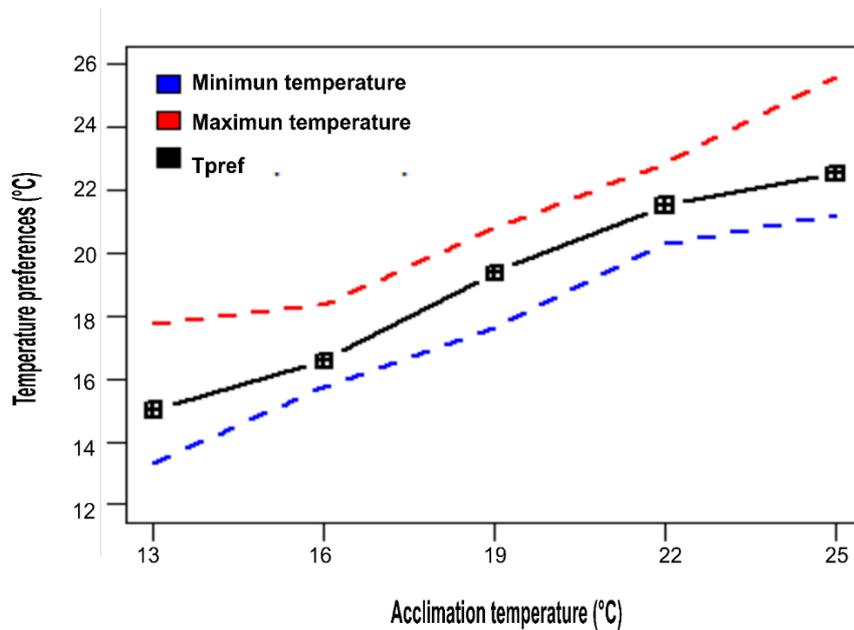


Figure 2.5. Behavioural thermal range for southern calamari acclimated at different temperatures (13, 16, 19, 22, and 25 °C). The solid black line represents the T_{pref} in relation with acclimation temperature where the box is the mean T_{pref} for treatment. The dashed blue line represents the T_{min} where the squid is found per treatment and the dashed red line corresponds to the T_{max} that individuals experienced per acclimation treatment.

As ocean warming continues, range shifts will be more likely to occur if species do not have the innate phenotypic plasticity to adapt faster to the environmental challenges. Even highly plastic species like squid are vulnerable to increases in environmental temperature, as shown here in their energetic budget, and behavioural thermoregulation could offer a faster response for thermal refugia than adaptation. Squid are opportunistic species, voracious predators and important prey in marine ecosystems. In south-east Australia, squid have been identified as a key link between food webs in different habitats, having a strong effect on ecosystems (de la Chesnais et al., 2019), so significant redistribution of squid range or abundance are likely to have significant ecological effects across trophic cascades. Consequently, understanding biological responses in this species could be critical to predicting and managing marine ecosystems in this climate change hot spot.

Considering the ecological importance of squid around the globe, and the limited information available on their thermal biology, future research should be pursuing the study of this critical

group, for example, examining the behavioural responses under thermal stressors. Additionally, the study of squid's physiological and behavioural thermal limits will improve our capacity to understand and predict cephalopod species distribution limits. For instance, our results indicate that squid would also be energetically constrained at cold temperatures, however, the capacity of acclimation at lower limits in this species is yet to be examined, and future studies of cold tolerances and adaptation may be key to understanding possible future distribution of this species (Wolfe et al., 2020). As our results indicate, to be able to more accurately understand and predict species responses to future changes, it is necessary to integrate different measures of individual performances.

2.5 Conclusion

The current study suggests that southern calamari choose to behaviourally thermoregulate, moving to more optimum water temperatures according to their metabolic capacities. As temperature increases, the energy necessary for basal and maintenance performance increases with a reduction in the aerobic energy available for other vital biological processes (e.g. growth and locomotion), and survival appears to be reduced. Thus, southern calamari could reach their metabolic threshold in a future warming scenario and potentially actively seek more favourable habitats, extending or shifting their distribution. However, the limited habitat and resources south of Tasmanian waters may favour the squid staying and keeping their internal balance by responding to environmental stress in other ways – such as increasing feed intake, biochemical buffering or genetic adaptation.

Chapter 3: Understanding species performance in a changing world by examining the predatory behavioural responses to temperature of southern calamari

Abstract

Predator–prey interactions are key drivers in structuring communities, with the potential to substantially impact the whole ecosystem when important predators and prey are involved. Thus, the study of the predator behavioural responses of these important species under different temperatures could help us to understand species responses to future environmental conditions, as well as to forecast trends in ecosystem dynamics under climate change. Squid are voracious predators and also important prey for other top predators. To date, the available data suggests that under current and projected ocean warming, the behaviour of ectotherms could be modified (for example, through individual movement, predator avoidance and escape speed), yet little is known of the influence of temperature on the predatory behaviour of cephalopods. Here, the effect of water temperature on the predatory behaviour of adult southern calamari (*Sepioteuthis australis*) was examined using video recording demonstrating that squid exhibited different behaviour and performance capabilities across temperature treatments. Overall, responses of squid to capture prey were faster and more persistent at higher temperature treatments (25 °C), suggesting that individuals need to increase their food consumption rate, presumably associated with the higher energetic cost of living at elevated temperatures. However, findings of a possible decrease in capture efficiency and increased prey handling time suggest that further

research should consider the energetic balances and trade-offs for this need for increased predation by squid at elevated temperatures. As cephalopods are ecologically important species acting as key links in food webs around the world, the results here could have important implications for the dynamics of marine ecosystems in future.

Key words: acclimation, cephalopods, climate change, locomotion, temperature, predatory behaviour, species interactions.

3.1 Introduction

Marine ecosystems across the world are being affected by climate change, with impacts ranging from changes in species' life histories (e.g. growth and development), distribution and interactions, through to shifts in ecosystem composition, stability and function (Hoegh-Guldberg and Bruno, 2010; Doney et al., 2012; Vergés et al., 2014; Payne et al., 2016). Ectotherms are particularly vulnerable to ocean warming, as temperature plays a vital role in physiological regulation and behavioural performance such as locomotion or feeding intake (Amarasekare, 2015) with the potential to impact ecological communities by altering the strength and stability of trophic interactions (Rall et al., 2010; Grigaltchik et al., 2012; Gilbert et al., 2014). Modification of these interactions could have significant consequences for whole marine communities when important predator or prey species are affected. Interactions of key species can determine the flux of nutrients among individuals though communities and ecosystems (Dell et al., 2014; Horwitz et al., 2020).

Cephalopods are considered marine keystone species, serving as important predators and prey around the world (Wood and O'Dor, 2000; Rosa and Seibel, 2008; Pimentel et al., 2012). They are voracious carnivores and opportunistic animals, feeding on a wide variety of live prey including fish, cephalopods, gastropods, bivalves or crustaceans (Hanlon and Messenger, 2018b). Furthermore, as fast-growing animals with the potential to respond quickly to

environmental changes, they are likely to play a significant role in the response of marine ecosystems to climate change (Pecl and Jackson, 2008; de la Chesnais et al., 2019). Therefore, the study of how temperature affects behavioural performance (e.g. predation and locomotion) (Gilman et al., 2010), in ecologically important species such as squid, could improve our capacity to more accurately forecast the dynamics of their associated ecosystems.

Changes in water temperature substantially alter marine organisms' physiology, for example metabolism, and thermal limits (Nguyen et al., 2011; Pimentel et al., 2012), and behaviour such as escape speed, feeding rates and movement (Malavasi et al., 2013; Nay et al., 2015; Gandhi and Cecala, 2016). In previous studies of fish and marine invertebrates, predator behaviour has also been shown to be affected by temperature (Kidawa et al., 2010; Grigaltchik et al., 2012; Sentis et al., 2012; Horwitz et al., 2020), and some species of squid have responded to other environmental stressors like acidification (Spady et al., 2018). However, studies examining the behavioural responses of cephalopods to warming waters have been under-represented in the literature (Higgins et al., 2012). To date, few studies have examined how changes in temperature affect the anti-predatory behaviour or escape speeds of cephalopods (Neumeister et al., 2000). However, no studies have examined the effect of acclimation temperature on their predatory behaviour. This is an important gap in our understanding of how future warming conditions could affect ecologically important species, with potential flow-on effects for community stability.

Predation is a key process in structuring communities and has cascading effects across trophic levels (Steffan and Snyder, 2010; Warren et al., 2017). Furthermore, predation pressure can alter prey morphology, and regulate population size, as well as prey species composition by trophic cascades (Warren et al., 2017). Any encounter between predator and prey depends on a complex interaction of physiological and behavioural capacities such as locomotion, escape

speed and foraging pattern (Lienart et al., 2014; Öhlund et al., 2015). Yet, different factors may affect the motivation to make an attack, including predation risk, hunger or prey availability (Sentis et al., 2012). Moreover, the behavioural functional response still mainly depends on two parameters – search rate and handling time (the time that a predator takes to kill and consume the prey), and both parameters are directly related to the water temperature (Sentis et al., 2012). Handling time is the time taken to capture and kill the prey, and the rate of gut clearance (Jeschke et al., 2002), whereas searching is an active predator's behaviour, directly associated with locomotor performance. For example, in *Stylocheilus striatus* (sea hare), an increase in water temperature (from 28 to 31 °C) leads to a reduction in locomotion speed (Horwitz et al., 2020), or in the predator *Macquaria novemaculeata* (Australian bass), the number of attacks increase under warm conditions (25 °C) in comparison with cold acclimation (15 °C) (Grigaltchik et al., 2012).

Ocean warming, induced by anthropogenic CO₂ emissions, is expected to increase by between 2 °C and 4 by the end of the century (IPCC, 2021) (Bindoff et al., 2019), yet the velocity and magnitude of change differs regionally around the world. In south-eastern Australia, ocean warming is occurring almost three times faster than the global average due to the intensification and poleward extension of the East Australian Current (Ridgway, 2007; Oliver et al., 2015), creating a regional 'hotspot'. Such fast-warming regions can act as natural laboratories for climate change as climate-driven biological and ecological changes may be accelerated, occurring ahead of such changes in other regions (Hobday and Pecl, 2014).

In waters around south-eastern Australia, particularly Tasmania, extensive alteration of marine ecosystems has been linked with temperature increase, for example in the loss of habitat-forming species like kelp and the range extensions of many 'new' species (Johnson et al., 2011; Last et al., 2011; Pecl et al., 2019). However, most of the research effort has focused on

understanding species current and projected distributional responses to warming. Very little work has been undertaken on how species might perform within their existing ranges and interact with existing or with novel species in response to warming waters.

Southern calamari (*Sepioteuthis australis*) are a large loliginid species, endemic to southern Australia and northern New Zealand. They are commonly found in shallow waters (< 20m deep) over seagrass meadows or sandy habitats with a lifespan of an approximately one year (Moltschaniwskyj and Steer, 2004). Within their geographical range, this species experiences average summer temperatures of 19 °C with a maximum of 21 °C. Information regarding how ocean warming may affect the performance of this economically and ecologically valuable species around Tasmanian waters is limited. In this part of the world, squid have been identified as having a strong effect on ecosystems, acting as a link between food webs in different habitats (de la Chesnais et al., 2019). As a result, squid performance under different thermal regimes could alter the stability and strength of the biotic interactions, leading into modification of whole marine community dynamics under warming conditions.

The main objective of this study was to examine the effect of acclimation temperature on the predatory behaviour of southern calamari. Specifically, I examined their predatory interactions with a common prey, Australian salmon (*Arripis trutta*), under different thermal scenarios, simulating current and possible future environmental conditions (13 °C, 16 °C, 19 °C, 22 °C, and 25 °C). The study of how environmental temperature affects a species' behavioural performance in key predator–prey interactions could help us to identify any climate-driven major changes in species interactions, which may in turn influence future population trends and ecosystem dynamics in response to climate change.

3.2 Materials and methods

3.2.1 Squid collection and holding conditions

A total of 100 adult squid were caught (mantle length 110–247 mm with a mean of 244 ± 91.67 g in weight) by hand-jigging from the south-east coast of Tasmania ($43^{\circ}00'27.0''S$ $147^{\circ}19'32.5''E$) from December 2018 to May 2019, with groups of 10 individuals captured within 24-hour periods. Collection depths varied from 5 to 15 m, as southern calamari are a largely inshore species. For acclimation to temperature and the experimental procedures, squid were transported to the IMAS Taroona research facilities at the University of Tasmania. During the animals' transport, as well as their initial time in the holding tanks, water temperature was maintained at the same temperature as that of collection (± 0.5 °C), which ranged between 14 °C and 19 °C over the capture months. In total, five temperature treatments were conducted (13 °C, 16 °C, 19 °C, 22 °C, and 25°C), representing current and possible future conditions, with 20 squid acclimated per treatment.

Once at the research facility, 10 individuals were placed in a holding tank (2.2 m diameter x 0.8 m high, two per temperature treatment) connected to a recirculating system, which contained multiple stages of filtration (drum filter, foam fractionator, biofilter, and UV treatment) and a heater/chiller unit (Aquahort heat pump LWH030SC). After the first 12 hours of the squid being in the holding tank, temperatures were increased/decreased progressively by 1 °C every 12 hours until the treatment temperature was reached to avoid any possible thermal shock to the animals. Squid were acclimated to the treatment conditions for a full week prior to any procedures being undertaken. Water quality parameters in the holding tanks (checked 3 times per day) were kept constant and at a suitable level for the squid; pH 7.7–8.2; salinity 34–36‰; $NO_3 < 10$ mg L⁻¹; $NO_2 < 0.1$ mg L⁻¹; $NH_4 < 0.25$ mg L⁻¹; and oxygen saturation < 110 %, and the water delivery was maintained at a constant 30 ± 2 L/h. Additionally, a photoperiod

cycle of 13 hr/11 hr light–dark was established, with 30 minutes each of programmed sunset and sunrise. Squid were fed daily (approximately 1:30 pm) with small live fish (body size >150 mm), including locally caught Australian salmon (*Arripis trutta*) or garfish (*Hyporhamphus melanochir*).

3.2.2 Experimental set-up

The experimental behavioural arena (Figure 3.1) consisted of a circular tank (1.0 m diameter and 0.5m high) receiving water supply at ~ 20 l/h. Temperature in the arena was controlled by a heater/chiller unit (Aquahort heat pump VL130R), and an air stone kept the dissolved oxygen at around 100%. The arena was divided by a transparent acrylic window, with a GoPro HERO 4 camera mounted behind the window to record a lateral view of the squid during trials. A GoPro HERO 5 was mounted on top of the tank at a height of 0.5 m and two lamps (10 W RGB LED) were mounted parallel to but 5 cm higher than the GoPro HERO 5 to eliminate the shadow of the squid. The arena was completely covered with a black curtain, to isolate it from any external stimuli.

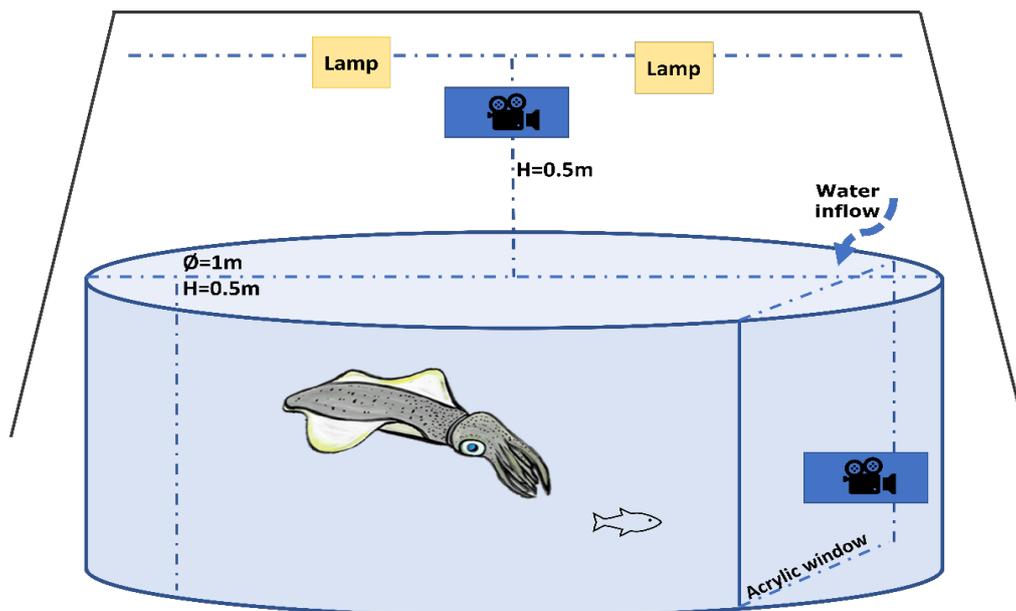


Figure 3.1. Diagram of the experimental arena used to measure predatory behaviour in southern calamari (examples of the video recording of interaction predator–prey trial: <https://cloudstor.aarnet.edu.au/plus/s/vcc9jqrG89yi1g2>).

3.2.3 Experimental trial

Prior to behavioural trials, squid were exposed to metabolic activity trials for 22 hours (Chapter 2). During these trials, squid were exhausted in order to obtain the maximum metabolic rate (MMR), with squid swimming until they could no longer maintain equilibrium. As a result, mortality increased at higher temperature treatments, reducing the sample size to 37 squid across all the treatments, resulting in an unbalanced design ($n = 6, 10, 10, 5$ and 7 individuals at $13\text{ }^{\circ}\text{C}, 16\text{ }^{\circ}\text{C}, 19\text{ }^{\circ}\text{C}, 22\text{ }^{\circ}\text{C}$ and $25\text{ }^{\circ}\text{C}$, respectively). Additionally, individuals that showed unusual behaviour in the arena, such as curled arms or resting on the bottom for periods longer than two minutes were discarded from the trials.

To begin each trial, a single squid was moved from the respirometry chamber, placed in the behavioural arena and allowed to acclimate for 1 hour. Predatory trial started within 13-14 hours after exhaustive exercise was conducted. Furthermore, to increase hunger levels they were fasted for 47 hours (24 h starvation + 22 h of metabolic experiments + 1 h acclimation) prior the predator-prey trial commencing. Cameras began recording two minutes before the prey was introduced to record squid activity. Following that, a single live prey (Australian salmon) was placed into the arena, and the resulting interaction was recorded for a further 15 minutes. Individual fish were always introduced into the tank in the same position with less than a second of air-exposure before the individual was placed in the behavioural arena. Once the fish was introduced into the tank, squid could immediately attack the prey. In the event that a squid did not eat the prey during this period, it was removed immediately and placed back into the holding tank. This period was chosen due to our previous observations as well as other studies (Sugimoto and Ikeda, 2013; Spady et al., 2018), where squid are generally able to capture their prey within 10 minutes. All the Australian salmon used in the experiments were of similar size (103 ± 27.6 mm in length) and were acclimated for one week to the same water temperature and conditions (pH, DO, and salinity) as the particular squid treatment in question.

3.2.4 Behavioural analysis

The behavioural parameters chosen for this study were established in previous studies as good proxies of squid predation behaviour (Jantzen and Havenhand, 2003; Sugimoto and Ikeda, 2013; Spady et al., 2018).

Individual behavioural parameters observed included:

- Number of attacks – number of attempts the squid made to capture the prey.
- Latency time to attack (min) – time between the introduction of the prey in the arena and the first attack.
- Attack distance (mm) – the distance between the end of the arms (immediately before the tentacle's extension) and the prey (Figure 3.2).
- Tentacle elongation (mm) – the length of the tentacles when fully extended to capture the prey.
- Attack direction/position – the orientation of the squid's body in relation to the prey when the attack occurred. Attack position was classified as 'rigid arms', 'downward pointing' and 'upward pointing'. The 'rigid arms' position is when the arms and tentacles are held together in a horizontal body plan. 'Downward pointing' and 'upward pointing' postures are defined as the orientation when the whole body exceeded 45° from the horizontal plane in the corresponding direction (Jantzen and Havenhand, 2003) (Figure 3.2).
- Body pattern – display of body pigmentation once in the attack position, categorised as 'clear (transparent)', where chromatophores are reduced in size, and 'dark', where most of the chromatophores are expanded (Jantzen and Havenhand, 2003, York and Bartol, 2016).

- Handling time – the time that squid took to kill the prey.

Additionally, I also recorded the proportion of squid that attacked the prey, captured the prey, and captured the prey on the first attempt.

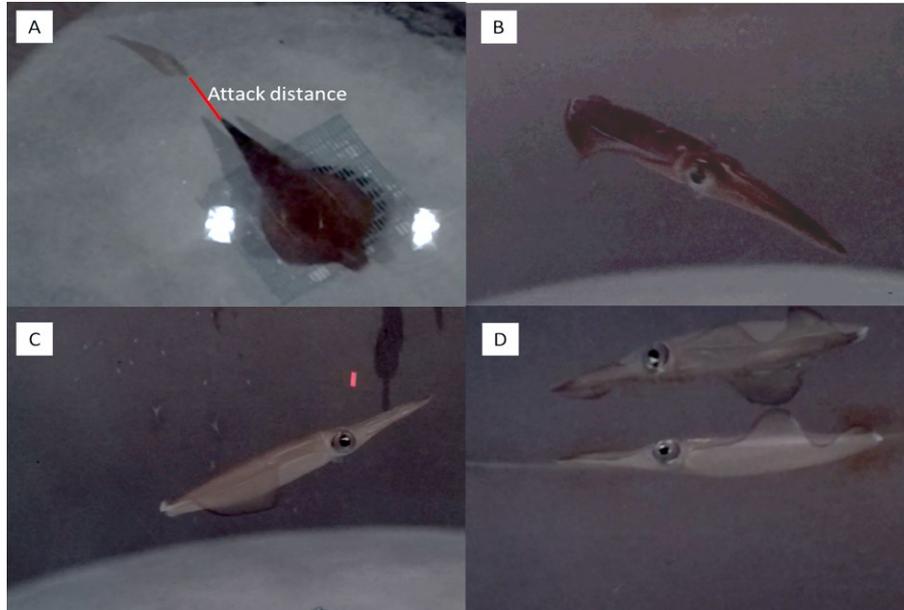


Figure 3.2. southern calamari parameter observed in the experimental arena; A) Attack distance and body pattern 'dark', B) 'downward pointing' attack position, C) 'upward pointing' attack position, D) 'rigid arms' attack position.

3.2.5 Data analysis.

R software (www.r-project.org) was used to perform all the statistical analysis in this study.

Generalised linear models (GLMs) with a binomial distribution and a logit link function were used to investigate the relationship between acclimation temperature (13 °C, 16 °C, 19 °C, 22 °C and 25 °C) and the proportion of squid that attacked the prey and the capture success. GLMs with a negative binomial distribution were used to compare the effect of acclimation temperature on the number of strike attempts. Preliminary analyses were performed for those variables using generalised linear mixed models (GLMM), including capture group (date and location caught) and acclimation days as random effects. The variance and standard deviation were zero and consequently, they were dropped from the model, fitting simple GLM models.

For the remainder of the behavioural performance measures (attack position, distance, body pattern, latency and handling time) results were only examined for the individuals that successfully captured the prey. As a result of the small numbers of squid attacking the prey ($n=1$) at lower temperature treatments (13 °C and 16 °C), both these treatments were necessarily eliminated from further statistical analysis. GLMs with a binomial logistic regression were used to examine the relationship between acclimation temperature, the ability to capture the prey at the first strike, and body pattern, where the attack position was examined using a multinomial logistic regression. To investigate the relationship between attack distance, and tentacle elongation, GLMs with a gaussian distribution and a log link were used, and GLMs with a gamma distribution and a log link were used to model latency and handling time.

Additionally, squid mantle length and total weight were included together with the acclimation temperature as a factor in all the models. Akaike information criterion (AIC) values were used to establish the best fit of the model for each measure of performance (data not show), and model assumptions were verified by examining residuals compared to the fitted values by inspection of the residual-fit plots.

3.3 Results

Acclimation temperature had a significant effect on the probability of a squid making an attack ($X^2=12.4$; $df=1$, $P <0.0001$), as well as the capture success ($X^2=7.1$; $df= 1$; $P <0.001$). The proportion of squid that attacked the prey increased substantially between treatments. Around 30% of the squid that acclimated to lower temperatures (13 °C and 16 °C) made an attack strike, increasing to 80% at 19 °C and 22 °C, and reaching 100% at 25 °C (Figure 3.3). Similar percentages were observed in the proportion of squid that successfully captured the prey, with 16.6% and 10% of the squid capturing the prey at 13 °C and 16 °C respectively, compared to 50% at 19 °C, 80% at 22 °C and 100% at 25 °C. The number of strikes needed to achieve

capture success also increased significantly with acclimation temperature ($X^2 = 4.8$; $df=1$, $P < 0.05$) (Figure 3.3).

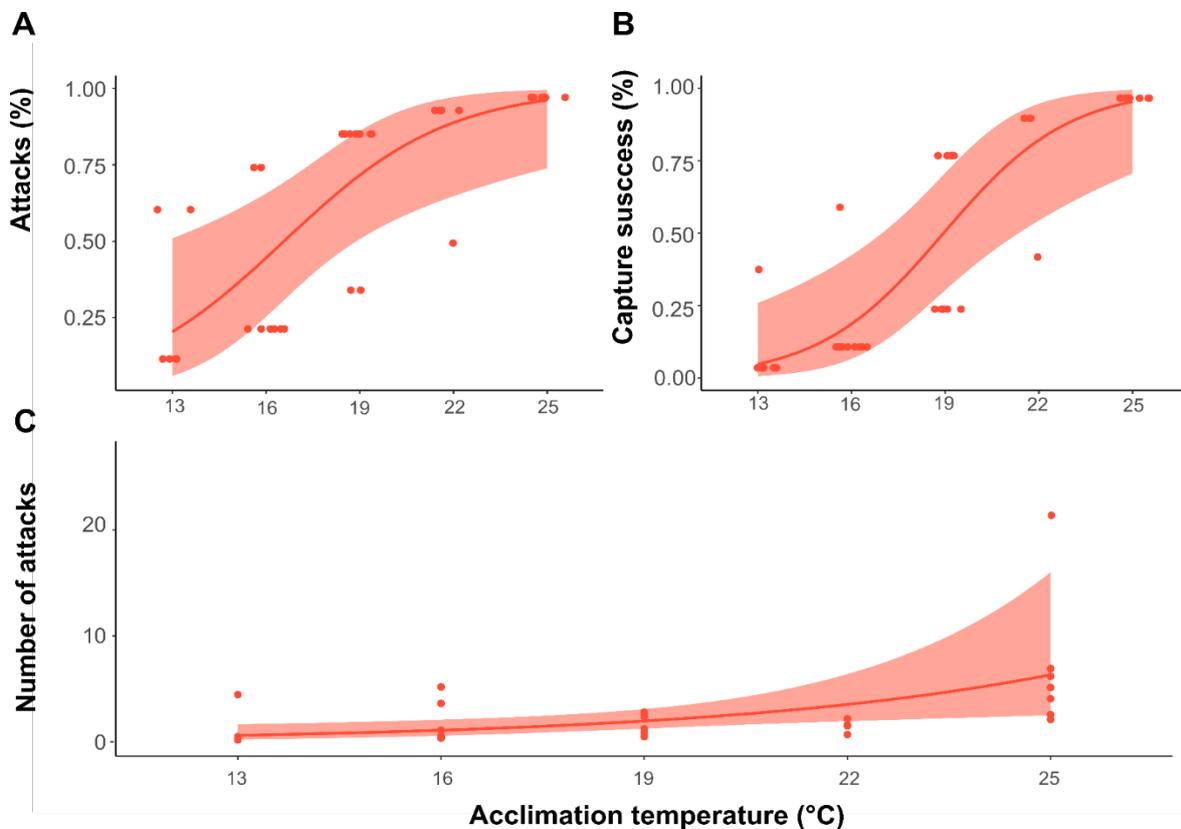


Figure 3.3. Relationship between behavioural responses of southern calamari and the five different acclimation temperatures (13, 16, 19, 22 and 25 °C). A) GLM with binomial distribution of the probability that individual squid will make an attack. B) GLM with binomial distribution, of capture success of squid per treatment C) GLM with a negative binomial distribution modelling the total number of strike attempts, including when squid did not get the prey. Thick line represents the expected value of the model for the variable measured. The shadow band corresponds to the 95% confidence interval, and the individual squid values are represented by the red points.

For individuals that caught the prey at 19 °C, 22 °C and 25 °C, there were not statistical differences in the proportion of squid that successfully captured the fish at the first strike attempt ($X^2 = 1.4$; $df=1$, $P > 0.05$). Although an overall decrease occurred with temperature from 60% (3 out of 5 individual) at 19 °C, and 75% (3 out of 4 individuals) at 22 °C, to 28.5% (2 out of 7 squid) at 25 °C. The lack of statistical significance could be related to the low sample size. Latency and handling time were also different among treatments ($X^2 = 57.3$; $df=1$, $P < 0.001$; $X^2 = 24.3$; $df=1$, $P < 0.001$). At higher temperatures (22 °C and 25 °C), the time that squid took to initiate the attack was reduced compared to individuals acclimated at 19 °C, in contrast to

the increase in handling time at higher temperatures (Figure 3.5). At 19 °C, squid took between 1 and 11 min to attack, whereas squid at 22 °C and 25 °C started the attack only seconds after the prey had been placed in the arena (0.15 min \pm 0.08; 0.07 min \pm 0.01, respectively). Furthermore, individuals at 19 °C only handled the prey for a mean of 0.5 min (\pm 0.3 SD), rising to 1.5 min (\pm 0.6 SD) and 2.2 min (\pm 0.7 SD) in squid acclimated to 22 °C and 25 °C, respectively. The distance from which squid started the attack, and the elongation of tentacles, also significantly differed across temperature treatments ($X^2=10.3$; $df=1$, $P <0.01$; $X^2=8.7$; $df=1$, $P <0.01$), increasing with acclimation temperature (Figure 3.4). Individuals at 19 °C chose to be closer to the prey when the attack occurred (66.1 \pm 30 mm), compared with individuals at 22 °C (96.5 \pm 30.9 mm) and 25 °C (106.1 \pm 24.3 mm). Moreover, the elongation of the tentacles was also shorter at 19 °C (78.9 \pm 36.1 mm), while it was 102.1 mm (\pm 24.6) at 22°C and 108.5 mm (\pm 14.6) at 25 °C.

Neither attack position nor body colour choice differed between treatments ($X^2=2.0$; $df=2$ $P=0.7$; $X^2=0.11$; $df=1$ $P=0.73$). However, the preferred body pattern of southern calamari during the attack, across the three treatments, was the dark colour display, with 80%, 75% and 71% of the squid choosing it at 19 °C, 22 °C, and 25 °C respectively (Figure 3.4). Additionally, there was no effect of mantle length or squid body weight for any of the behavioural parameters (data not shown).

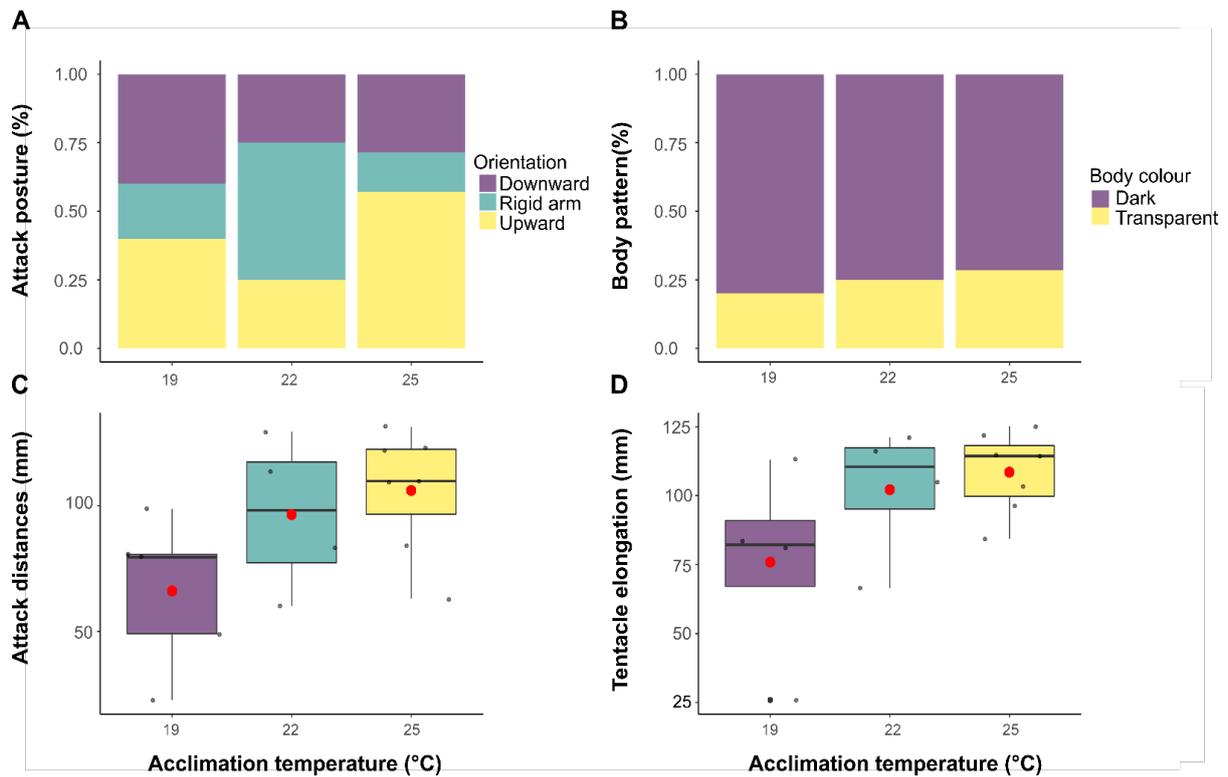


Figure 3.4. Behavioural measures of southern calamari that successfully attacked prey at different temperatures of acclimation (19, 22 and 25 °C). A) proportion of squid exhibiting the different positions of attack (downward, rigid arms, and upward). B) the proportion of squid exhibiting different body display patterns (dark and transparent). C) boxplots of the attack distance (mm) and D) tentacle elongation (mm). In the boxplot, points indicate individual values and boxes represent the first and third quartiles. Within each box, the median is represented by the solid line and the mean by the red point.

3.4 Discussion

In several of the parameters investigated in this study southern calamari predatory behaviour differed depending on the acclimation temperature. Two of the most notable differences between treatments was the proportion of squid attacking the prey and the capture success, with both measures increasing with the temperature of acclimation. All individuals acclimated to 25 °C successfully attacked and captured 100% of prey provided, meanwhile at low temperatures (13 °C and 16 °C) only 30% of squid attacked the prey and with less than 16% success. These results suggest that southern calamari will need to increase food consumption rates to compensate for the energetic costs associated with living at elevated temperatures. Similar results have been found in other species of marine fish (Bethea et al., 2007; Grigaltchik

et al., 2012) and invertebrates (Morón Lugo et al., 2020), where individuals at elevated acclimation temperatures were more motivated to attack than those at lower temperatures.

The decrease in the predation rate at lower temperatures (13 °C and 16 °C) may also be a consequence of the reduction in metabolism of southern calamari at those temperatures (Chapter 2), having reduced energy/feeding requirements and possibly saving energy by not trying to capture the prey. It has also been suggested that similar reductions in other species of squid could be linked with the depression of metabolic rates due to environmental factors (Spady et al., 2018). The ‘aerobic scope protection hypothesis’ suggests that the reduction in an individual’s food consumption may serve to intentionally conserve a portion of their available aerobic scope by limiting energetic expenditure on prey capture or digestion (Jutfelt et al., 2021). This hypothesis could explain the results found here at lower temperature treatments, yet future investigation would be necessary.

Behavioural differences were also found in the squid which attacked the prey at the different temperature treatments (19 °C, 22 °C and 25 °C). Time until first attack and numbers of attacks could be interpreted as measures of predation motivation (Grigaltchik et al., 2012). Increased acclimation temperature greatly reduced the latency time of squid to attack the prey. At 22 °C and 25 °C, individuals reacted in seconds, whereas at 19 °C squid took between 1 and 10 min to attack. This indicates that in squid the motivation to make an attack is directly influenced by temperature, probably associated with hunger levels and energetic requirements. In contrast, the handling time that squid needed to kill the prey increased with temperature, rising to over a min at 22 °C and above 2 min at 25 °C compared with a 0.5 min at 19 °C. Furthermore, the capacity of squid to successfully strike the prey on the first attempt was also reduced at 25 °C, with only 28% (2 out of 7) of squid capturing the prey in the first attempt compared with 60% and 75% at 19 °C and 22 °C, respectively. These results might indicate that the physical

capabilities of squid might be impacted at elevated temperatures, reducing their ability to handle (capture and kill) the prey effectively. In other marine invertebrates, for example the Antarctic sea star (*Odontaster validus*), some physical abilities such as motor coordination or speed were also reduced at increased temperatures (Kidawa et al., 2010). Findings of a possible decrease in capture efficiency and increased prey handling time suggest that further research should consider the energetic balances and trade-offs involved in the need for increased predation among squid at elevated temperatures. Additionally, the effects of acclimation temperature on the prey could potentially be linked with the increase in handling time and the success rate of strikes on the first attempt, as higher temperatures may increase prey mobility. Future studies exploring these effects could examine how changes in water conditions affect the prey, to determine if those effects would be influencing squid ability to capture their prey.

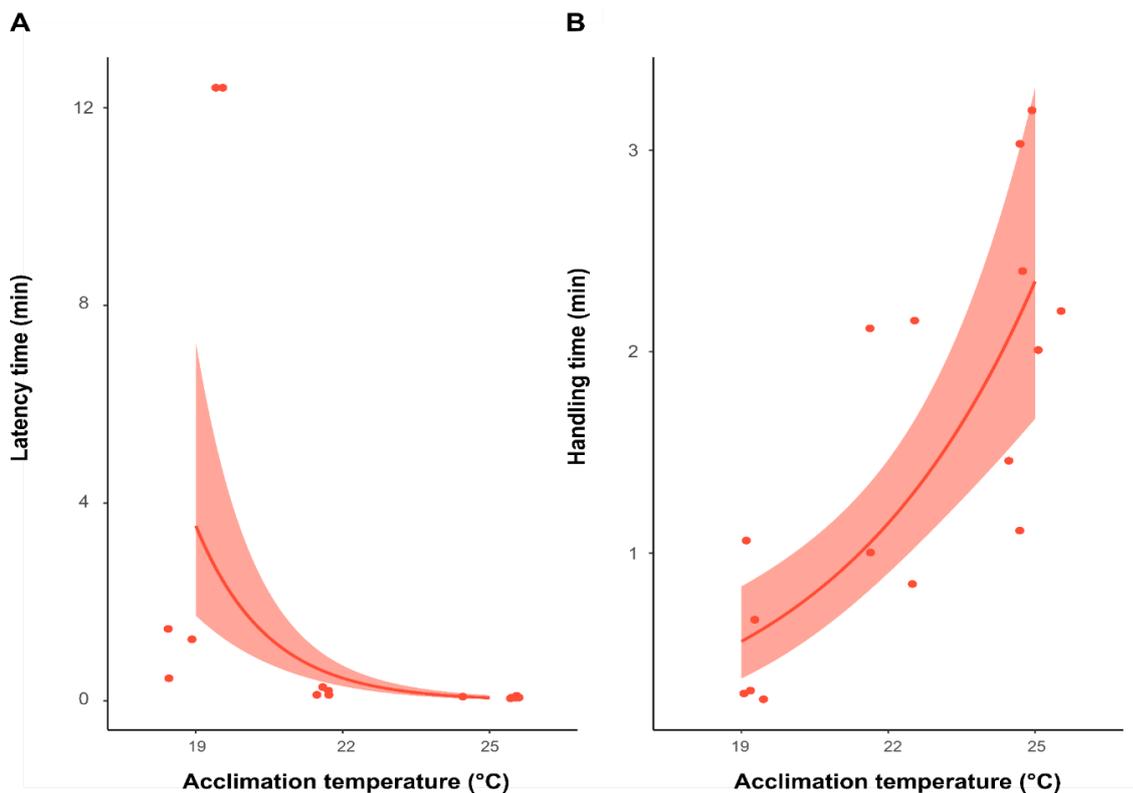


Figure 3.5. Southern calamari which successfully attack prey at different temperatures of acclimation (19, 22 and 25 °C). A) GLM model with a gamma distribution of latency time (min) that squid took to make the first attack B) GLM with a gamma distribution of the handling time (min) that squid took to capture and kill the prey. Thick line represents the expected value from the model for the variable measure. The shadow band corresponds to the 95% confidence interval, and the individual values are represented by the red points.

Attack distance and tentacle elongation of squid increased at higher temperatures; this could also be related to the impatience of squid to capture the prey instead of a predatory strategy per se. In some cephalopod species, environmental stresses like acidification can lead to an increase in striking distances (Spady et al., 2018). Greater attack distance could be beneficial for the squid if prey mobility is increased in warmer waters by better disguising the incoming attack and reducing the chances of escape. Regarding their other predatory tactics, neither the attack direction nor body pattern showed differences between temperature treatments. However, across treatments 19 °C, 22 °C and 25 °C, the most preferable body pattern was a dark color. This pattern (banded or totally dark) possibly acts as a disrupting coloration, distracting the attention from the extending tentacles (York and Bartol, 2016; Hanlon and Messenger, 2018b) which might confuse the prey, and confer an advantage to the squid.

Cephalopods are well known for having a high metabolic rate (O'Dor and Webber, 1986; O'Dor et al., 2002), and in a previous study (Chapter 2), metabolic activity (standard, routine metabolic rates) had been identified as increasing with temperature in southern calamari. Squid will have a higher energetic requirement (~ 30%) to support basal and maintenance processes at 25 °C, than at lower temperatures, supporting the suggestion that an increase in the consumption rate will be required to maintain energetic balance at higher temperatures.

As the metabolic demands of squid are likely to increase due to ocean warming, *in situ* individuals might have different options for maintaining their energetic balance and overall fitness including, for example, consuming prey with higher energetic/nutritional content or increasing their feeding rates (Horwitz et al., 2020). If the individuals choose the latter option, squid may be more willing to actively search for prey, perhaps resulting in their being bolder and exposing them to a higher predation risk (Biro and Stamps, 2010; Killen et al., 2011; Careau and Garland Jr, 2012; Cornwell et al., 2020). If individuals are more active, more energy

would be allocated to locomotion, limiting energy available for other important performance such reproduction or growth. Furthermore, the metabolic thermal optimum (T_{opt}) indicated by the aerobic scope for this species is between 19 °C and 22 °C (Chapter 2), beyond that point individual performance maybe be limited (Peck et al., 2009; Pörtner and Peck, 2010).

Modifications to predatory performance could alter the outcome of predator–prey interactions, potentially resulting in a cascade effect throughout food webs (Warren et al., 2017). Cephalopods are important and opportunistic predators, where changes in their feeding rates could have significant consequences for the structure of ecosystems (Spady et al., 2018; de la Chesnais et al., 2019). Southern calamari are likely to increase feeding intake rates as ocean warming continues in Tasmanian waters, putting greater pressure on their prey and potentially triggering a cascade effect on the food web, altering community and ecosystem stability.

To date, in an ocean warming context, information on feeding rates (especially in the wild), energy budgets and prey preferences in cephalopods is limited and future research could further examine these key concepts to better understand species performance under the various climate change scenarios. Although our sample sizes were smaller than intended, trends across treatments were clear and consistent. However, further work could expand our results by larger sample sizes as well as exploring differences with sex, life stages and growth rate. This will all be important in more accurately predicting the responses of this critical animal group to future environmental challenges. Moreover, marine ecosystems are not only being altered by ocean warming, including changes in size structures (Audzijonyte et al., 2020), but also acidification, pollution, changes in productivity, and oxygen content, as well as overfishing (Hoegh-Guldberg and Bruno, 2010; Payne et al., 2016). The physical capacities of other species of squid to capture their prey were reportedly not impacted by environmental stressors such as acidification, although their decision-making processes and strategies have been shown to be

affected (Spady et al., 2014; Spady et al., 2018). However, we have an extremely limited understanding of how multiple stressors will combine to impact the predatory behaviour of this group – an important gap in our knowledge of how future squid populations will respond to climate change over coming decades.

3.5 Conclusions

Ocean warming will modify ecosystems by affecting the interactions of existing predator–prey combinations, as well as creating novel ones as new species enter regions and existing species depart (Pecl et al., 2017). In this context, it is important to examine how species predatory performance alters due to environmental changes. Our study showed that environmental temperature has a major impact of the behaviour of southern calamari. Overall, our findings indicate that elevated temperatures in line with those predicted for the future could alter the predatory behaviour of southern calamari by increasing consumption rates, as well as increasing the number of attempts they need to capture their prey, and their prey handling times. Individuals would then need to be more active to capture prey to maintain energetic balance and as a result they will be more exposed to predation risk, as well as experience greater trade-offs between essential and non-essential performance.

4

Chapter 4: Critical thermal limits and the molecular pathways underpinning adaptation to temperature in southern calamari (*Sepioteuthis australis*)

Abstract

As ocean warming rates increase, understanding organisms' thermal tolerances and their capacity to adapt to new environmental conditions is essential for predicting how species may respond to future climate challenges. Cephalopods are highly phenotypically flexible species, meaning they can potentially adapt rapidly to environmental challenges. However, it is unclear what mechanisms are behind their high phenotypic plasticity and the relationship with the specific environmental stressors such as temperature. *Sepioteuthis australis* (southern calamari) is an iconic, economically valuable and ecologically important species common around the Tasmanian coast, yet the information regarding their likely responses to climate change and their thermal biology is still limited. The main aim of this study was to determine if acclimation temperature affects squid thermal tolerance limits. Additionally, transcriptomics (RNA-seq) was used to examine potential mechanisms involved in their thermal adaptation. For that, I investigate the influence of five thermal acclimation scenarios on the upper thermal limit (CT_{max}) of southern calamari by exposing individuals to a rapid increase in water temperature. Results here show that acclimation temperature has a profound effect on the upper thermal limit for southern calamari. Individuals acclimated to lower temperatures (13 °C and 16 °C) achieved a CT_{max} of 24.4 °C as average, while squid acclimated to elevated temperatures (25 °C) reached an average CT_{max} of 29.5 °C. These findings indicate that southern calamari are likely

capable of activating mechanisms to improve their chances of survival given rapid changes in water temperature, such as may occur under marine heatwave scenarios.. Our transcriptomic results suggest that squid modulate RNA splicing activity as a main response to rapidly shift and adapt to changing thermal conditions, with apoptotic, stress or immune responses involved as well.

Key words: Adaptation, cephalopods, critical thermal maximum, gene expression, RNA-seq, squid, ocean warming.

4.1 Introduction

Anthropogenic greenhouse emissions and other human pressures like overfishing and pollution, are strongly influencing ocean stability, affecting species and their associated ecosystems (Hoegh-Guldberg and Bruno, 2010; Doney et al., 2012; Poloczanska et al., 2013; Payne et al., 2016). These changes are not only having significant consequences at ecosystem levels but also on human wellbeing through altering fish stock distribution and abundance, food security and ultimately influencing the livelihoods of the communities that depend on these marine resources (Pecl et al., 2017). It is therefore paramount that we better understand how future environmental conditions could potentially affect species of particular importance to fisheries and for food security.

Ocean warming could affect marine ectotherms as temperature plays an important role in the regulation of their physiological and cellular processes (Pörtner, 2002; Pörtner and Farrell, 2008; Pörtner and Peck, 2010). Ectotherms have the capacity to adjust their physiology and behaviour to the natural daily and seasonal variation in water conditions. However, species may fail to adapt to the climate change-driven increases in warming, if temperatures exceed species-specific thermal tolerances, resulting in individual constraints at temperature limits, and ultimately affecting their survival (Pörtner and Peck, 2010). Not surprisingly, marine

species are being affected as a result of ocean warming, leading to changes in body size (Audzijonyte et al., 2020), reproduction (López-Galindo et al., 2019) and geographical distribution (Last et al., 2011; Sunday et al., 2012), which may in turn change ecosystem function, composition and stability (Vergés et al., 2014). Consequently, the study of thermal tolerance mechanisms, and their potential for adaptation, are crucial to more accurately predicting long-term trends in natural ecosystems (Munday et al., 2017).

Global warming rates have intensified around the world with projections indicating that ocean temperature could rise between 2 °C and 4 °C by the end of the century (IPCC, 2021). In addition to long-term trends, marine heatwaves are also predicted to increase in intensity and frequency (Oliver et al., 2019). The magnitude and rapidity of those changes surpass most shifts over geological time (Blois et al., 2013; Millar et al., 2017), yet the pace of change is not uniform around the world. Waters off the coast of south-eastern Australia have been identified as one of the fastest changing regions of the world, where warming rates are increasing three to four times faster than the global average due to the intensification and poleward extension of the East Australian Current (Ridgway, 2007; Hobday and Pecl, 2014). Aside from creating an urgent need to understand impacts in this region specifically, this potentially creates a ‘natural laboratory’ as climate-driven impacts on modification in species and communities may be accelerated (Hobday and Pecl, 2014). In Tasmanian waters, in particular, changes to biodiversity and accelerated habitat loss have been linked to long-term warming trends (Johnson et al., 2011; Last et al., 2011), and short-term events (e.g. heatwaves) (Oliver et al., 2017; Oliver et al., 2018). However, most studies have examined climate-driven changes in the distribution of species, and our knowledge regarding how species within their existing ranges respond to changing conditions now and into the future is still very limited. Identifying the potential impacts of climate change in key species is necessary to understand not only the effect

of long-term trends in climate, but also extreme short-term events like heatwaves as these events can have devastating consequences at population levels (Oliver et al., 2019).

Sepioteuthis australis (southern calamari) is an ecologically and economically valuable species from southern Australia and northern New Zealand. Throughout these waters, squid have been identified as having a strong effect on the ecosystem dynamics, providing strong links between trophic levels due to their voracious prey consumption and high production rates (de la Chesnais et al., 2019). Southern calamari are a benthic species associated with seagrass meadows or sandy habitats and they have a short life cycle of approximately a year (Moltschaniwskyj and Steer, 2004). Throughout their distribution in Tasmanian waters, squid experience an average in summer temperatures of 19 °C during summer months with maximum of 22 °C, with projections indicating that summer inshore temperatures could reach 25 °C by the end of the century (CSIRO and Bureau of Meteorology, climate change in Australia - <http://www.climatechangeinaustralia.gov.au/>). To date, study of the thermal biology in southern calamari is minimal, posing a key gap in our knowledge of likely species adaptation to future environmental conditions in Tasmanian waters.

Physiological studies can be used to determine thermal limits, identify which mechanisms set those limits, and establish how species vary in acclimation capacities as a result of modification of their thermal tolerances (Vinagre et al., 2016). Species thermal tolerances define fundamental thermal niches (Ern et al., 2016) and their limits define the species' ability to persist under extreme acute thermal events (Magozzi and Calosi, 2015). Additionally, stage-specific thermal tolerance limits can influence selection of spawning grounds, recruitment levels, and spatial temporal ranges in individuals (Vijai et al., 2015). Therefore, the study of those limits under different thermal scenarios can help inform our understanding of ectotherm performances under changing conditions, establishing the capacity of species to acclimate

(Vinagre et al., 2016; Illing et al., 2020) and informing our ability to forecast species future distribution patterns (Sunday et al., 2012).

The critical thermal maximum limit (CT_{max}) is a measure commonly used to determine the upper limit of the thermal tolerance window, beyond which performances decrease rapidly and chances of mortality increase (Manríquez et al., 2019). The capacity of marine invertebrates to modify their thermal limits in response to acclimation temperature appear to be species-specific and variable, with studies in marine invertebrates reporting modification of their thermal limits (Ravaux et al., 2012; Rosa et al., 2012), or no effect on their thermal tolerances (Delorme et al., 2020). In cephalopods, information regarding their ability to modify thermal limits by phenotypic plasticity is still limited (Xavier et al., 2015). In early squid life stages, acclimation temperature has shown a positive relationship with thermal limits, increasing their thermal window (Rosa et al., 2012), yet information regarding this effect on the CT_{max} in adult squid is lacking. Even if the early life stages are ultimately more vulnerable than the adult stages, adults failing to adjust to changed conditions could negatively impact the population if they cease reproduction.

Acclimation is one of the main mechanisms for species to survive under future changing conditions. Acclimation is defined as a form of adaptative phenotypic plasticity altering performance and is likely to improve species overall fitness (Angilletta, 2009; Gerken et al., 2015). Species can potentially acclimate and modify their thermal window by changing mitochondrial densities, and the molecular pathways of their functional capacities (Pörtner and Knust, 2007). Thus, modulating gene expression is critical in the adaptation to short and long-term environmental stressors (Place et al., 2012; Veilleux et al., 2018) by encoding proteins involved in physiological regulation, cellular stress responses, apoptosis, cell cycle, RNA editing or modification of the lipid membrane composition (Garrett and Rosenthal, 2012a;

Gerken et al., 2015; Zheng et al., 2019). For example, elevated expression of chaperones (proteins that guide other proteins along the proper pathways for folding) such as heat shock proteins (HSPs), have often been identified as a common response in different species to environmental changes (Garrido et al., 2001; Li et al., 2021). In fish, regulation of biochemical, metabolic, and physiological pathways, including modifying the fatty acids and lipid content of cellular membranes, production of specific isozymes, and engaging muscle fibre types which synthesise molecular chaperones, have also been documented (Gerlach et al., 1990; Long et al., 2013; Murzina et al., 2020). Nevertheless, the study of the molecular mechanisms associated with cephalopod responses to environmental temperature and their capacity of acclimation and adaptation is very new, and information is very limited.

In recent years, high RNA editing activity by deamination of adenosine to inosine (A-to-I) has been observed in cephalopods where the genomic sequence is also evolutionarily conserved, meaning that RNA editing could confer a selective advantage in this group (Liscovitch-Brauer et al., 2017). Thus, this process could be beneficial in cephalopods under future climate scenarios, potentially mediating their high phenotypic plasticity (Garrett and Rosenthal, 2012b). RNA editing is a post-transcriptional process which modifies the transcribed mRNAs into novel protein isoforms (Garrett and Rosenthal, 2012b). This process allows the diversification of the proteomes, enabling individuals to potentially respond rapidly to changes in their environments. However, the potential involvement of RNA editing in the response to environmental stressors is still not clear due to the lack of empirical studies.

For cephalopod, their life history strategy of ‘live fast, die young’, with short generation times may allow them to adapt rapidly to environmental conditions, improving their chances of survival (Pecl et al., 2004a; Pecl and Jackson, 2008; Rosa and Seibel, 2008). Their strategy and the potential ability to respond rapidly to challenging conditions make them ideal models to

investigate mechanisms of resilience to environmental stressors. RNA-seq analysis (also referred to as transcriptomics) is a powerful tool which is used for identifying the genes associated with species-specific responses to environmental challenges (Harrington et al., 2020; Pérez-Portela et al., 2020), providing a powerful new insights into the molecular mechanisms of adaptations (Bay et al., 2017). To date, only three species of the Cephalopoda class have an assembled and annotated genome (Albertin et al., 2015; Kim et al., 2018; Belcaid et al., 2019), which could be one of the reasons for the limited information in this area in cephalopods. However, the development of *de novo* assembly techniques, where genomes can be constructed from a large number of DNA fragments, with no *a priori* knowledge of the correct sequence or order of those fragments, offers new and efficient strategies to perform a comprehensive analysis at the molecular level especially in non-model organisms (García-Fernández et al., 2019). Currently, there are few studies on transcription in cephalopods (Salazar et al., 2015; Juárez et al., 2019; Benoist et al., 2020), and to the best of my knowledge no study has observed the effect of acclimation temperature on the transcription of any species of the Cephalopoda class to understand potential impacts of ocean warming.

The main objectives of this study were: 1) to test if acclimation temperature influences the physiological thermal tolerance of squid, by assessing the critical thermal maximum limits (CT_{max}); and 2) to identify the molecular mechanisms involved in thermal tolerance and phenotypic plasticity responses throughout the transcriptome. This is the first study to address how acclimation temperature influences the thermal maximum limits of tolerance as well as the transcriptome in squid. By linking animal performance traits to transcriptomic changes, we can increase our understanding of the molecular pathways involved in the observed physiological traits (Windisch et al., 2014), which is crucial in forecasting species responses under continuing climate change.

4.2 Materials and methods

To examine phenotypic plasticity in squid, upper thermal limits and transcriptional responses to temperature were investigated in wild squid acclimated to different temperatures for a seven-day period. Upper thermal limits were evaluated, assessing the ability of an individual to survive rapid changes in water temperature. A subgroup of the acclimated squid were then euthanized and samples collected for further RNA extraction and sequencing.

4.2.1 Squid collection and holding conditions

Collection of southern calamari by hand-jigging occurred in the south-east of Tasmania (43°00'27.0"S, 147°19'32.5"E) between December 2018 and June 2019, when water temperatures oscillated between 14 °C and 19 °C. A total of 100 adult squid (mantle length 110–247 mm with a mean of 244 ± 91.67 g in weight) were caught, with 10 individuals collected per trip. After capture, animals were transferred to the Institute for Marine and Antarctic Studies at the Taroona research facility and placed into a 4000-litre holding tank connected to a recirculated water system. Squid were held at the same temperature as the collection (± 0.5 °C) for the first 12 hours, followed by an increase/decrease of 1 °C every 12 hours until the treatment temperature was reached. Per treatment, 20 squid were acclimated for seven days at the required temperature before any measurements were taken. Actual acclimation periods ranged between 7 and 9 days due to the need for staggering of individual experiments. Five different acclimation treatments were examined (13 °C, 16 °C, 19 °C, 22 °C and 25 °C), representing current and future temperature water scenarios. Water flow into the tank was maintained at 30 ± 2 l/h and at suitable levels of: pH 7.7–8.2; salinity 34–36‰; $\text{NO}_3^- < 10$ mg L⁻¹; $\text{NO}_2^- < 0.1$ mg L⁻¹; $\text{NH}_4^+ < 0.25$ mg L⁻¹; and oxygen saturation <110%. Squid were exposed to a photoperiod of 13 h:11 h L:D, including a programmed 30 minutes of sunset and sunrise. An AI LED System C2 (Aquaillumination, USA) connected to a TM618 timer

(SINOTIMER, Wenzhou, China) controlled the photoperiod in the holding tanks. Individuals were fed every 24 hours (always ~ 1:30 pm) on a diet of live fish (body size >150 mm) including Australian salmon (*Arripis trutta*) or garfish (*Hyporhamphus melanochir*), locally caught by beach seining.

4.2.2 Critical thermal maximum (CT_{max})

4.2.2.1 Trial and set-up

Critical thermal maximum (CT_{max}) was measured with individuals acclimated to the five temperature treatments (13 °C, 16 °C, 19 °C, 22 °C and 25 °C), however, numbers of squid varied between temperature treatments as mortality increased at higher temperatures (22 °C and 25 °C (during metabolic activity trials described in Chapter 2). Our experiments across thesis chapters were designed so that the same squid were used in different trials. Before the CT_{max} trial, individual squid were used to perform metabolic activity (Chapter 2) and predator–prey interaction experiments (Chapter 3). The CT_{max} trial was performed after 30 minutes to 1 hour after predatory behaviour. Additionally, individuals which exhibited abnormal behaviour (such as lying on the bottom of the tank or curling their arms) in the experimental arena were excluded from the CT_{max} trial. Exact numbers of squid used per treatment are shown in Table 4.1.

The experimental arena consisted of a circular tank (0.5 m diameter and 0.3 m high) with an air stone to maintain oxygen at suitable levels. To control water temperature in the chamber, two water inflow lines were connected. One inflow line was connected to a heater/chiller unit (Aquahort heat pump VL130R) which delivered water at a flow rate of 10 l/h during the acclimation period to keep treatment temperature constant. The second inflow line was attached to a buffer tank with two immersion heaters (Istra Elements and Engineering, Caringbah, Australia) to increase the temperature during the trial by 0.1 °C min⁻¹. The fast rate of

temperature increment increase was chosen to avoid the possibility of acclimation of squid during the experimental phase (Komoroske et al., 2014). Each individual squid was placed into the critical thermal arena at the same temperature as the treatment one and was allowed to adjust for 30 minutes prior the start of temperature ramping. Once in the experimental arena, animals were monitored every 5 min until the temperature reached 20 °C, thereafter squid were constantly monitored. CT_{max} was defined as the temperature at which squid lost equilibrium (LOE) over at least 10 seconds, losing the ability to escape, and react to external stimuli (Ern et al., 2016).

4.2.2.2 Data analysis

Statistical analysis was performed using R version 3.6.1 statistical software. A generalised linear mixed model (GLMM) with a gaussian distribution was used to analyse the relationship between CT_{max} and temperature of acclimation using the lme4 package in R (Bates et al., 2015). Acclimation temperature was selected as a factor, with differences in acclimation days (7–9 days) and capture/holding groups (date and location caught) assigned as random effects. Squid body weight and mantle length were initially included in the model as fixed factors. However, they were not significant ($P > 0.05$), resulting in the simplification of the model with their elimination. Normality and homoscedasticity were visually verified using the diagnostic plot from the sjPlot package in R version 2.8.7, and log transformation of the CT_{max} was used because of the absence of normality. An Akaike information criterion (AIC) number was used to select the model with best fit to the data.

4.2.3 RNA-seq

4.2.3.1 RNA extraction

Transcriptomic analysis was conducted on a separate subset of squid acclimated to temperatures of 13 °C, 19 °C and 25 °C. Individuals (n = 2 per temperature treatment) were randomly selected from the holding tank and put into an anaesthetic bath of MgCl₂ (3.5%) until unresponsive, and then euthanised by brain dissection (Fiorito et al., 2015). At the moment of euthanasia, the systemic heart, brachial hearts (x 2) and gills were collected for transcriptomic analysis. Gill samples were always collected from the posterior end of the squid's left gill. Meanwhile, whole systemic and brachial hearts were stored – with an average weight of 0.2 gr. and 0.05 gr. respectively. Samples were flash frozen with liquid nitrogen and kept in –80 °C until further RNA extraction.

Total RNA was isolated by RNAzol[®] RT (Merck) according to the manufacturer's protocol (Chomczynski et al., 2010). RNA samples were sent to Novogen (Hong Kong) for cDNA library construction, normalisation and high-throughput sequencing. A total of 18 RNA samples (n = 2 individuals per treatment x 3 tissues x 3 treatments) were sequenced.

4.2.3.2 cDNA library construction and sequencing

Prior to cDNA library construction, quality control of the RNA was completed to ensure the reliability of the data. A Nanodrop 2100 spectrophotometer (Thermo Fisher, USA) was used for preliminary quantification, and Agilent 2100 Bioanalyzer[™] (Agilent Technologies, CA, USA) for verification of RNA integrity and quantitation. This procedure was followed by agarose electrophoresis to test for degradation and possible contamination.

Libraries were generated using NEBNext Ultra[™] RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations. For the cDNA library construction, mRNA was enriched with oligo (dT) beads, followed by random fragmentation and cDNA

synthesis applying random hexamers and reverse transcriptase (RNase H⁻¹). A second-strand synthesis was generated by nick translation, and purification of cDNA was executed by AMPure XP beads (Beckman Coulter, Beverly, USA). A further purification, terminal repair, A-tailing, ligation of sequencing adaptors and PCR enrichment were completed to obtain the final cDNA library. For that, library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies), then library quality and quantity were assessed using the Agilent 2100 system and a quantitative PCR (Q-PCR). Sequencing was achieved via Illumina HiSeq 2500 (150 paired end, generating at least 6 Gb per sample) with a total of 18 RNA-Seq libraries.

4.2.3.3 *De novo* assembly and functional annotation

The FASTQ sequenced files were logged in FASTQC for quality analysis. Low quality bases (N >10% and Phred score \leq 30) and adapter contamination were eliminated for further transcriptomic reconstruction. Trinity software (Grabherr et al., 2011; Haas et al., 2013) was employed to produce *de novo* assembly due to the lack of a reference genome for southern calamari. Corset software was then used for clustering contigs based on shared reads to eliminated possible redundancy (Davidson and Oshlack, 2014).

To establish a comprehensive functional annotation, the *de novo* assembly was mapped against seven different databases which included the NCBI non-redundant protein sequences (NR), NCBI nucleotide sequences (NT), Protein family (Pfam), eukaryotic Orthologous Groups (KOG), Swiss-Prot, Kyoto Encyclopedia of Genes and Genome (KEGG), and Gen Ontology (GO). Diamond 0.8.22 was used for annotation in NR, SwissProt and KOG with a threshold E-value of 10^{-5} for Nr and SwissProt, and 10^{-3} for KOG. NCBI blast 2.2.28+ was used to map NT with an E-value threshold of 10^{-5} . HMMER 3.0 and KEGG Automatic Annotation Server

(KASS) was used to map Pfam and KEGG respectively. NR and Pfam resulting annotations were then mapped against GO by Blast2GO v2.5 (Götz et al., 2008).

4.2.3.4 Differential gene expression and enrichment analysis

Clean reads, after trimming by Corset, were mapped using Bowtie2 v. 2.2.1 (Langmead and Salzberg, 2012) and expression levels were quantified by RSEM (Li and Dewey, 2011). To examine similarity among samples, Pearson correlations were used and visualisation via heatmap with Euclidean distances and a principal component analysis (PCA) were performed on the 18 samples using the R package pcaExplorer (Marini and Binder, 2019). Following that, differentially expressed genes (DEG) analyses were completed using the DESeq R package (Anders and Huber, 2010), where read counts were first normalised and a negative binomial distribution model fitted. Significance values were adjusted using the Benjamini-Hochberg approach to controlling the false discovery rate, where the threshold was set at $p.\text{adj} < 0.05$ and $\log_2 \text{FoldChange} > 2$. A GO enrichment analysis was implemented with the Goseq package (Young et al. 2010), and the threshold was applied as $p.\text{adj} < 0.05$. KOBAS software was then used to test the statistical enrichment of DEGs in the KEGG pathways with $p.\text{adj} < 0.05$ (Kanehisa et al., 2008).

4.3 Results

4.3.1 Critical thermal maximum (CT_{max})

Acclimation temperature had a significant effect on the CT_{max} in southern calamari ($\chi^2 = 69.3$; $df = 3$; $P < 0.001$). Squid maximum thermal limits were positively influenced by temperature of acclimation with an increase of ~20% between coldest (13 °C and 16 °C) and highest (25 °C) treatments (Figure 4.1). Individuals acclimated to a treatment temperature of 13 °C or 16 °C exhibited similar patterns with an average CT_{max} of 24.4 °C (± 0.7) and 24 °C (± 1) respectively.

CT_{max} increased to 25.6 °C ± 1.4 °C, 27.4 ± 0.7 °C and 29.5 ± 0.5 °C at treatments 19 °C, 22 °C and 25 °C, respectively (Table 4.1). All the values are presented as mean ± standard deviation.

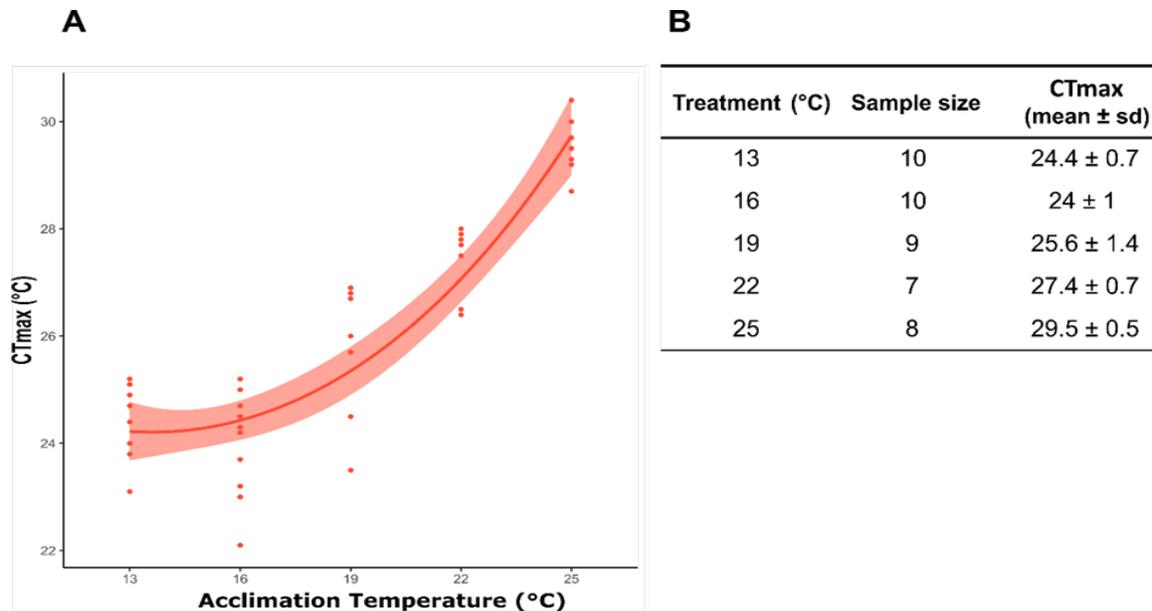


Figure 4.1. Critical thermal maxima (CT_{max}) of southern calamari acclimated at five different temperatures, A) GLMM model of the data: the thick line represents the expected value of the model for the variable measured. The shadow band corresponds to the 95% confidence interval, and the individual squid values are represented by red points. B) table of CT_{max} (mean ± sd) of southern calamari at sample size (N).

4.3.2 RNA-seq

4.3.2.1 RNA-sequencing and *de novo* assembly

RNA-seq analysis was used to examine the molecular pathways in squid in response to acclimation temperature (13 °C, 19 °C and 25 °C). Illumina sequencing supplied a total of 859,591,496 raw reads, of which 97.03% corresponded to clean reads with a mean output of 6.9 G per library and 38% GC content. *De novo* transcriptome assembly produced a total of 218,997 transcripts (N50 = 1157 bp and N90 = 359 bp), where the longest transcripts of each cluster were selected as unigenes (218,946 in total) for further analysis in southern calamari. The list of unigenes (average length 820 bp, ranging from 201 bp to 30,263 bp), referred to herein as transcripts, was considered as the transcriptome. Of the transcripts, 49.52% were <500 bp in length, with 42.89% between 500 bp and 2k bp and 7.78% longer than 2k bp, where

28.35 % (62,083) of them were successfully annotated in at least one of the seven databases; 21.28% (46,606) were successfully annotated on Nr, revealing a low level of annotation that is common in this class (Benoist et al., 2020). More details regarding the annotation of the seven databases for the southern calamari transcriptome can be found in the supplementary materials (Appendix 2.2.).

To gain greater insight into the underlying biology of squid thermal acclimation, KEEG annotation was used to identify the most relevant pathways across southern calamari transcription at a functional level, where 14,905 transcripts were annotated in 231 unique pathways (Table S.2.2.3). The most represented categories were endocytosis, spliceosome, and RNA transport with 272, 236 and 218 associated transcripts, respectively. Other major pathways represented were ubiquitin mediated proteolysis and protein processing in the endoplasmic reticulum as well as purine metabolism. Moreover, PI3K-AKT signaling and the MAPK signaling pathway were identified as environmental information processing categories with more associated transcripts (Table 4.2).

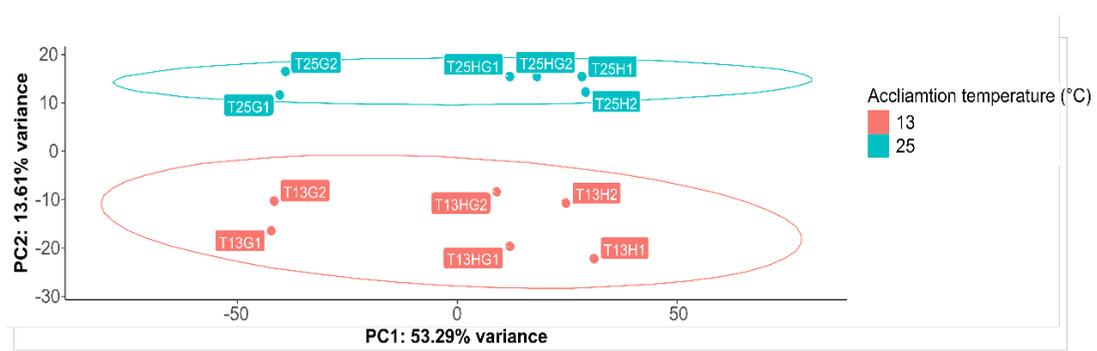
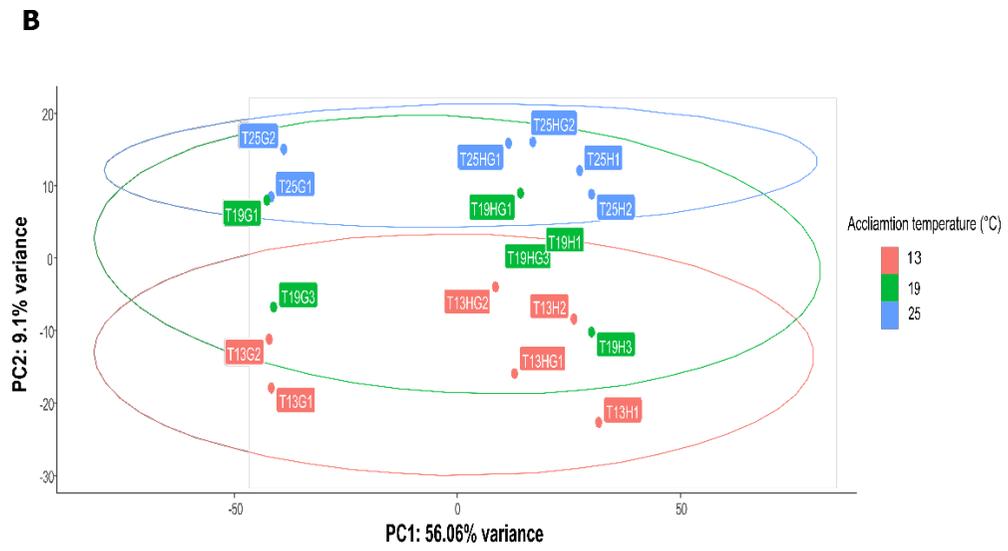
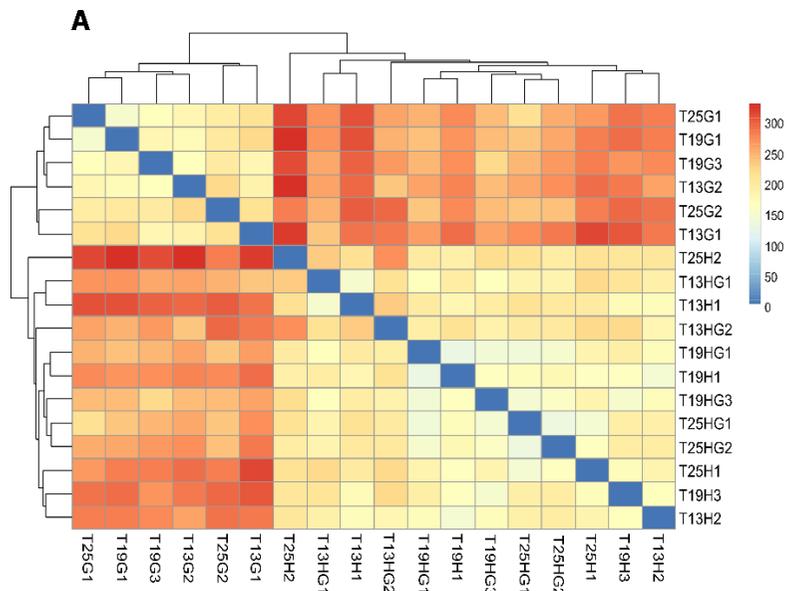
Table 4.1. KEEG pathways with more annotated genes in the southern calamari transcriptome

	Id pathway	Pathway description	Annotated genes
Cellular Processes	ko04144	Endocytosis	272
	ko04510	Focal adhesion	201
Genetic Information processing	ko03040	Spliceosome	236
	ko03013	RNA transport	218
	ko04120	Ubiquitin mediated proteolysis	209
	ko04141	Protein processing in endoplasmic reticulum	207
	ko03010	Ribosome	203
Environmental Information Processing	ko04151	PI3K-Akt signaling pathway	214
	ko04010	MAPK signaling pathway	209
Metabolism	ko00230	Purine metabolism	213

4.3.2.2 Differential gene expression analysis

The results of the pairwise comparison of all 18 samples can be found in supplementary material Figure S.2.3.2, where the Pearson coefficient between samples varied between 0.71 and 0.95. The PCA and heatmap showed the relationship of gene expression between temperature treatments and replicates (Figure 4.2). In here, treatment 13 °C and 25 °C are clearly divided in distinct groups, whereas treatment 19 °C overlaps between treatments. A clear separation of the two experimental groups (T13 and T25) highlighted the existence of fundamental differences resulting from the acclimation conditions (Figure 4.3). As a result, treatment 19 °C was eliminated from further differential expression analysis to identify possible pathways associated with acclimation to elevated and cold temperatures. The Pearson correlation and PCA plots also clearly signify that the gills are distinct from the branchial and systemic hearts, whereas the latter two tissues are indistinguishable in their expression patterns.

A total of 247 genes were DEGs between treatment 25 °C vs 13 °C. Of those, 102 genes were upregulated at 25 °C and 145 upregulated at 13 °C, where 163 (65.9%) of the genes were functionally identified, 69 upregulated at 25 °C and 92 at 13 °C. The most significantly upregulated genes at 25 °C with known function were small glutamine-rich tetratricopeptide repeat protein beta (SGTA), MAP3K12-binding inhibitor protein 1 (MBIP), thymidine phosphorylase (TYMP), proteasome-associated protein ECM29, and lipopolysaccharide-induced TNF-alpha factor (LITAF). At 13 °C, the most significant genes encoded NADH pyrophosphatase zinc ribbon domain (zf-NADH_PPase), E3 ubiquitin-protein ligase (MIB1), histone H4 transcription factor (Hinfp), 60S ribosomal protein L31 (RpL31), and Collagen alpha-1(IV) chain. A complete list of the DEGs for the T25 vs T13 comparison can be found in supplementary material (Appendix 2.3, Table S.2.3.2).

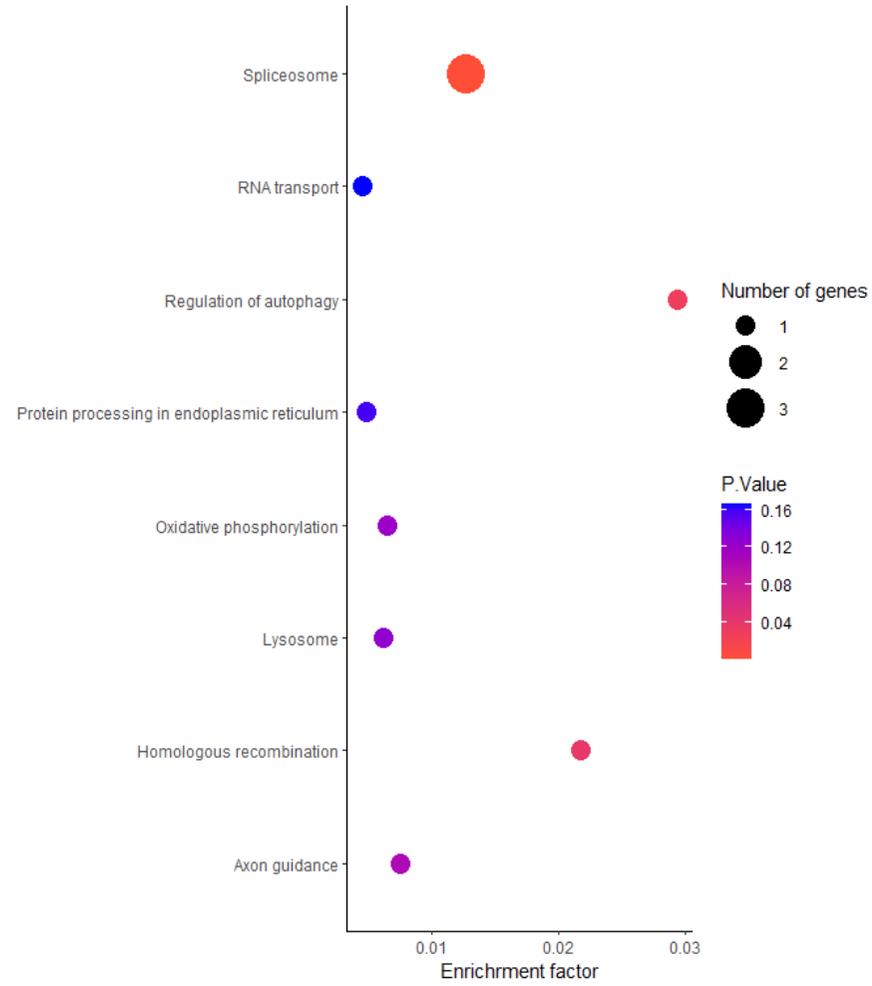
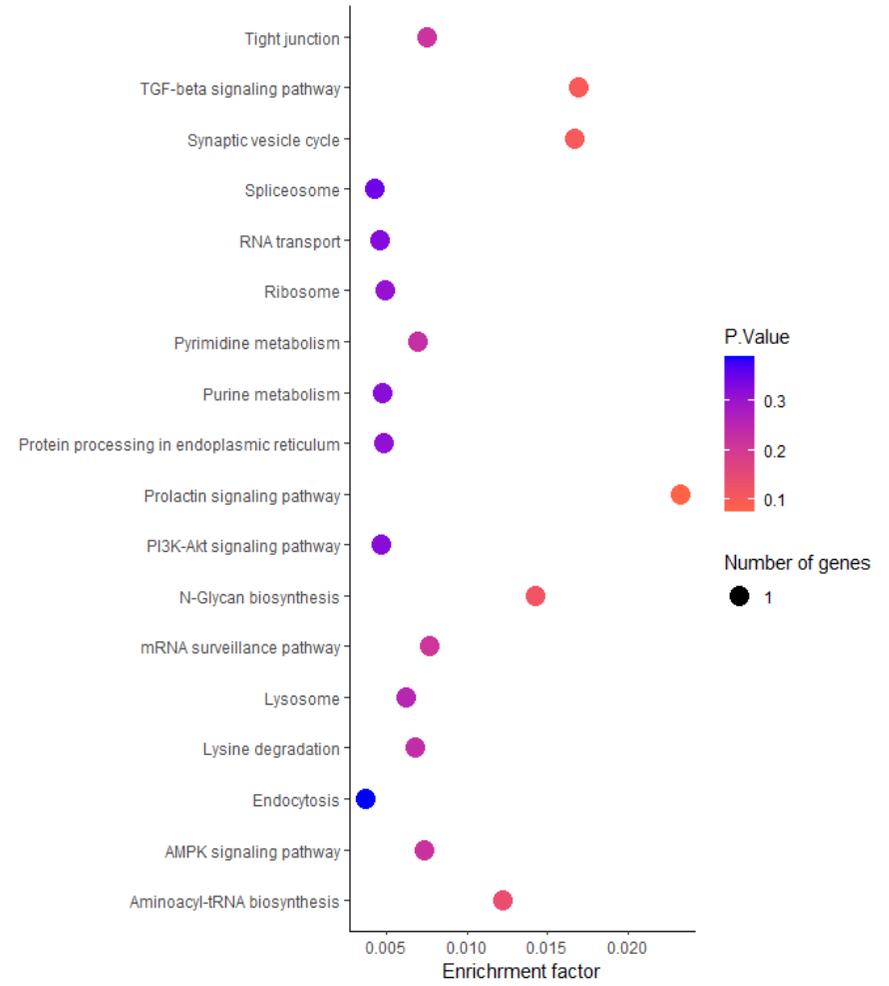


Enrichment analysis for Gene Ontology (GO) did not identify any significantly enriched GO terms between DE and non-DE gene sets ($p_{\text{adj}} > 0.05$) (Supplementary material, Table S 2.3.3.). A spliceosome pathway (KEGG ID: ko3040) was significantly enriched at treatment 25 °C, in which three DEGs (out of the 236 transcripts found in the transcriptome) were upregulated (Figure 4.4): heterogeneous nuclear ribonucleoprotein 87F (HNRNPs), calcium homeostasis endoplasmic reticulum protein (CHERP), as well as pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15/PRP43 (PRP43) which is responsible for driving rearrangements of the spliceosome's RNA-RNA and RNA-protein networks required for splicing (Hoskins and Moore, 2012). Additionally, upregulated genes were identified encoding RNA transport (eukaryotic translation initiation factor 3 subunit H 'EIF3H'), oxidative phosphorylation (NADH dehydrogenase 1 beta subcomplex subunit 11 'NDUFB11'), or homologous recombination (DNA topoisomerase 3-alpha 'TOP3A') pathways. The last one being essential for the accurate repair of DNA double-strand breaks (DSBs). At 13 °C, no KEGG pathways were significantly enriched ($p_{\text{adj}} > 0.05$), yet an encoding gene, serine/threonine protein phosphatase 2A (PPP2R1A), was found associated with two major pathways related to environmental information processing. These were the PI3K-AKT and AMPK signaling pathways which play a significant role in the regulation of mitochondrial biogenesis, autophagy, cell growth and proliferation. DEGs involved in spliceosome and RNA transport pathways were also found at upregulated at 13 °C (Figure 4.4).

Overall, the vast majority of annotated DEGs encode for proteins involved in RNA regulation/expression via transcription, translation or post-transcriptional modification with 33 upregulated genes at 25 °C (~50% of the DEGs with known function) and another 30 at 13 °C (~ 30% of the DEGs with known function) (Figure 4.6). For instance, transcription factors including TCF12 or zinc finger C3HC4 type were upregulated at treatment 25 °C. Meanwhile,

at temperature 13 °C, transcription elongation factor B polypeptide 3 (tceb-3), zinc finger 782 (ZNF782), transcription initiation factor TFIID, transposon Ty3-I Gag-Pol polyprotein (TY3B-I) and two zinc finger, C2H2 type were upregulated. Additionally, genes involved in ribosome biogenesis like 60S ribosomal protein L31 (RpL31) and sulfate transporter (SLC26A2) were identified in individuals acclimated at 13 °C. Some of the cold-induced genes have also been shown to play a role in splicing activity, like serine/arginine-rich splicing factor 12 (SRSF12), and tRNA-splicing endonuclease subunit Sen54 (TSEN54), as well as in translation of proteins such as leucine-tRNA ligase (LARS2) or eukaryotic initiation factor 4A-II (eIF4A), that are involved in the initiation of the mRNA translation promoting cell proliferation such as apoptosis, cell cycling or differentiation.

DEGs were also characterised by regulation of the stress-defence, immune, proteolysis, apoptosis and metabolism mechanisms which are often related with the organismal responses to thermal regulation, corresponding with 35% and 42% of the DEGs with known functions at 25 °C and 13 °C respectively (Table 4.3). Stress-defence response upregulated at 25 °C genes included NUP153, ALDH3A2, FBXL14, TRIM2, YLPM1 and ARM-repeat, where E3 ubiquitin-protein ligase (RFWD2), inactive Ufm1-specific protease (UFSP1), SET and MYND domain-containing protein 5 (Smyd5) were some of the genes involved in immune responses. TRIM2 in association with RFWD2, for instance, mediate the ubiquitination pathway, playing a neuroprotective function. Interestingly, a protein of the family of mitogen-activated protein kinases, MAP3k2, was upregulated at 25 °C, which directly relates to cellular responses to stimuli like heat shock. A unique gene-encoding function of the heat shock protein family (HSPs), AHSA1, which has a role in the activation of the HSP90, was found at cold temperatures (13 °C). However, HSPs expressed uniformly across treatments (Figure 4.7 and Supplemental material Table S.2.3.1).

A**B**

Genes encoding immune responses after cold acclimation were also found, including interferon regulatory factor (IRF1), leucine-rich repeat (LRR) or IKK (inhibitor of nuclear factor kappa- β). The IKK gene is found in other cephalopods species playing a significant role in their innate immune response (Salazar et al., 2015). Proteins encoding for proteolysis processes were also DEG at 13 °C, for example, PEST proteolytic signal-containing nuclear protein isoform X1 (PCNP) or ubiquitin carboxyl-terminal hydrolase 32 (USP32). These proteins are both involved in the ubiquitin pathway which control metabolic responses to thermal stress, where PCNP is also involve in the regulation of cell cycle. Related to apoptosis processes, upregulated genes at 25 °C including serine/threonine protein kinase (WNK1) and tetratricopeptide repeat (TPR), whereas sin3 histone deacetylase (SDS3), STE20-related kinase (STRADA) or Septine-7 (Sept7) were upregulated genes at cold acclimation temperature.

4.4 Discussion

Due to the current rates of global climate change, assessing the adaptation potential of species to ocean warming is essential to better forecast species responses to future conditions and ultimately their associated communities and ecosystems. Here, the effect of acclimation temperature on southern calamari was examined for the first time by the integration of physiological traits (CT_{max}) and RNA-seq methods with the aim of determining the fundamental mechanisms behind the purported resilience of squid to changes in environmental temperature.

Table 4.2. DEGs between T25 vs T13, that encode defence/stress response functions.

T25 vs T13	Symbol	Putative description	Log2Folchange
Upregulated 25 °C	NUP153	Nuclear pore complex protein	6.8
	ALDH3A2	Fatty aldehyde dehydrogenase	inf
	FBXL14	F-box/LRR repeat protein 14	4.7
	TRIM2	Tripartite motif-containing protein 2	inf
	YLPM1	YLP motif-containing protein 1	inf
	ARMC5	Armadillo repeat-containing protein 5	inf
Upregulated 13 °C	ECoRII	EcoRII C terminal//tRNA synthetases class I (E and Q), catalytic domain	inf
	MIB1	E3 ubiquitin-protein ligase	-3.1
	XPC	XPC-binding domain	inf
	AHSA1	Activator of 90 kDa heat shock protein	-4.8

4.4.1 Critical maximum limit in response to thermal acclimation

In the present study, acclimation had a profound effect on squid upper thermal limit (CT_{max}), which increased with temperature of acclimation. These results suggest that in a short period of acclimation (7–9 days), squid have the ability to increase their thermal window via phenotypic plasticity, supporting the idea that thermal limits are plastic and sensitive to individual thermal history (Wang and Overgaard, 2007). Similarly, in other cephalopods, upper thermal limits are associated positively with temperature, for example, in the early life stages of *Loligo vulgaris* (common squid) (Rosa et al., 2012) or *Enteroctopus megalocyathus* (Patagonia red octopus) paralarvae (Uriarte et al., 2018).

These findings suggest a high phenotypic capacity in cephalopod species which may allow for faster evolutionary adaptation under long-term change in environmental conditions. Additionally, acute CT_{max} experimental approaches, as examined in the present study, provide insight into a species' ability to survive under a rapid short-term extreme event (Bates and Morley, 2020). Consequently, southern calamari potentially have the capacity to adapt quickly to rapid environmental changes, conferring an advantage for this species to survive under short-term extreme climate events such as heatwaves. Nevertheless, for populations to persist through changing conditions, individuals also need to be able to perform activities such as growth and reproduction (Chen et al., 2018). Previous studies (Chapter 2) indicated that southern calamari metabolic performance would probably decrease at higher temperatures (22 °C and 25 °C). Thus, individuals might have the ability to rapidly acclimate and survive for short periods of time but overall, over longer time periods, performance may be impaired resulting in a constraint in more complex traits. Further studies should investigate the impact of acclimation time on complex performances to provide insight into evolutionary adaptation of this seemingly plastic taxa.

4.4.2. Molecular response to thermal acclimation

The development of *de novo* transcriptomic techniques has offered the unique opportunity to study genetic expression at the tissue or organismal level in species without a sequenced genome, such as southern calamari. Here, the transcriptome of southern calamari previously acclimated to three thermal scenarios (13 °C, 19 °C and 25 °C) were obtained with a 28% annotation success which is comparable to transcriptomes previously published for cephalopod species (Liu et al., 2016; Tian et al., 2018; García-Fernández et al., 2019).

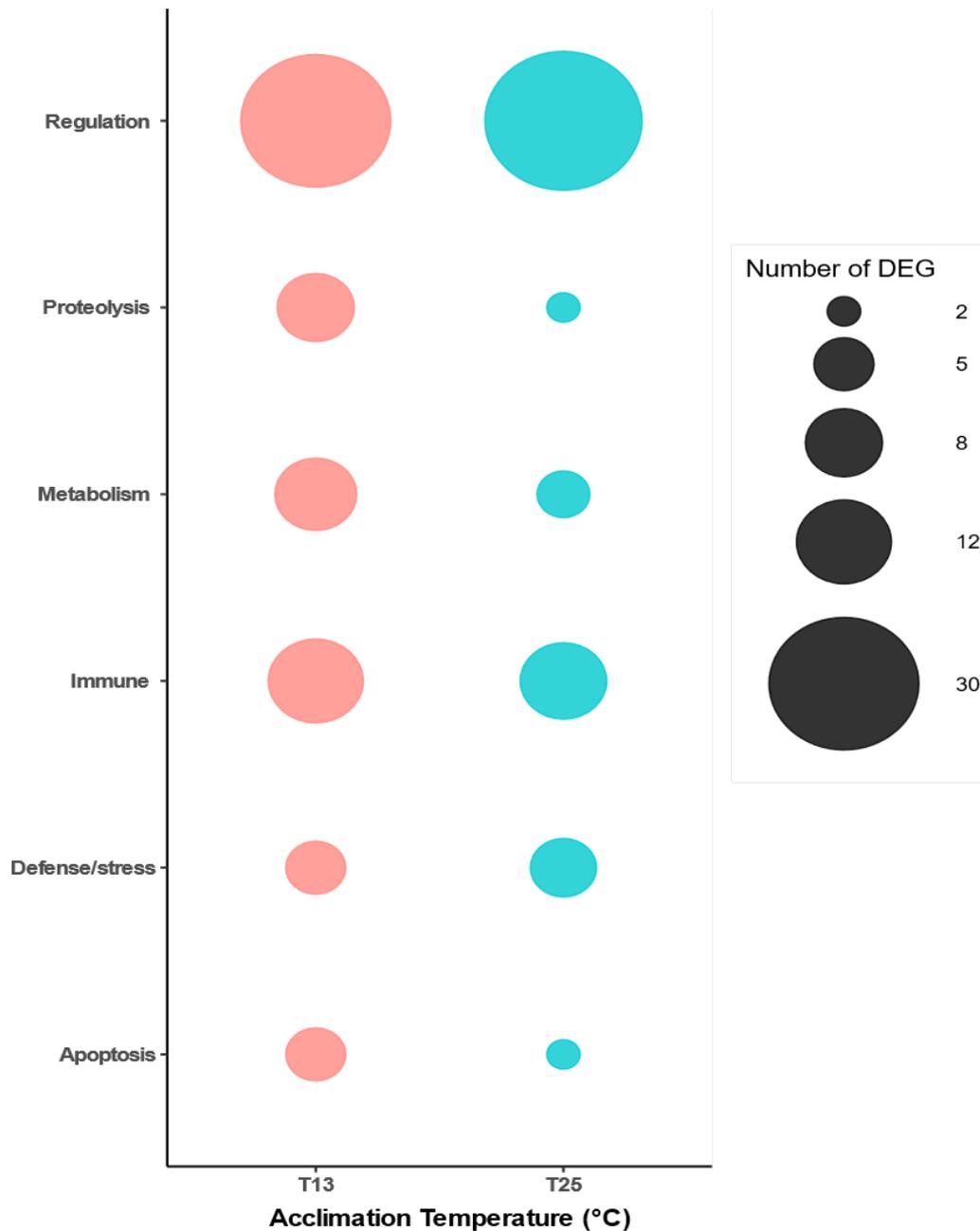


Figure 4.5. number of DEGs with known function group by functional responses including regulation (transcription, translation and post-transcription modification), proteolysis, metabolism, immune system, Defence/stress mechanisms and apoptosis process.

Modification in gene expression at multiple levels, including transcriptional, post-transcriptional and translational regulation, is believed to be one of the major cellular adaptive responses to changes in environmental conditions (Lackner et al., 2012; Anttila et al., 2014).

Results here revealed that southern calamari appear to have high capacity to modify RNA expression, either through regulating the transcription rate of specific genes (through transcription factors and transposons) or by splicing activity. The majority of the differentially expressed genes (DEGs) are involved in RNA expression or modification, 50% and 30% at 25 °C and 13 °C, respectively. Overall, these results indicate that squid rely on mechanisms of RNA regulation/expression to rapidly acclimate and cope with sudden changes in temperature. Species modify their RNA by different mechanisms, with post-transcriptional modification by splicing probably one of the most commonly used in eukaryotes (Garrett and Rosenthal, 2012b). Increasing evidence suggests that alternative splicing is important for adaptation responses to different stress conditions (Fujikake et al., 2005; Ali and Reddy, 2008; Hoskins and Moore, 2012; Gu et al., 2018). Not surprisingly, here, spliceosome (ko03040) was found to be one of the main pathways with 236 associated transcripts in the southern calamari transcriptome. Furthermore, different splicing factors were DEGs at either elevated or cold temperatures, with KEGG enrichment analysis demonstrating that the pathway was significantly enriched at elevated temperatures (25 °C). These results indicate that splicing activity likely plays a crucial role in squid thermal adaptation to future warming, conferring flexibility to respond rapidly to stressful environmental conditions.

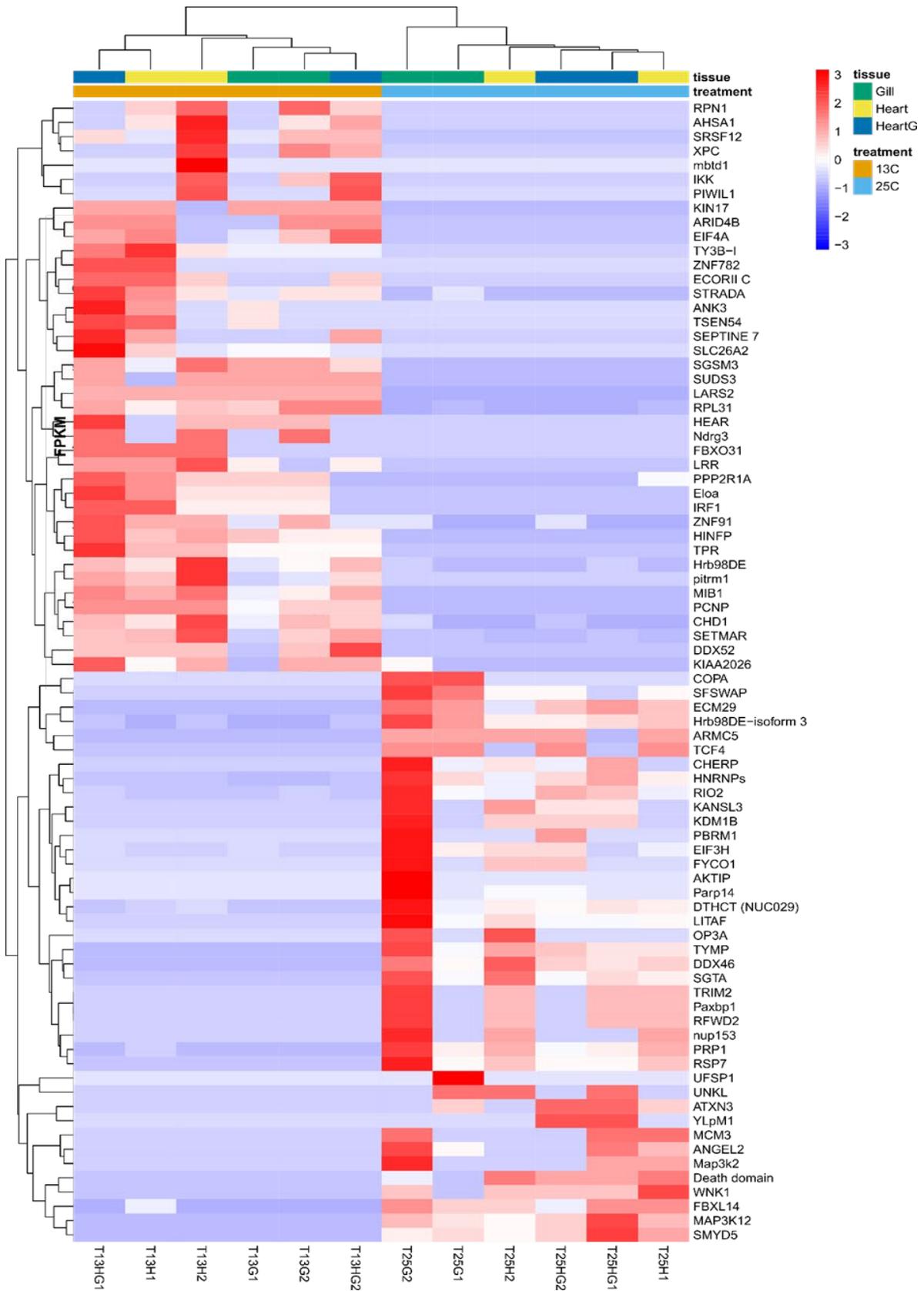


Figure 4.6. heatmap of the DEGs between temperature of acclimation 25 °C vs 13 °C involved in RNA expression, immune, stress/defence, and apoptosis responses per treatment and tissue.

In cephalopods, another post-transcriptional mechanism, RNA editing by deamination of adenosine to inosine (A-to-I), has been also proposed as a mechanism which could confer advantages under challenging conditions (Garrett and Rosenthal, 2012b). This mechanism offers to individuals the ability to turn on and off their proteins conferring some benefits in comparison with DNA editing (Garrett and Rosenthal, 2012), as well as recoding mRNAs, giving them the option to express diverse, and functionally distinct protein isoforms (Garrett and Rosenthal, 2012b; Alon et al., 2015). The ADAR (adenosine deaminase that acts on RNA) family of enzymes are responsible for A-to-I RNA editing (Alon et al., 2015), and ADAR 1 was found expressed across tissues and treatments with similar expression in southern calamari (Figure 4.7). In polar octopus, it was also found that the RNA editing process accentuated adaptation of the potassium channels(K^+ channels) to cold temperatures (Garrett and Rosenthal, 2012a). These results suggested that A-to-I RNA editing is probably associated with the mechanisms leading to the high phenotypic plasticity observed in cephalopods. Tissues examined in the present study were the systemic heart, branchial hearts, and gills, and high A-to-I RNA editing activity has also been recorded in the squid nervous system (Liscovitch-Brauer et al., 2017). This implies that A-to I RNA editing is not only limited to the nervous system and that squid might also use it across different tissues, probably to diversify their functionality. Further research examining the phenotypic plasticity of the squid nervous system, as well as other tissues under different climate scenarios, will provide a better understanding of the role of RNA editing in plasticity and thermal regulation.

Further genes encoding C2H2 zinc finger transcription factors were also found expressed in the squid transcriptome in the present study. The upregulation of these DEGs at 13 °C suggested that this family has a major role in the response to changes in temperatures, in particular for cold adaptation, and makes them a potential candidate for thermal adaptation in this species. Another mechanism that seems to be more likely associated to cold acclimation in

squid was ribosome biogenesis. Genes encoding this function were upregulated at 13 °C, for example HEAT repeat, yet no genes related to this process were DEGs at elevated temperatures. Ribosome biogenesis and in particular this gene have been associated with cold acclimation in previous studies on zebra fish (Long et al., 2013). This process is necessary for cellular adaptation, growth and proliferation (Leary and Huang, 2001) which seem to be beneficial for squid under unfavourable cold conditions.

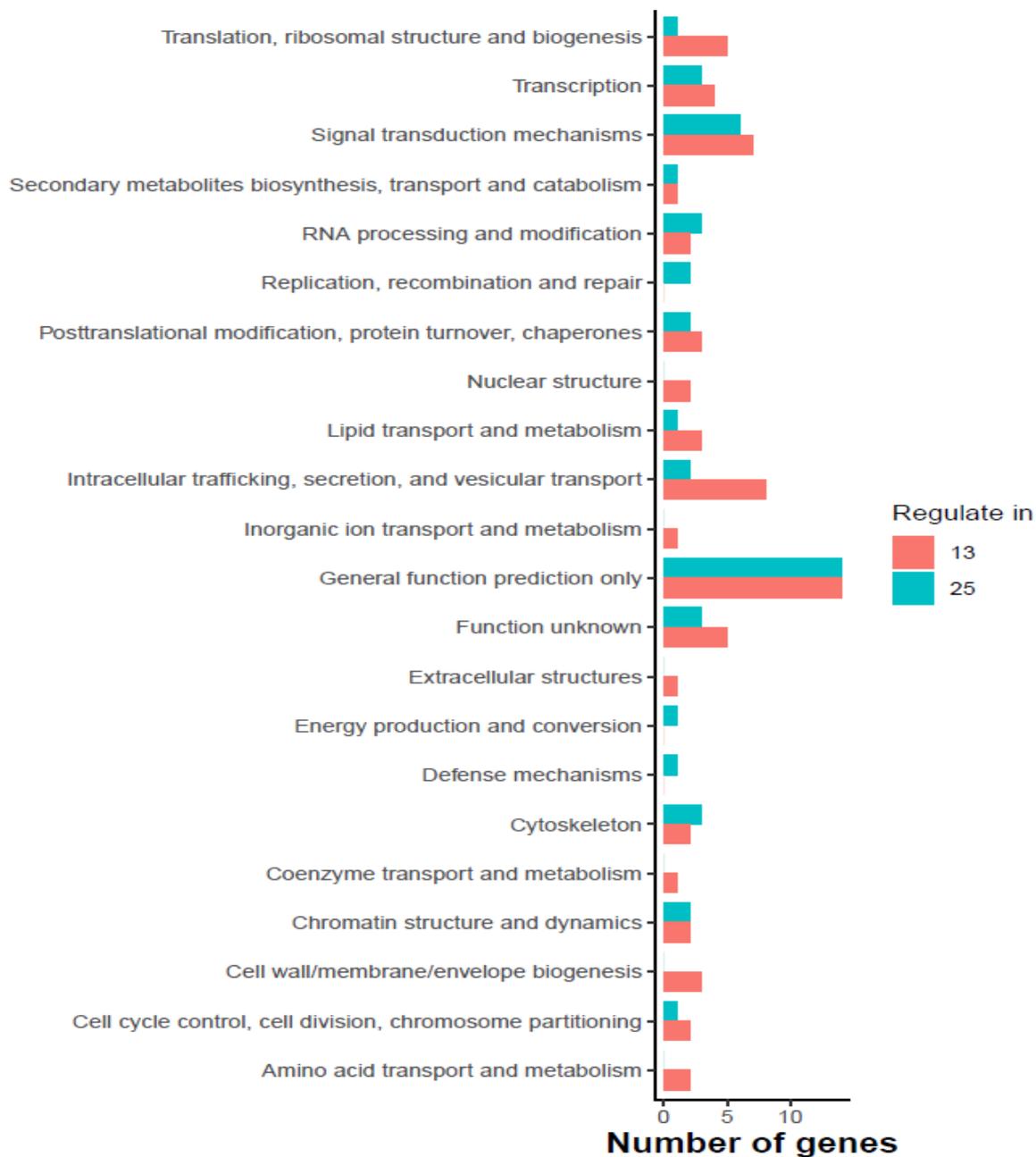


Figure 4.7. classification of the DEGs of the annotated transcripts by KOG at both temperatures of acclimation.

Interestingly in the present study, GO results did not show any significant enriched GO terms of the DEGs and only Spliceosome in the KEGG analysis, which could be indicative of highly functional use of the pathways by squid in response to both low and elevated temperatures, as well as a broader plastic transcriptional response. In other marine invertebrates, the lack of differentiation in enrichment terms has been associated with a wide acclimation ability of species (Clark et al., 2021), which could be indicative of plastic transcriptional responses and linked to higher thermal tolerance of the organisms (Bernal et al., 2020). Still, two pathways involved in environmental information processing were found to be highly representative in the southern calamari transcriptome; phosphatidylinositol 3-kinase (PI3K)-AKT and the MAPK signaling pathway, both of which have previously been found to be associated with thermal responses (Anestis et al., 2007; Moustafa et al., 2014; Wang et al., 2015; Chen et al., 2016; Liu et al., 2017). Previous studies have shown that the (PI3K)-AKT signaling pathway could be activated by many types of cellular stimuli, including changed environmental conditions, which regulate fundamental cellular functions such as transcription, translation, proliferation, growth, and ultimately survival (Tian et al., 2018). Meanwhile, MAPK signaling pathways play an important role in complex cellular programs like cellular proliferation, differentiation, or apoptosis (Zhang et al., 2019) and have been implicated in the forming of temperature tolerance in different organisms (von Mering et al., 2002; Long et al., 2013; Tian et al., 2018). Additionally, this pathway plays an important role in the immune process of different organisms, particularly in their innate immune response (Zhan et al., 2018). Here, 213 genes were annotated in the PI3K-AKT and 209 genes in the MAPK signaling pathway across all 18 libraries, implying that both pathways might have a significant role in squid thermal resilience to changes. Interestingly, serine/threonine protein phosphatase 2A (PP2A) was regulated at cold temperatures of acclimation, which is indeed known to be involved in the

PI3K-AKT and AMPK signaling pathways (Figure 4.4), playing a major role in cell homeostasis to keep a stable internal environment (Janssens and Goris, 2001).

Genes encoding defence and stress functions is a classic species response to deal more efficiently with changes in temperature and have been previously shown in different marine species (Gerken et al., 2015; Tian et al., 2018; Zheng et al., 2019). These responses involve the expression of a variety of evolutionarily conserved stress-responsive genes whose function include protein folding and repair, DNA repair, removal of damaged proteins or genes controlling the cell cycle (Somero, 2010; Komoroske et al., 2015). In this study, DEGs related to stress and defence functions were upregulated at both cold and elevated temperatures of acclimation (Table 4.2). For example, tripartite-motif-containing protein 2 (TRIM2), which can mediate autoubiquitylation, was upregulated in individuals acclimated at 25 °C. By contrast, the EcoRII gene and E3 ubiquitin-protein ligase (MIB1) were upregulated at cold acclimation (13 °C). For instance, MIB1 is well known to be involved in DNA repair and ubiquitin mediated proteolysis (Lyzenga and Stone, 2011; Wang et al., 2018). Furthermore, apoptosis-related genes are likely to encode proteins involved in programmed cell death, acting as a defence strategy (Sleight et al., 2018). For instance, at elevated temperatures, the death receptor of tumour necrosis factor (TNF), which is involved in the apoptosis mechanism and had been previously identified as a defence mechanism in response to thermal stress was upregulated at elevated temperatures (Moya et al., 2012). The use of these stress and defence mechanisms by southern calamari is in accordance with those previously reported in other species of marine invertebrate (Pérez-Portela et al., 2020; Clark et al., 2021) underpinning resilience to changes in temperature conditions.

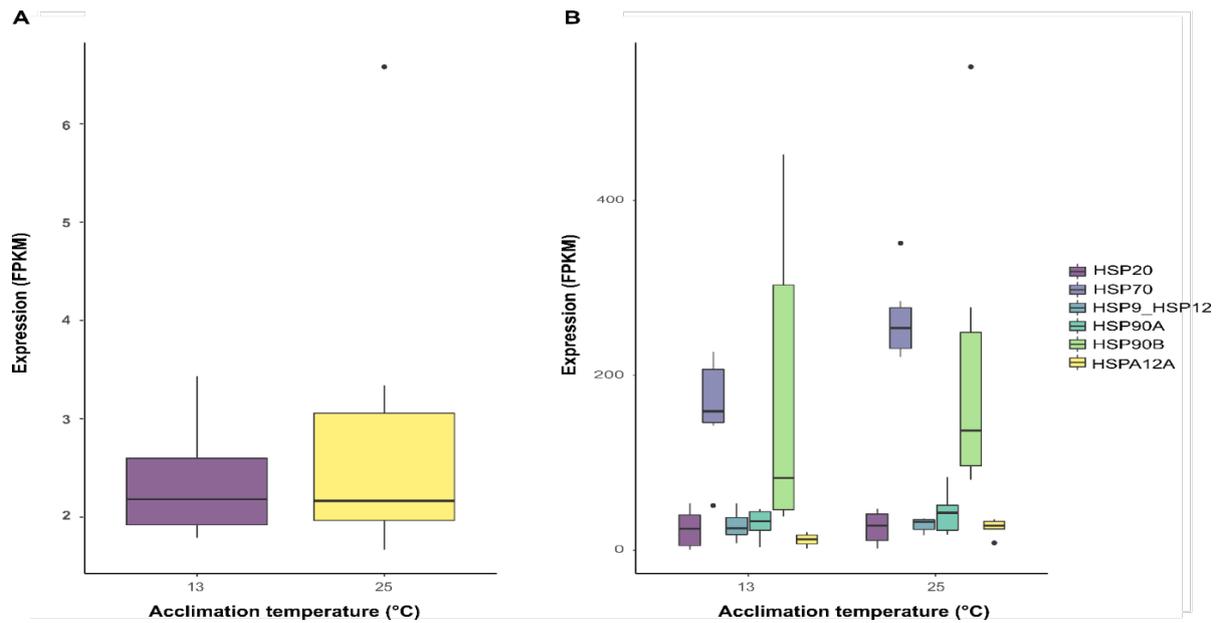


Figure 4.8. expression at lower (13C) and higher (25C) temperature treatments of A) expression of gene-encoding ADAR-1 in squid, B) examples of the expression (FPKM) of different proteins of the HSPs family.

Heat shock proteins (HSPs) are one of the major protein families known for their role in response to thermal stress, mediating different cellular processes, such as protein translocation or apoptosis regulation (Garrido et al., 2001). In the present study, multiple HSP encoding genes were found in squid at both the high and low temperature treatments, where HSP70 and HSP90B were the most highly expressed (Figures 4.7, supplementary material Appendix 2, Table S.2.3.1). Interestingly, an activator of the HSP90 (AHSA1) was the only protein upregulated at the low temperature (13 °C) in comparison with the high temperature. The activation of the HSP family in response to stress has been documented widely in marine animals (Komoroske et al., 2015; Chen et al., 2018; Pérez-Portela et al., 2020), however, even if these can be interpreted as a general response, the regulation of particular genes is usually context-dependent (Clark and Peck, 2009; Sleight et al., 2018). For example, in marine invertebrates, the identification of a massive expression of the HSPA12 heat shock protein 70KD gene family, suggested that these proteins might act as regulators highlighting adaptation to heat (Clark et al., 2021). In cephalopods, the function of these proteins is not entirely clear.

For instance, in previous studies of squid (*Loligo vulgaris*) at early life stages HSPs were found in paralarvae under thermal stress conditions but not in embryos, suggesting that perhaps the HSP family is life stage-specific or may not play an essential role in cephalopod responses under stress conditions in comparison with other species (Rosa et al., 2012). Our results suggest that the proteins in this family possibly have a significant role in the thermal regulation of squid. However, further studies should be conducted to fully understand the function of HSPs in cephalopods to identify if HSPs confer a higher adaptation to external temperatures, as well as further resolving their function across life stages. Additionally, HSPs have been identified to play an important role in innate and adaptive immune responses in organisms (Xie et al., 2015).

Cephalopods, like other protostomes, lack an adaptive immune system and rely on an innate immune system which refers to non-specific physical, chemical and cellular defence mechanisms, responding immediately to the pathogen/stressor (Benoist et al., 2020). Genes encoding proteins assigned with innate immune responses in other cephalopods (Tian et al., 2018; Benoist et al., 2020) were found to be DEGs across both acclimation temperatures. For example, the inhibitor of nuclear factor κ -B kinase subunit (IKK) was upregulated at 13 °C or F-box/LRR repeat protein 14 upregulated at T 25 °C. Similarly, proteins associated with leucine-rich repeat (LRR) have been expressed under thermal stress in other organisms (Sleight et al., 2018). Additionally, F-box proteins are regulatory proteins playing key roles in proteasomal degradation which has been linked to regulation of low temperature stress tolerance in plants (Venkatesh et al., 2020). The regulation of immune-related genes by southern calamari suggested that cephalopods could rely on innate immune capacity to cope with changes in thermal scenarios.

The enhanced energetic costs associated with living at elevated temperatures is considered a major potential impact that climate change could have on species, due to the increase in oxygen

consumption and the decrease in aerobic scope (Pörtner and Farrell, 2008). Results here show two DEGs involved in the electron transport chain, NADH dehydrogenase and cytochrome C, at both temperatures of acclimation (13 °C, and 25 °C), suggesting that the individuals were respiring aerobically. Similar results were also found in other marine invertebrates in response to acute warming (Clark et al., 2017) .

4.5 Conclusions

Phenotypic plasticity is an essential part of the responses of individuals to environmental challenges. These responses are typically associated with modification of gene expression (Healy and Schulte, 2018). According to our results, southern calamari appear to have the capacity to increase their thermal tolerance limits following exposure to higher or lower temperature regimes, which might be beneficial to facing current and future warmer conditions. Transcriptomic analysis revealed the significance of mechanisms of RNA regulation, such as spliceosome as a primary pathway to diversify their proteome and be able to shift their gene expression rapidly to handle the new environmental conditions. DEG analysis also highlighted other biological processes that are likely to be important in squid thermal responses such as ribosome biogenesis, proteolysis, apoptosis, or innate immune responses. Overall, these findings provide insights regarding the underlying mechanisms of thermal acclimation of cephalopods. However, a lack remains of a broader approach examining the molecular and proteomics pathways involved in the evolutionary and developmental response in cephalopods. These studies could be highly informative in understanding how highly plastic species with fast growth rates are likely to respond to climate change, as these attributes may provide an advantage for rapid adaptation to environmental changes.

Chapter 5: General discussion

This thesis provided a novel and integrative approach to examining the effect acclimation temperature may have on squid performances in the near future. To date, this is the only study integrating the effect of acclimation temperature on physiology and behaviour as well as at molecular level in any cephalopod species. Individuals can respond to changes in environmental conditions in different ways, from biochemical buffering, through to physiological mechanisms and genetic adaptations, and behavioural modifications (Peck et al., 2014). In terms of climate change, the most relevant of these responses has been argued to be phenotypic plasticity, primarily through the acclimation of physiological processes to altered conditions, and through genetic adaptation (Somero, 2010). Cephalopods, due to their inherent phenotypic plasticity and fast-paced life history strategies, might respond to future changing environmental conditions faster than other species and could be ‘winners’ under future climate change scenarios (Doubleday et al., 2016).

Chapter 2 investigated the effect of temperature on metabolic performance and behavioural thermal preferences in individuals acclimated at five different thermal scenarios (13 °C, 16 °C, 19 °C, 22 °C and 25 °C). The results suggested that squid would actively seek to move to more optimal temperatures according to their metabolic capacities. A direct relationship was found between acclimation temperature and oxygen consumption, where standard, routine and maximum metabolic rates increased with temperature, leading to a decrease in aerobic scope at 22 °C. This is in accordance with the OCLTT hypothesis (Pörtner and Farrell, 2008) that suggests the oxygen supply to the tissues would not be enough to meet the increasing demands. This hypothesis assumes that the thermal optimum of the aerobic scope coincides with the

temperature where performance and long-time fitness is maximised, and beyond this temperature overall fitness reduces (Pörtner and Knust, 2007). Squid mortality also increased at the higher temperature treatments (22 °C and 25 °C) in further support of the OCLTT hypothesis that predicts performance will decrease once the thermal optimum is exceeded. Coincidentally, the mortality occurred during or just after the exhaustion protocol had been performed. This is not surprising, as the jet propulsion mode used by squid to escape is well known to be energetically inefficient (O'Dor and Webber, 1986). This result suggests that the cost of energetically expensive jet propulsion is higher than squid may be able to sustain in the future, as energy requirements for maintaining metabolism will increase with ocean warming, limiting the energy available for other activities such as hunting, escape and growth. Furthermore, results indicated that individuals acclimated to optimal metabolic temperatures (19 °C) have a higher EPOC and longer recovery times, suggesting that those individuals have a higher anaerobic capacity for sustained swimming which could be associated with the use of more glycogen or production of lactate. This also could be linked to the increase in mortality at higher temperature treatments due to reduction in the anaerobic capacity and subsequent decrease in performance after exhaustive exercise.

The thermal preferences of southern calamari indicated that individuals actively seek thermal conditions that are more beneficial for their metabolic performance. For instance, individuals acclimated to 25 °C sought cooler temperatures close to 22 °C, meanwhile individuals at 13 °C moved to warmer temperatures around 15 °C. This could have significant implications for the future distribution of this species, given climate change projections. Currently, eastern Tasmanian waters can reach a maximum of 22 °C with an average of 19 °C during summer. As ocean warming continues to intensify, southern calamari might be exposed to water temperatures higher than their metabolic thermal optimum – during marine heatwaves for example. Individuals may respond and move to more favourable conditions for thermal refugia

as a rapidly interim response until evolutionary adaptation can occur (Nay et al., 2015; Habary et al., 2016; Gervais et al., 2018). However, capacity for behavioural thermoregulation may be limited by lack of coastal habitat poleward of Tasmania. Consequently, the species may need to trade-off between more physiologically favourable conditions and other factors like habitats or availability in resources.

Chapter 3 presents an examination of predatory responses at five different acclimation temperatures representing current and future climate scenarios in Tasmanian waters. Here, the behavioural responses of southern calamari differed between treatments. The motivation to attack prey, as well as capture success increased with acclimation temperature, with 80% to 100% of the squid attacking their prey at the higher temperatures (22 °C and 25 °C), compared to only 30% at lower temperatures (13 °C and 16 °C). Furthermore, other differences in the responses of individuals which attacked prey were also found across temperature treatments (19 °C, 22 °C and 25 °C). One of the most notable differences was the latency time to attack prey, which was significantly reduced to only a few seconds at 22 °C and 25 °C, whereas it took minutes to initiate the attack at 19 °C. In contrast, handling time (time that squid took to manipulate and kill the prey) increased at higher temperatures, as did the number of strikes needed to successfully capture prey at 25 °C. Overall, these results suggested that squid need to increase their feeding rates to meet the increased energy requirements at higher temperatures, in order to maintain their energetic balance of to meet the elevated cost of living in a warming world. The requirement for increased feeding rates at higher temperatures is potentially supported by other aspects of their predation strategies, such as body patterns and colour being unaffected by temperature but counteracted by their ability to capture prey being limited at higher temperatures. However, the decrease in squid ability to capture their prey at first attempt, as well as the increase in handling time, could also be related to the effect of temperature conditions on the prey. The Australian salmon prey were exposed to the same thermal scenarios

as the squid, which could have also altered their behaviour as temperature increased or decreased. This may reduce the potential for squid to capture the prey successfully at first attempt as the increase in water temperature could increase activity and locomotion on the fish. Future research should include the effect of the acclimation temperature on the prey to understand how their behaviour may be influenced biotic interactions.

Squid are opportunistic species with high food consumption rates, that play an important role linking trophic levels. The changes in their predatory behaviours found here and the possible increase in food consumption as temperatures increase, could have significant consequences at the community and ecosystems levels by way of cascading effects. If feeding rates increase with temperature, this could lead to more pressure on their prey, potentially affecting the stability of the prey population and potentially impacting ecosystem stability. On the other hand, their higher motivational responses as well as their reduction in ability to capture the prey at elevated temperatures suggest that squid will be more willing to continuously search for prey, leading to bolder behaviours and potentially an increased predation risk. This could possibly help regulate the population and assist with maintaining ecosystem stability.

Chapter 4 examined the thermal acclimation capacity of southern calamari in terms of the upper thermal limits (CT_{max}) in individuals acclimated to the same five different temperatures as for thermal preferences, metabolism and predatory behaviour. Additionally, the possible molecular pathways associated with their responses to temperature were examined at 13 °C, 19 °C and 25 °C. Acclimation temperature had a positive effect on squid CT_{max} , suggesting their capacity to rapidly alter their thermal window thru phenotypic plasticity. This could be beneficial for southern calamari during a short-term climate event such as a marine heatwave. RNA-seq was used to identify pathways involved in thermal acclimation. The results of the RNA-seq study here suggested that squid used post-transcriptional RNA modifications (ADAR,

splicing activity) as the main mechanism of molecular response to changes in environmental temperature, which could confer to squid the ability to quickly modify their RNA expression. Other classical mechanisms of thermal response were also observed across temperatures treatments, including proteolysis, and defence responses such as apoptotic and immune responses.

Understanding species responses to changes in environmental conditions, such as ocean warming, are critical for accurately predicting long-term trends in natural ecosystems. As such, individual physiological and behavioural traits under different thermal scenarios could be incorporated into ecosystem models to forecast responses of this species under future conditions, for example, models of intermediate complexity (Twinn et al., 2020), process-based/mechanistic SDMs (Fernandes et al., 2013; Queirós et al., 2015) or Age-structure end-to-end (Pethybridge et al., 2019).

5.1 Limitation and future directions

Cephalopods are generally a challenging group to maintain for long periods in captivity, which is likely to be a contributing factor in the general lack of information for this group in terms of continuing climate change. Advances in the culture of cephalopods is therefore essential to determining what the future holds for this important group. Many cephalopods exhibit sexual dimorphism in size and growth, often maturing at different age and sizes. Therefore, the study of sex differences of cephalopod physiology and behavioural performance under various climate change scenarios would be highly informative for future projections, which was prohibited here due to logistic limitations.

The results here indicate that southern calamari actively seek out temperatures more favourable for their metabolic performance. However, suitable shallow habitat for this species is limited further south. Given this geographical or spatial restriction and the predatory response that squid showed at higher temperatures, a trade-off might occur between available resources and preferred temperatures. Future research should investigate the relationship between

temperature, food and habitat preferences, which could have implications for future distribution of individuals.

Previous studies had reported temperature to have a direct effect on CT_{max} in cephalopods at early life stages (Rosa et al., 2012; Uriarte et al., 2012) – similar to the results reported here for adults. However, the current understanding of thermal limits is minimal in this critical group. Establishing critical thermal minimums in addition to CT_{max} will be necessary for determining thermal tolerance windows. This could help to better predict the future distribution of cephalopod species. Here, the metabolic performance of southern calamari suggests that the species may be more energetically limited at low temperatures than at high temperatures, so understanding the role of cold adaptation could be also important to identifying possible poleward species redistribution.

Cephalopods are known to be a highly plastic group in terms of many different life history characteristics, particularly in response to temperature (Jackson and O'Dor, 2001; Pecl et al., 2004a; Pecl and Jackson, 2008), and thermal plasticity is likely to play a major role in determining species performance (including biotic interactions or physiological capacities) in a changing world (Warren et al., 2017). Thermal acclimation could be modified across the life cycle (i.e. within one individual life span) (Donelson et al., 2016; Fox et al., 2019), which could confer potential for evolutionary adaptations. In cephalopods, how acclimation may occur throughout the life stages had not been previously examined. Future studies should consider exploring phenotypic plasticity across life stages and generations to understand the potential of cephalopods for adaptation, which could be key for their survival under future climate change scenarios.

Oceans are not only experiencing warming, but also acidification, decreasing dissolved oxygen, pollution, overfishing and change in productivity, resulting in further risk for organisms. It will

be important to determine if multiple stressors have synergetic, antagonistic or additive effects on the physiology and behaviour of cephalopods, to better predict how populations will respond in the future.

5.2 Conclusions

Overall, the results suggest that squid populations could face consequences under future warming scenarios in Tasmanian waters, as increasing energetic demands could limit their performance and overall fitness, affecting their survival rates. To support the energetic cost of living at elevated temperatures, it appears that southern calamari could increase their feeding intake as they were highly motivated to attack and capture their prey at higher temperatures. They could nonetheless also actively seek thermal refugia, choosing temperatures more favourable to their physiological optimum to alleviate their physiological challenges, possibly even shifting their distribution. Southern calamari show a high phenotypic plasticity in their CT_{max} , which is probably correlated with the use of molecular mechanisms, like post-transcriptional modification (A-to-I RNA editing or splicing activity) or changing RNA expression by transcription factors (including zinc finger helix loop helix, or EF-hand domain) allowing them to diversify their proteome. This could potentially facilitate an increase in their thermal tolerance window, leading to a faster evolutionary response as a species' ability to cope with temperature changes will rely on their inherent thermal tolerance, acclimation and evolutionary adaptation capacity.

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Appendix 1

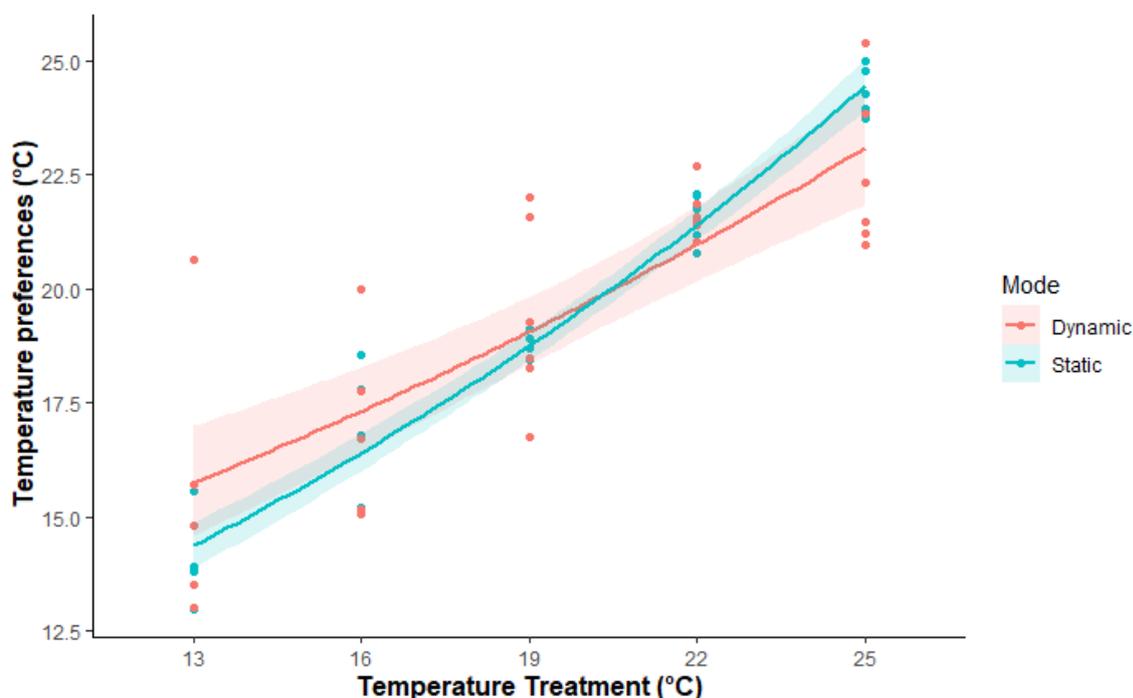


Figure S.1.1 Temperature preferences of per acclimation treatment separated in static, and dynamic phase.

Table S.1.1. GLMMs model summary of the metabolic traits (Excess post-exercise oxygen consumption and recovery time) and temperature range in relationship with acclimation temperature. CI correspond with the confidencial interval (2.5% to 97.5%) and statistic correspond t value

Predictors	EPOC				Recovery.time			
	Estimates	std. Error	CI	Statistic	Estimates	std. Error	CI	Statistic
(Intercept)	4.67 ***	0.35	3.98 – 5.35	13.42	-0.14	0.20	-0.53 – 0.26	-0.67
poly(Temperature, 2)1	0.03	2.47	-4.81 – 4.87	0.01	-1.33	1.44	-4.15 – 1.50	-0.92
poly(Temperature, 2)2	-3.30	2.41	-8.01 – 1.42	-1.37	-1.49	1.40	-4.25 – 1.26	-1.06
Random Effects								
σ^2	1.63				0.55			
τ_{00}	0.62	capture_time			0.21	capture_time		
ICC	0.28				0.28			
N	9	capture_time			9	capture_time		
Observations	35				35			

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Table S.1.2. GLM summary of the metabolic traits (standard metabolic activity (SMR), routine metabolic activity (RMR), maximum metabolic rates (MMR), and aerobic scope (AS)) and temperature range in relationship with the temperature of acclimation. CI correspond with the confidence interval (2.5% to 97.5%) and statistics correspond to t value.

<i>Predictors</i>	SMR			RMR			MMR			AS						
	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>Statistic</i>	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>Statistic</i>	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>Statistic</i>				
(Intercept)	5.37 ^{***}	0.09	5.20–5.54	63.03	5.61 ^{***}	0.07	5.47–5.76	77.21	6.19 ^{***}	0.03	6.12–6.25	199.86	5.08 ^{***}	0.07	4.95–5.22	75.22
Temperature	0.12 ^{***}	0.02	0.07–0.17	5.00												
log(Temperature)				0.32 ^{***}	0.06	0.20–0.45	5.25									
poly(Temperature, 2)1								1.09 ^{***}	0.23	0.63–1.55	4.80	0.93	0.50	-0.07–1.94	1.88	
poly(Temperature, 2)2								-0.50 [*]	0.22	-0.94–-0.06	-2.30	-1.26 [*]	0.47	-2.22–-0.30	-2.66	
Observations	49			49				40				40				

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Table S.1.3. GLMMS of the behavioural performance (Temperature preferences, maximum/min avoidance and temperature range) in relationship with the temperature of acclimation. CI correspond with the confidence interval (2.5% to 97.5%) and statistic correspond t value.

<i>Predictors</i>	Tpref			T max avoidance			T min avoidance			T range						
	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>Statistic</i>	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>Statistic</i>	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>Statistic</i>				
Intercept	1.06***	0.30	0.47–1.66	3.51	1.49***	0.31	0.88–2.09	4.84	0.78***	0.18	0.42–1.14	4.26	1.15***	0.10	0.95–1.35	11.18
Temperature	0.64***	0.10	0.44–0.84	6.22	0.53***	0.10	0.33–0.74	5.07	0.71***	0.06	0.59–0.83	11.48				
poly(Temperature, 2)1													-0.28	0.54	-1.34–0.77	-0.52
poly(Temperature, 2)2													1.06	0.54	-0.00–2.13	1.96
Random Effects																
σ^2	0.01				0.00				0.00					0.07		
τ_{00}	0.00	capture_time			0.00	capture_time			0.00	capture_time				0.08	capture_time	
ICC	0.20				0.51				0.09					0.52		
N	10	capture_time			10	capture_time			10	capture_time				10	capture_time	
Observations	28				28				28					28		
Marginal R ² / Conditional R ²	0.659 / 0.728				0.643 / 0.827				0.850 / 0.864				0.229 / 0.631			

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Appendix 2

Appendix 2.1. Quality control and RNA-seq assembly

Table S.2.1.1. Quality control of the 18 RNA-seq libraries. Raw reads correspond with the original sequencing reads counts and clean reads is the number of reads after filtering. Clean bases are defined as the clean reads number multiply read length and save in G unit. The error rate is the average of sequencing error rate which is calculated by $Q_{phred} = -10 \log_{10}(e)$. Q20 and Q30 are the percentage of bases whose correct base recognition rates are greater than 99% and 99.9% respectively. GC is the percentage of guanine (G) and cytosine (C) total bases

Acclimation temperature (°C)	Sample	Raw Reads	Clean Reads	Clean bases	Error (%)	Q20 (%)	Q30 (%)	GC Content (%)
13	T13G1	43,306,866	41,962,962	6.3G	0.03	97.65	93.31	42.71
13	T13G2	44,630,402	43,370,838	6.5G	0.03	97.23	92.53	41.69
13	T13HG1	51,798,732	49,904,332	7.5G	0.03	96.89	91.83	37.92
13	T13HG2	41,471,056	40,336,634	6.1G	0.03	96.8	91.59	35.26
13	T13H1	46,768,846	44,960,786	6.7G	0.03	97.15	92.34	38.32
13	T13H2	48,653,306	47,808,620	7.2G	0.03	96.33	90.5	36.71
19	T19G1	43,328,808	41,970,666	6.3G	0.03	96.82	91.56	39.89
19	T19G3	48,325,028	47,152,626	7.1G	0.03	96.81	91.41	41.9
19	T19HG1	49,193,302	47,780,272	7.2G	0.03	96.34	90.4	36.3
19	T19HG3	55,709,356	54,202,834	8.1G	0.03	96.59	91.02	35.81
19	T19H1	46,315,998	45,263,740	6.8G	0.03	96.59	91.03	36.43
19	T19H3	46,119,032	44,997,460	6.7G	0.03	96.22	90.23	36.54
25	T25G1	54,823,740	53,408,398	8G	0.03	96.55	90.95	40.83
25	T25G2	51,259,278	50,134,928	7.5G	0.03	96.32	90.53	37.15
25	T25HG1	52,797,924	51,075,232	7.7G	0.03	96.4	90.58	36.39
25	T25HG2	51,901,170	48,928,266	7.3G	0.03	96.58	90.89	36.81
25	T25H1	42,349,518	40,495,246	6.1G	0.03	96.77	91.43	35.83
25	T25H2	40,839,134	40,383,526	6.1G	0.03	95.95	89.58	38.89

Table S.2.1.2. *De novo* assembly statistics of southern calamari full transcriptome

Raw reads	859,591,496
Clean reads	834,137,366
% GC	38
Assembly	
Number of transcripts	218,946
Min transcript size (bp)	201
Mean transcript size (bp)	820
N50 (bp)	1,157
Longest transcript size (bp)	30,263
Total assembled bases (bp)	179,619,063
Number of transcripts between 200-500bp	108423
Number of transcripts between 500bp -1K bp	64041
Number of transcripts greater than 1 K bp	46482

Appendix 2.2. Gene functional annotation

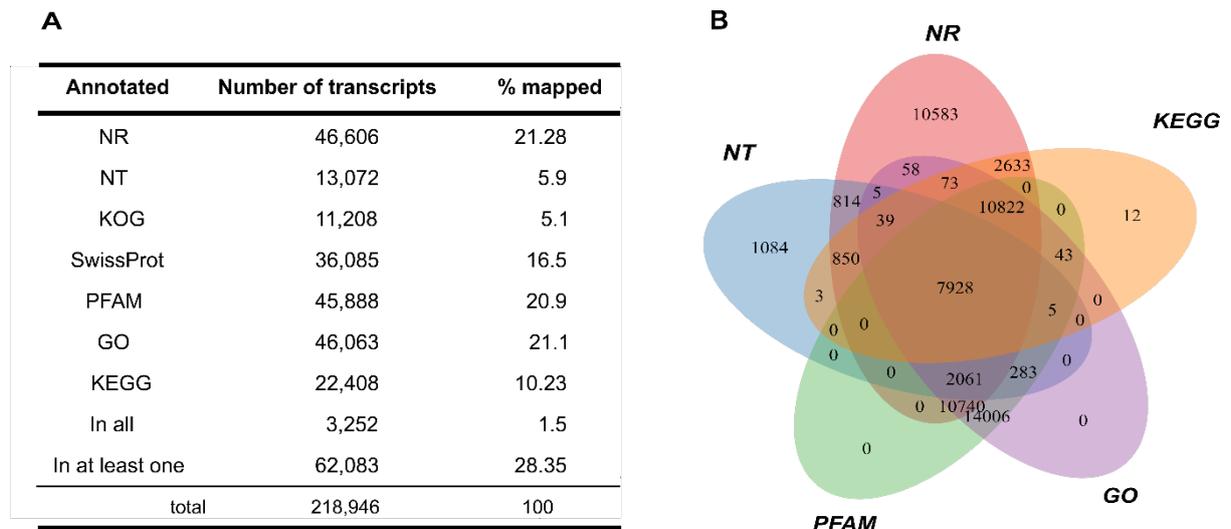


Figure S.2.2.1 The ratio of successful annotation of the assembly in southern calamari A) number of transcript and percentage of transcripts successfully mapped against seven databases, which included NCBI non-redundant protein sequences (NR), NCBI nucleotide sequences (NT), Protein family (Pfam), eukaryotic Orthologous Groups (KOG), SwissProt, Kyoto Encyclopedia of Genes and Genome (KEGG), and Gen Ontology (GO). B) Venn diagram of the successfully annotated transcripts of the five databases.

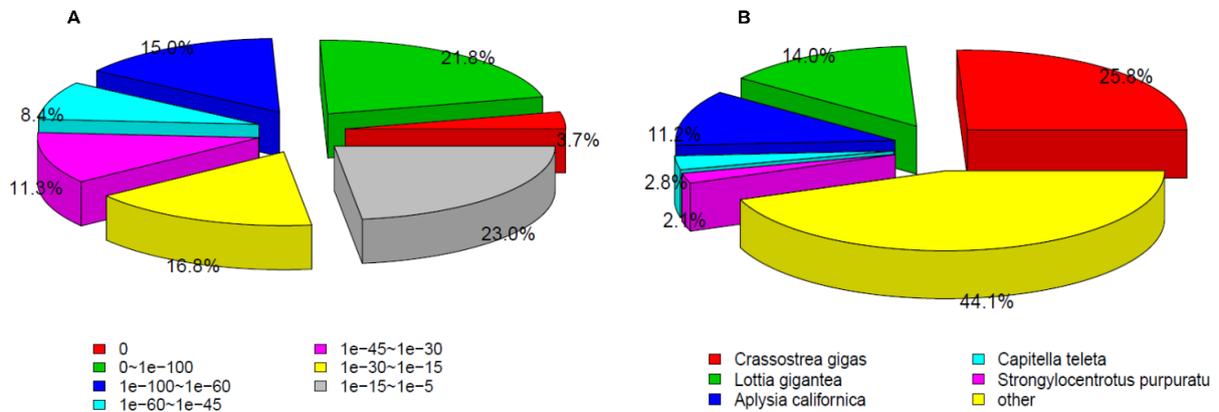


Figure S.2.2.2. Blast to NR database A) E-value distribution of the transcripts mapped against Nr, B) % of the transcripts mapped to NR database classified on the different species with the transcripts results for the blast.

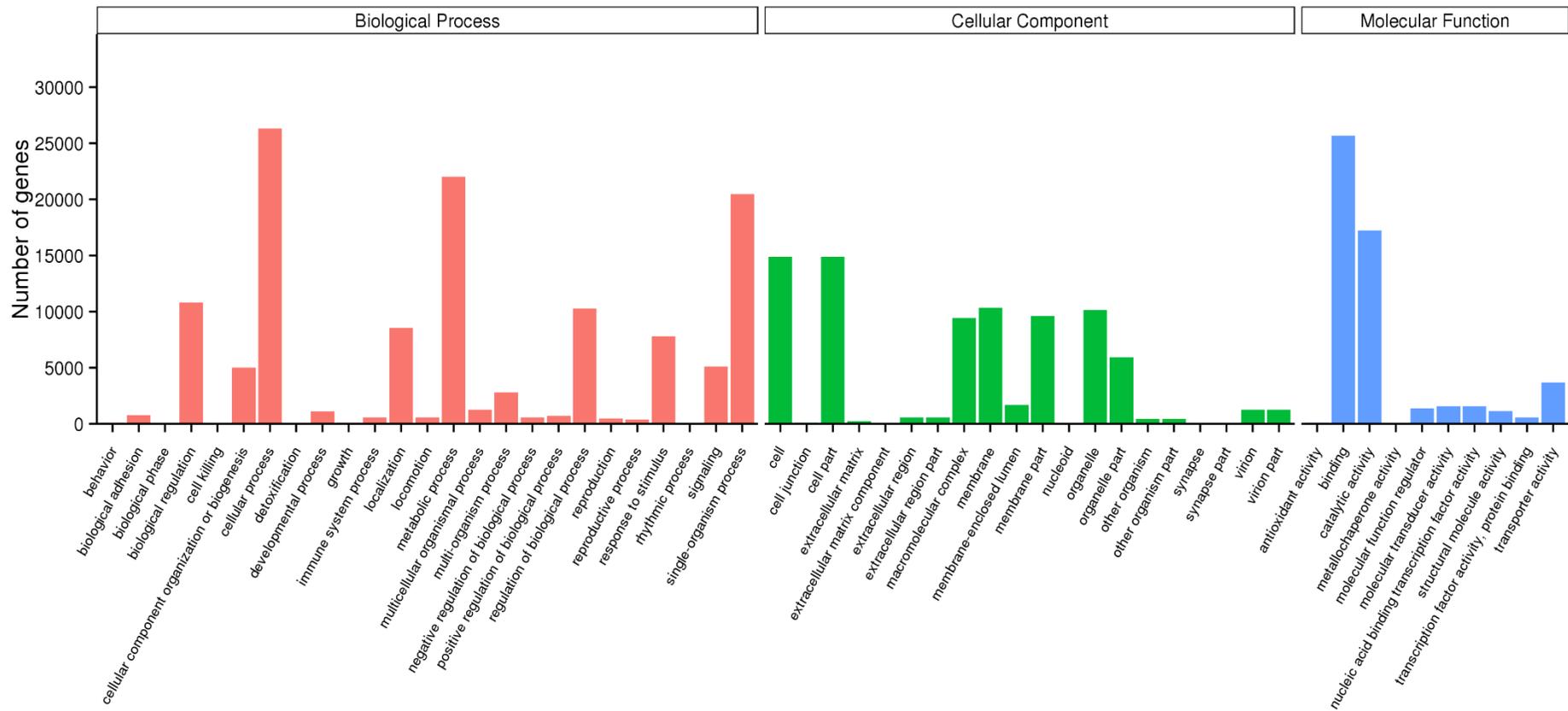


Figure S.2.2.3. Gene function annotated in GO (Gene Ontology) of southern calamari full transcriptomic divided in biological process, molecular functions and cellular components. Biological process (48.3%) represents the biological targets that the gene or gene product contributes to. Molecular functions define biochemical activity. Cellular components indicate the location where a gene product is active.

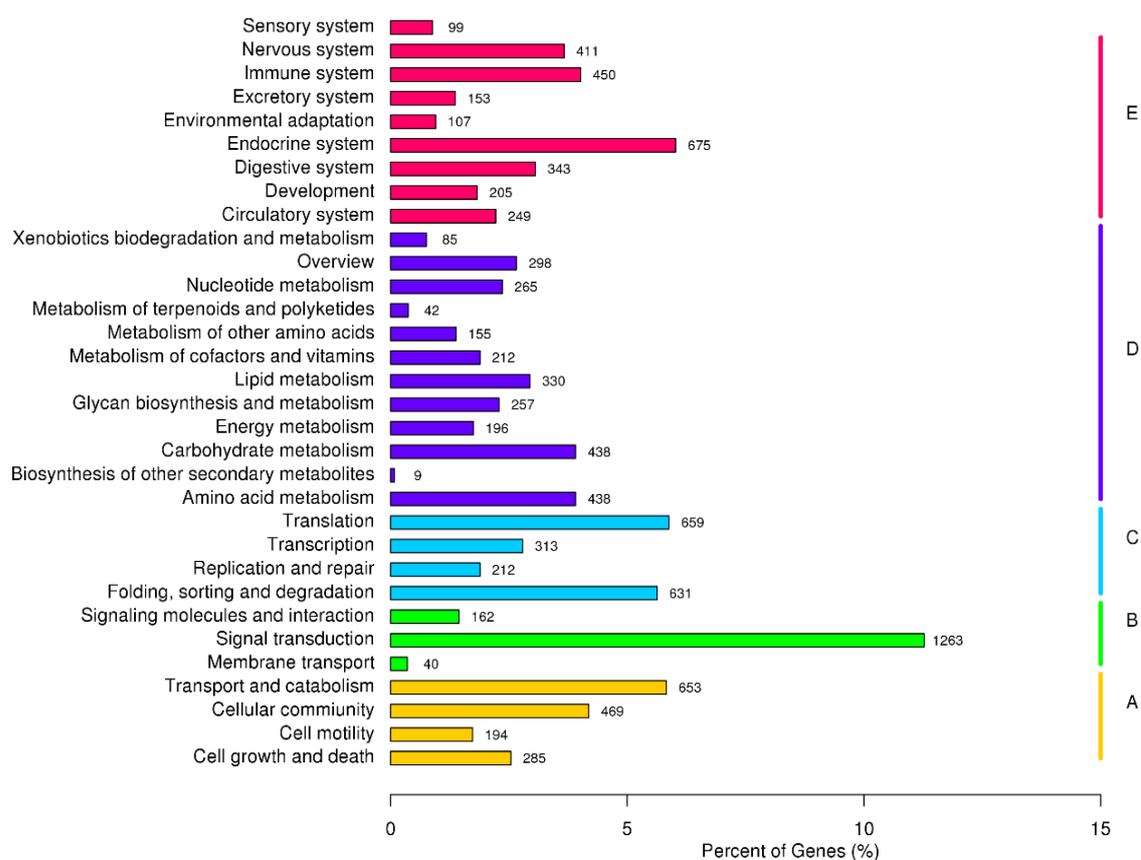


Figure S.2.2.4. Transcripts successfully annotated in KEGG. The KEGG pathways are divided into: A) cellular processes, B) environmental information processing, C) Genetic information processing, D) metabolism, E) organismal system.

Table S.2.2.3 List of annotated transcripts in KEGG database for the full transcriptome on southern calamari (<https://cloudstor.aarnet.edu.au/plus/s/kolv6yKMUAfTNIK>).

Appendix 2.3 Gene expression, DEG analysis and enrichment analysis

A

Acclimation temperature (°C)	Sample name	Total reads	Total mapped
13	T13G1	41962962	31996922(76.25%)
13	T13G2	43370838	31664240(73.01%)
13	T13HG1	49904332	36288608(72.72%)
13	T13HG2	40336634	30702862(76.12%)
13	T13H1	44960786	33570074(74.67%)
13	T13H2	47808620	34425036(72.01%)
19	T19G1	41970666	28910070(68.88%)
19	T19G3	47152626	34761238(73.72%)
19	T19HG1	47780272	35376016(74.04%)
19	T19HG3	54202834	40180894(74.13%)
19	T19H1	45263740	33361650(73.71%)
19	T19H3	44997460	32907652(73.13%)
25	T25G1	53408398	37302890(69.84%)
25	T25G2	50134928	31970148(63.77%)
25	T25HG1	51075232	38539676(75.46%)
25	T25HG2	48928266	38268878(78.21%)
25	T25H1	40495246	30148258(74.45%)
25	T25H2	40383526	30590376(75.75%)

B

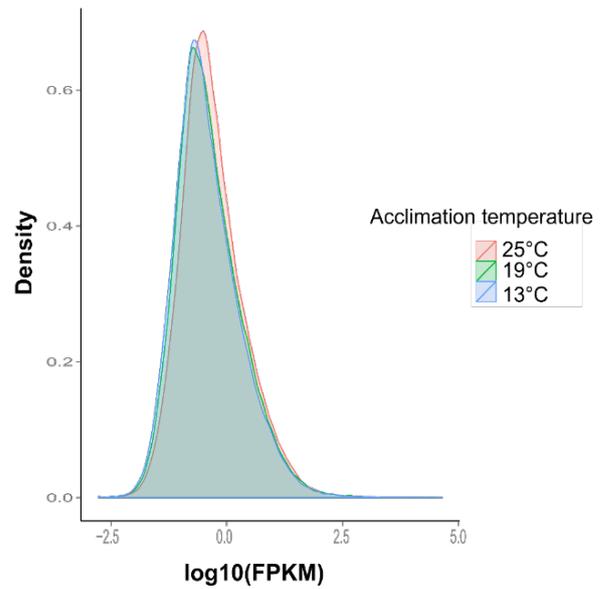


Figure S.2.3.1. A) overview of the reference alignment per sample, B) comparison of gene expression levels across the different acclimation temperatures.

Table S.2.3.1. List of genes encoding ADAR and proteins of the heat shock protein family in southern calamari transcriptome (<https://cloudstor.aarnet.edu.au/plus/s/eD1NwbZq0TIQiaI>).

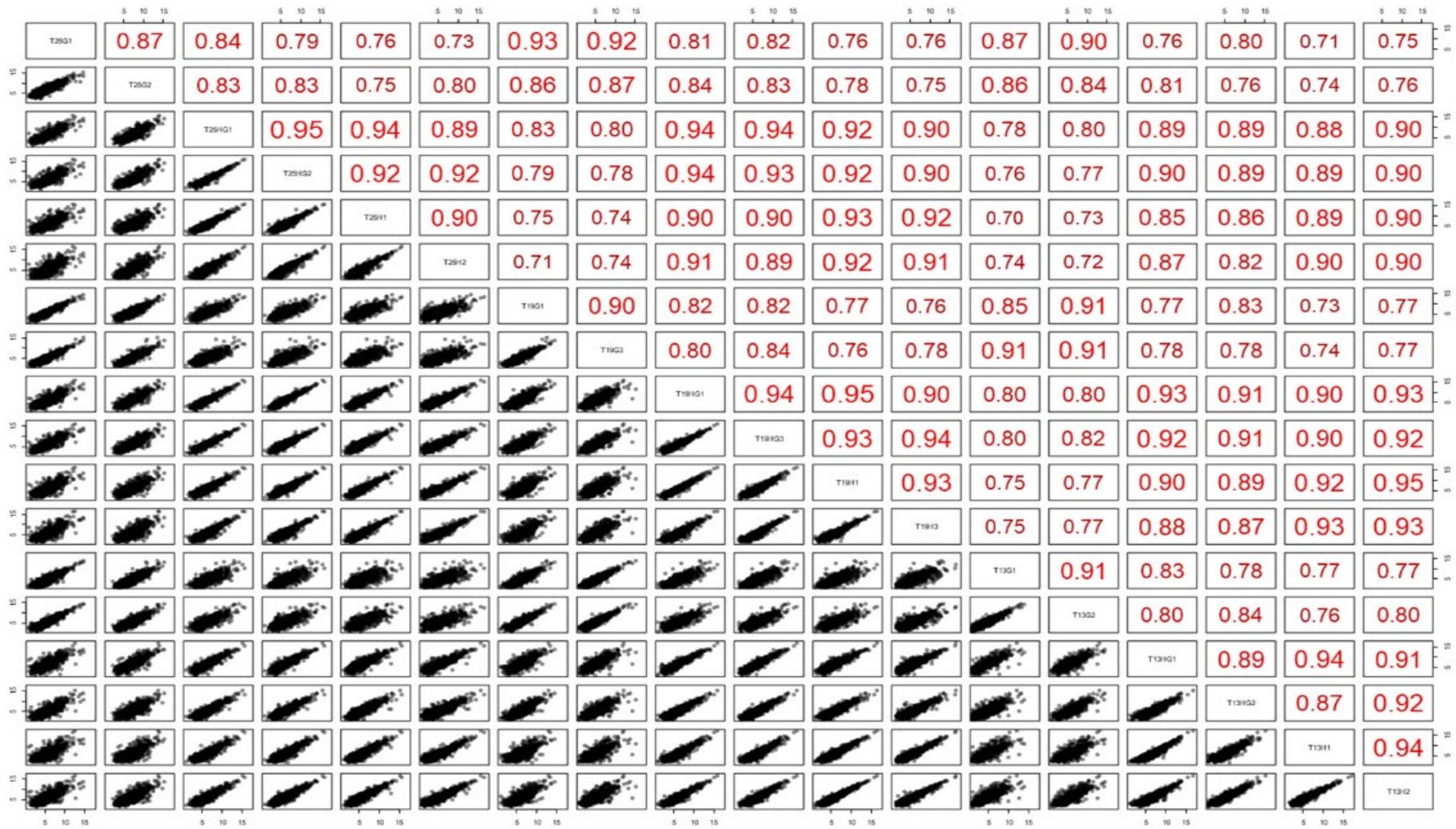


Figure S.2.3.2. Pearson correlation between the 18 samples showing R^2 (Pearson coefficient), with scatterplot showing the expression between samples (\log_{10} FPKM)

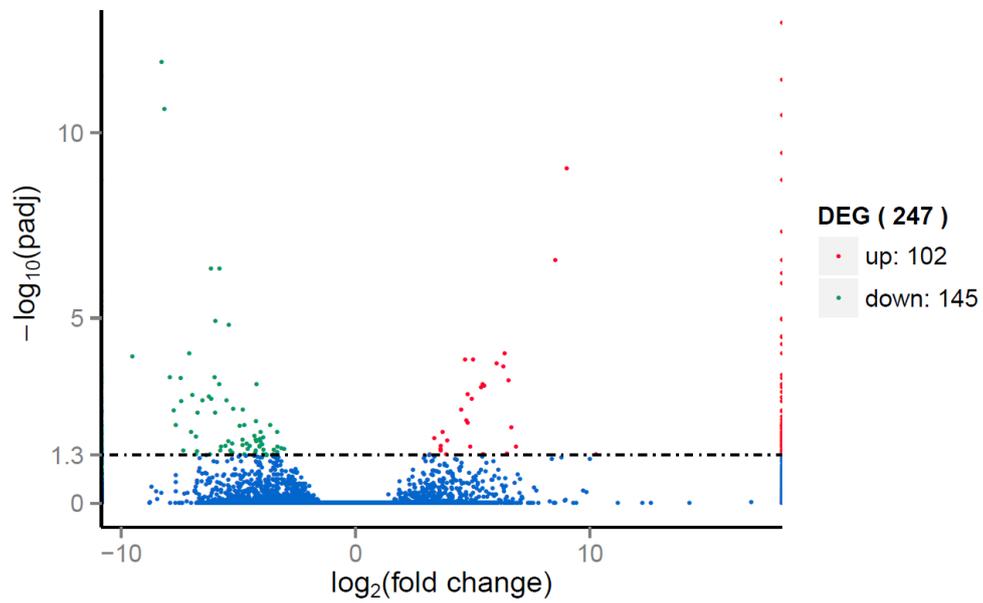


Figure S.2.3.3. Volcano plot of the DEGs between high acclimation temperature and cold (T25 °C vs T13 °C).

Table S.2.3.2. DEGs between temperatures 25 °C vs 13 °C (<https://cloudstor.aarnet.edu.au/plus/s/XYnGhTMbnIB1TuV>)

Table S 2.3.3. GO and KEGG Enrichment analysis (<https://cloudstor.aarnet.edu.au/plus/s/A7kVKoliQLrd5u6>)

