Growth and reproduction of a short-lived cephalopod: Mechanisms that facilitate population success in a highly variable environment.

by

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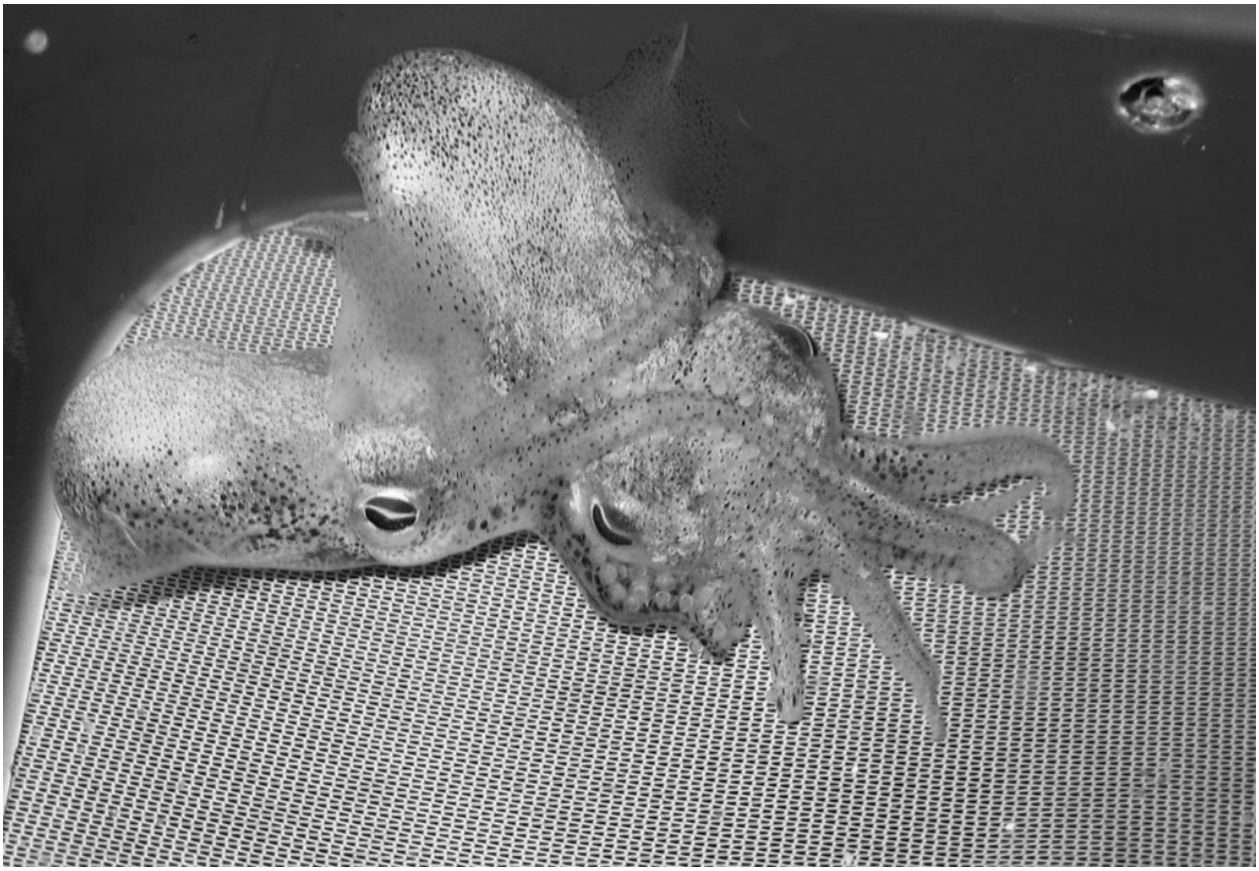
Frontispiece



Mating pair of *Euprymna tasmanica*

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Abstract

For short-lived species seasonal fluctuations in environmental conditions plays a major part in shaping population structure and dynamics. Cephalopods are known for their short life span, rapid growth, and early maturation. Small changes in environmental conditions have significant effects on life history characteristics, such as the timing and size at hatching, growth rates, longevity, and size and age at maturation. For cephalopods that live for less than a year annual environmental cues are not used to synchronise life events (e.g. timing of gametogenesis and spawning), instead individuals grow and reproduce throughout the majority of the year. As a result cephalopod populations are typically made up of multiple cohorts with differing life history characteristics, making it difficult to establish generalizations about the structure and dynamics of populations both within and among species. To date, both laboratory and field based studies have been useful in understanding some of the mechanisms responsible for the plastic characteristics that has allowed cephalopods to be so successful in response to the variable environmental conditions. The aim of this research was to use a small cephalopod species with a short life span, *Euprymna tasmanica*, to explore the relationships between growth and reproductive output from laboratory held populations alongside growth estimates from the field to explain some of the mechanisms that may be responsible for the seasonal variability found within populations of *Euprymna tasmanica* in northern Tasmania.

In captivity *E. tasmanica* hatched at approximately 0.3g and the percent increase in body mass per day followed a biphasic growth pattern, starting with a fast exponential growth model followed by slower almost linear growth. Changes in temperature and ration had significant

impact on the growth and reproductive characteristics, however in most the influence of temperature and ration were independent of each other. During the initial stage of growth higher water temperature was seen to significantly increase the rate of growth, while greater rations increased growth during the late phase of growth. An elevation of temperature of 5°C over the entire lifespan decreased the age and weight at first egg deposition by 16 days, and 0.89g respectively, and halved the average egg size. Females fed at a greater ration were 12 days younger at first egg deposition and produced eggs that were on average 25% larger; however, their size at first batch deposition (6.23g ± 0.19) was no different from those females fed a smaller ration. Only the number of eggs in the batch was affected by the interaction of temperature and ration, with individuals experiencing a combination of high temperature and ration producing average batch sizes of around 128 eggs which was approximately twice the size of the other treatments.

Within the population *of E. tasmanica* sampled, it was apparent that the temperatures experienced had a significant effect on the growth, maturity and reproductive condition. Growth in terms of size-at-age was sex specific, with the temperature experienced having no effect on the growth of males. Females on the other hand grew larger when experiencing cold water, but this was likely to be a factor of living longer rather than growing faster. Immature individuals that had experienced cooling or cold water temperatures were also larger, suggesting that maturity occurs at larger sizes during winter. It seems that the direction of change in water temperature, rather than the temperature range alone influenced the condition of *E. tasmanica*, with individuals of both sexes experiencing warming water

temperatures being in poorer reproductive and somatic condition compared to individuals who experienced cooling water temperatures.

Histological analysis of the mantle muscle dynamics showed significant differences of muscle block width, fibre frequency and fibre diameters from different regions of the mantle, indicating that growth is not uniform throughout the whole mantle. Differences in muscle growth dynamics of squid was largely dependent on the water temperature individuals experienced. Biochemical indices were also used to examine differences in growth among squid that experienced different water temperatures. In this study RNA, protein and RNA:Protein ratio showed that growth is faster in smaller individuals and individuals that experienced warmer environments. Additionally, the level of both reproductive investment and reproductive status of individuals had no effect on any of the biochemical indices, suggesting that variability in growth rates is not a factor on reproductive investment and that the process of growth and reproduction may be independent of each other. The large amount of unexplained variation in these results however, suggest that even when taking the environmental influence and reproductive condition into account the growth in *E. tasmanica* remains highly variable and difficult to explain.

By studying the influence of temperature and ration on the life history characteristics of individuals in the lab, alongside the assessment of a wild population, this study was successful in explaining how the structure and dynamics of a population of short-lived squid changes in response to short term environmental variability. While growth and reproduction progress together over much of an individual's life, it appeared that depending on the environment experienced individuals were able to switch between two main reproductive strategies, each

using a different method to maximise population fitness. Individuals that experience warming and warm water were able to growth fast, due to an increase in hypertrophic growth, increasing fitness by reducing the time between generations. Individuals adopting this strategy appeared to have similar reproductive characteristics to that of a terminal spawning species. In contrast, individuals that experienced cooling and cold water grew slowly mainly through a hyperplastic dominated growth, reaching maturity later. Although this strategy increased the risk of being preyed upon before spawning, individuals were able to spawn multiple times over an extended lifespan, increasing the chance that some of their offspring will experience conditions favourable for survival. While it is likely that the reproductive strategy of individuals will fall somewhere in-between these two strategies, the ability of this short-lived squid to survive in a variable environment owes its success to its flexible reproductive strategies.

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Utmost, as a Christian I thank God who I believe not only provided for all my daily needs but also gives me hope and a purpose in life, while exploring his creation over the past 4 years.

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Chapter 1 General introduction

1.1 Population fitness

A population is categorised as a group of individuals of the same species being maintained through reproduction in a defined area or habitat (Sinclair, 1988). For a population to survive in a given area the reproductive strategy used must insure successful recruitment in the environment in which individuals live. In biology, the ability of an individual to 'fit' into the environment it experiences is often referred to as 'fitness' (Heino and Kaitala, 1999). In a population sense, fitness can be modelled by generation interval and absolute fecundity which relies on the survival of an individual and its offspring (Roff, 1986; Brommer, 2000). Although fecundity and generation interval are dependent on the reproductive strategy of a species, simply increasing reproductive output does not increase the fitness of a species (Goodman, 1979), but rather requires a number of trade-offs among the life history characteristics (Roff, 1986). For example, fecundity increases with body size (Roff, 1981), but simply increasing body size will not necessarily increase fitness, as it takes longer to attain larger body size, which may compromise survival (Roff, 1986).

Measuring fitness of a population is complex and requires more than just measuring the life history strategy. In its simplest form, a measure of a population's fitness assumes that the environment is stable and that all individuals in the population use the same strategy (Brommer, 2000). Environmental variation however is a persistent feature in the life of organisms, with environmental conditions experienced by one stage of an organism being different to that experienced by subsequent stages (Beckerman*, et al.*, 2003). This is especially evident in a short-lived species living in a variable environment, where the conditions

experienced by one generation may be completely different to that of the next. The life history strategy of individuals living in a variable environment must therefore have some degree of flexibility to be successful (Rochet, 2000). The most successful strategy however will not always maximise fitness. For example, there are obvious benefits associated with fast growth, such as a shorter juvenile period, which increases the probability of surviving to reproduction (Roff, 1992; Stearns, 1992). However despite these advantages; individuals are often known to grow at lower rates than they are physiologically capable of (Arendt, 1997). This suggests that high growth rates have their own fitness costs, and in some instances it may be more beneficial for an individual to grow slowly (Gotthard, 2000). Hence life history characteristics are a result of 'strategic decisions' where the costs and benefits of one strategy is balanced against the other (Beckerman*, et al.*, 2002).

Environmental conditions experienced early in life can influence life history traits such as reproduction and survival later in life. For the water python, *Liasis fuscus,* the abundance of prey in its first year of life determines its lifetime growth rates, with individuals born in years of poor food supply having slower lifetime growth rates even when food supply increases (Madsen and Shine, 2000). These delayed life history effects have an important influence on an individual's performance and can give rise to 'cohort effects' at the population level (Beckerman*, et al.*, 2002; Moltschaniwskyj and Pecl, 2007). A cohort effect is a phenomenon where groups of individuals in a population differ in some average property, such as fecundity, body size or longevity (Lindstrom and Kokko, 2002). In some instances, cohort effects appear in the early adult phase (Cotton*, et al.*, 2004), however they are most often found in the embryonic developmental or juvenile phase of life (Mousseau and Fox, 1998; Lindstrom, 1999;

Beckerman*, et al.*, 2002; Gimenez, 2006). For example, plastic responses in growth during the larval phase of many marine species can strongly influence size-at-age and size-at-maturation, which ultimately influence survival and recruitment (Gimenez, 2010).

In longer lived species with overlapping generations, cohort effects may stabilise the genetic and phenotypic diversity of the population by introducing individual differences into the population (Bjornstad and Hansen, 1994; Doebeli and De Jong, 1998). For instance, during poor conditions if a few individuals are able to acquire enough resources, a population will not necessarily crash (Lindstrom and Kokko, 2002). In comparison, we know relatively little about cohort effects in short-lived species (Le Galliard*, et al.*, 2010). In some short-lived squid, spawning aggregations do not consist of well defined or stable population units, but rather a series of 'micro cohorts' which continually enter and leave (die) the population (Jackson and Pecl, 2003; Moltschaniwskyj and Pecl, 2007). Populations with one or two generations that have experienced very different environmental conditions will display large inter-annual variations in structure and size (Boyle and Boletzky, 1996).

1.2 Short-lived animals

Cephalopods are ideal organisms for studying the population variability of short-lived animals, as they are renowned for their fast growth rates and short lifespan which result in rapid turnovers in the population (Grist and Jackson, 2004; Pecl and Jackson, 2008). As the lifespan of many cephalopods, especially squid, is <1 year, there is a need for multiple spawning events throughout the year, resulting in different cohorts of individuals experiencing conditions very different from their parents (Guerra*, et al.*, 1992; Boyle and Rodhouse, 2005; Storero*, et al.*, 2010). For this reason, short term environmental variation is likely to affect a population of

cephalopods more so than longer lived fish. Cephalopods display considerable intra-population variability in growth rates and hence size-at-age and size-at-maturity (Jackson and Wadley, 1998; Semmens and Moltschaniwskyj, 2000). To date, some of the environmental conditions known to affect cephalopod life history characteristics are temperature (Villanueva, 2000; Vidal*, et al.*, 2002a; Steer*, et al.*, 2004), photoperiod (Paulij*, et al.*, 1990; Koueta and Boucaud-Camou, 2003), light intensity (Ikeda*, et al.*, 2004) and salinity (Cinti*, et al.*, 2004; Sen, 2005), however most of these studies have focused on environmental conditions on cephalopod embryos. After hatching, temperature (Forsythe and Van Heukelem, 1987; Forsythe and Hanlon, 1988; Forsythe, 1993; Pecl*, et al.*, 2004) and food (Moltschaniwskyj and Martinez, 1998; Jackson and Moltschaniwskyj, 2001b; Vidal, et al., 2002a; Steer, et al., 2004) are often cited as the key driving factors influencing the variability of life history characteristics. In general, increased temperature increases growth rate, reduces life span and size-at-maturity (Mangold, 1987; Boyle, 1990; Collins*, et al.*, 1995; Jackson*, et al.*, 1997; Raya*, et al.*, 1999; Jackson and Moltschaniwskyj, 2001a; Hatfield and Cadrin, 2002; Jackson and Moltschaniwskyj, 2002; Hendrickson, 2004), while the effects of ration in some species increases juvenile growth rate (Moltschaniwskyj and Martinez, 1998; Jackson and Moltschaniwskyj, 2001b), increases egg and offspring size (Steer*, et al.*, 2004), and improves offspring survival (Vidal, et al., 2002a; Steer, et al., 2004). Having such extremely flexible and plastic life history characteristics, cephalopods are often described as opportunistic organisms, allowing them to respond rapidly to small changes in environmental conditions (Jackson and O'Dor, 2001; Pecl and Moltschaniwskyj, 2006; Pecl and Jackson, 2008). These characteristics make cephalopods ideal organisms for

studying the ecology of short-lived species, especially with regard to how short term environmental change influences the structure and dynamics of a population.

1.3 Research to date

Studies between cephalopod populations and the environment are usually shaped by the type of data available, which in the past has been collected from trawl surveys, predator stomach contents, tagging and direct observations (see Pierce, *et al*., (2008), for review). As cephalopod catches are not as economically important as many teleost species they attract less resources and research interest; as a result, data is often collected in collaboration with teleost research activity (Pierce, et al., 2008). Due to the selective nature of fishing gear most of the data available does not include a complete representation of the population (Caddy, 1983). For example jig fisheries of *Loligo vulgaris reynaudii* under sample both the smallest and largest individuals (Lipinski, 1994), trawl fisheries uses mesh size to target individuals of a specific size, and faster swimming individuals may be able to avoid trawls altogether (Hanlon, 1998). Additionally fisheries that target spawning grounds are actually only targeting mature individuals that aggregate to mate or deposit eggs. Considering that cephalopods are highly influenced by environmental variation at all stages of their life (Guerra and Rocha, 1994; Pierce*, et al.*, 1994; González*, et al.*, 2005; Guerra, 2006) predicting the response of populations to environmental variability must be based on a species' full life history, with detailed information on the early life phase (Rodhouse, 2001). Therefore, solely relying on data from fisheries will not provide a complete picture of all life stages of the population, and alternate sources of data are needed.

Recently, based on age determination techniques (e.g. statolith (Jackson, 1994; Arkhipkin, et al., 2004), stylets (Leporati, et al., 2008), cuttlebones (Le Goff, et al., 1998; Bettencourt and Guerra, 2001), and beaks (Hernández-López, et al., 2001)) it is possible to back calculate hatch dates and estimate the environmental conditions experienced. However, such an approach may not be as useful in cephalopods as it has been in fish, as cephalopods respond rapidly to small changes in temperature (Forsythe, 1993; Jackson, et al., 1997) and knowledge of hatch dates may not provide sufficient detail on the immediate effects of environmental conditions. Many commercially important squid species are also highly mobile, migrating thousands of kilometres to spawn, and any correlation between the environment and an individual's life history will be constrained by a limited knowledge of both lateral and vertical migrations (Grist and Des Clers, 1999).

Experimental studies provide insight into the effects of temperature and ration on processes of growth and reproduction and in particular, have demonstrated that small changes in temperature experienced early in life strongly influence characteristics such as longevity and size-at-maturation which in turn affect the reproductive output of females (Forsythe and Hanlon, 1988; Semmens and Moltschaniwskyj, 2000; Forsythe*, et al.*, 2001). However, due to the difficulties of maintaining large, highly mobile, cephalopods in captivity (Hanlon*, et al.*, 1990), many experimental studies do not study individuals throughout their entire lifespan, but rather focus on one particular characteristic, such as hatching success or early development. While laboratory studies have been helpful in understanding how certain life history characteristics and reproductive strategies that shape the population, alone they cannot provide a picture of how the structure and dynamics of a wild population change in a variable

environment. Moreover, laboratory conditions cannot replicate the exact conditions experienced in nature and results should always be considered with some caution. This highlights the need to carry out laboratory studies alongside studies of the wild population.

To be able to infer sensible theories into what causes the variation observed in an individual's life history characteristics, there is a need to study the processes occurring at the sub-organismal level of biological organisation, including relative growth of tissues, proximal composition, and muscle tissue structure (Weatherley, 1990). Such studies provide insight into the mechanisms behind what is occurring at an individual based level and have successfully been used to assess the condition or health of fish populations (Fonseca and Cabral, 2007). Although these methods are relatively new to the field of cephalopod biology (Semmens and Jackson, 2005) they provide the important link in being able to comprehend the observed plasticity of life history traits (Ferron and Leggett, 1994), and begin to determine the overall life history strategy of a species (McGrath and Jackson, 2002).

1.4 The southern dumpling squid *Euprymna tasmanica*

The southern dumpling squid, *Euprymna tasmanica* is a small (30-40mm), nocturnal, short-lived squid species (approximately 6 months) that can attain weights of at least 15g (Norman and Lu, 1997; Jereb and Roper, 2005). Dumpling squid are a benthic species found in shallow coastal waters in soft sediments that border sea grass beds. During the day they burying into the sand where they remain relatively inactive, emerging at night to feed. Based on holding individuals in the laboratory, *E. tasmanica* feed on a large variety of invertebrates (copepods, crustaceans and marine worms) and fish, which is similar to adult cephalopods in the wild (Packard, 1972). At night individuals are generally found sitting on top of the substrate

sometimes blanketed in a layer of sand. If threatened *E. tasmanica* avoids detection by burying in the sand, rather than moving away from the threat using jet propulsion; a common predator avoidance strategy for most squid species (Calow, 1987). This burying behaviour makes capturing the animals reasonably easy, which is an important factor when sampling as every individual encountered can be captured, not just the slowest individuals in the population. Given their short lifespan, ease of capture and responsiveness to small changes in the environment, *E. tasmanica* is a good model species for studying how the environment affects population structure of short lived species. Additionally their small size and relatively sedentary lifestyle means that *E. tasmanica* is amenable to being held in laboratory conditions (Steer, et al., 2004; Sinn and Moltschaniwskyj, 2005; Moltschaniwskyj and Carter, 2010) for the use in experiments manipulating environmental conditions.

1.5 General Objectives

The objectives of this study were to:

1 Describe the temporal variation in life history traits in a population of short-lived dumpling squid, *Euprymna tasmanica*.

2 To explore the role the environment (i.e. temperature and food) plays in shaping the population dynamics of *Euprymna tasmanica*.

3 To identify the processes associated with the observed variation by describing the mechanisms underlying the flexible life history characteristics observed at the individual level.

1.6 Chapter summaries

This thesis consists of four data chapters. Each chapter can be considered as 'stand alone' manuscripts. Consequently there will be some inherent repetition in the methods.

1.6.1 Chapter 2: Population structure – how the environment shapes the population

Through monthly sampling over two consecutive years, this field study aimed to describe how different aspects (such as average weight, sex ratios, somatic and reproductive residuals) of the population changes with the varying environment. Cephalopods populations are not well defined stable units, but instead consist of a series of cohorts entering the population throughout one spawning season (Jackson*, et al.*, 2003; Moltschaniwskyj and Pecl, 2007). By sampling monthly over two successive years this study was successful in picking up temporal changes in the population structure. This chapter established the basis for the majority of the thesis, whereby descriptions from individuals of all stages of life were used to describe how different characteristics of the population changed with the varying environment conditions.

1.6.2 Chapter 3 - Individual life histories – a morphological approach to how the environment shapes an individual

This chapter further examined the environmental influences on individuals' life history characteristics, and focused on how temperature and ration influenced growth and reproductive output of captive females throughout their entire life. Temperature and ration are important environmental influences effecting the growth and reproductive capacity of cephalopods (Moltschaniwskyj and Jackson, 2000; Pecl, 2001; McGrath Steer and Jackson, 2004; Pecl, 2004). By study how temperature and ration affects growth and reproductive output at an

individual level provides insight into what factors influence the shape and dynamics of the population as a whole.

1.6.3 Chapter 4 - Growth mechanisms - an individual's strategy to maximize fitness in a given environment

In cephalopods, maturity and reproductive capacity are strongly related to size (Boyle*, et al.*, 1995; Jackson, et al., 1997; Jackson and O'Dor, 2001; Pecl, 2001; Steer*, et al.*, 2003a; Pecl and Jackson, 2008; Ceriola and Jackson, 2010), therefore studying the mechanisms behind growth is vital in identifying the cause of reproductive variability among individuals within the population. This chapter assessed the structure and organisation of mantle muscle tissue of individuals that experienced different environmental conditions, with the aim of providing insight into the mechanisms responsible for the flexibility found in life history characteristics.

1.6.4 Chapter 5 - Growth and reproduction – A biochemical approach in assessing the trade-off between growth and reproduction

Similar to the previous chapter, this chapter was designed to study the mechanisms behind growth and how the biochemical parameters in the mantle muscle changed in response to the environment. In many cephalopods growth and reproduction progress together throughout most of their adults life, with little evidence of a trade-off occurring between both processes (Mangold*, et al.*, 1993). This chapter uses biochemical parameters from the mantle tissue such as RNA and protein as an estimate of energy available for growth, with the aim to observe how instantaneous growth changes in response to sexual maturation among different environmental condition.

Chapter 2 Population structure – how the environment shapes the population.

2.1 Introduction

Over the past two decades, research exploring the relationship between short-term environmental variability and population dynamics of cephalopods has indicated that cephalopods populations consist of multiple cohorts with differing life history characteristics (Moltschaniwskyj and Pecl, 2007; Shaw*, et al.*, 2010). For many marine invertebrates, reproductive success largely depends on synchronised events of individuals within a population, such as gametogenesis and spawning (Mercier and Hamel, 2009). In a species that spawn annually these processes are generally controlled by environmental cues such as photoperiod and/or temperature (Bromage*, et al.*, 2001). Given that cephalopods are generally sub-annual, they cannot key into seasonal environmental cues to the same effect. As a result, the process of gametogenesis and spawning is asynchronous within the population (Boyle*, et al.*, 1995; Collins*, et al.*, 1995) leading to rapid declines when environmental conditions are unfavourable but allows populations to expand rapidly when conditions are favourable (Guerra*, et al.*, 1992; Rocha*, et al.*, 2001; Boyle and Rodhouse, 2005; Storero*, et al.*, 2010). Despite these inter-annual fluctuations in size, cephalopod populations are reasonably robust and mechanisms such as extended breeding seasons, batch spawning, sequential broods and variable growth rates provide them with a great capacity to deal with highly variable environments (Boyle and Boletzky, 1996). Fluctuation in biomass, together with the complex interaction between life histories and environmental variability are what make the assessment and management of cephalopod populations difficult (Rodhouse, 2010).

Traditionally, population studies are based on regular samples of a population over an extended period of time. However, population studies based solely on the date of capture only provide information of the collective environmental effects over the life history of an individual and provide little information on the specific effects on particular life stages (e.g. the influence of temperature on embryonic development) (Pierce*, et al.*, 2008). Increasingly, population studies are back-calculating hatch dates (Bower, 1996; Arkhipkin, 1997; Dawe and Beck, 1997; Jackson*, et al.*, 1997; Hatfield, 2000) providing some assessment of the life histories experienced by individuals. To back calculate hatch dates accurate estimation of age is required and several methods to estimate age in cephalopods have been explored, including the use of the statolith microstructure which has proven to be the most reliable and commonly used tool to age squid (Jackson, 1994; Arkhipkin*, et al.*, 2004). For some squid (e.g. sepiolids) and certainly octopus and cuttlefish, the crystalline structure of statoliths lacks visible increment (Moltschaniwskyj and Cappo, 2009) and other methods have been considered. These methods include examining the rate of increment formation in stylets (Leporati*, et al.*, 2008), cuttlebones (Le Goff*, et al.*, 1998; Bettencourt and Guerra, 2001), and beaks to infer age (Hernández-López*, et al.*, 2001). More recently biochemical measures such as RNA concentration have provided a means of estimating instantaneous growth rates (Moltschaniwskyj, 2004). All of these methods however are destructive, time consuming and are frequently unvalidated. The use of laboratory studies has also provided scientists with a good knowledge of the how specific environmental factors influence biological processes such as growth and reproduction (Forsythe and Van Heukelem, 1987; Forsythe*, et al.*, 2001). However, caution is needed when extrapolating from laboratory based results, as growth patterns of cephalopods held in aquaria generally do not reflect those observed in nature (Pecl and Moltschaniwskyj, 1999). Holding squid in captivity is thought to

accelerate sexual maturation (Hanlon*, et al.*, 1983), or at least stunt the growth rate (Hatfield*, et al.*, 2001) and hence size at maturation (Yang*, et al.*, 1986) compared to wild squid.

Despite the obvious merits of back calculating hatch dates, any correlation between the environment and an individual's life history will be constrained by limited knowledge of both lateral and vertical migrations (Grist and Des Clers, 1999). In addition to the 'passive' response to environmental variability, many cephalopod species also 'actively' migrate to environments that favour spawning and or feeding (Pierce, et al., 2008). Where these migrations are associated with reproduction, spawning aggregations are made up of multiple cohorts with new, younger animals consistently joining the aggregation (Forsythe, 2004; Moltschaniwskyj and Pecl, 2007). Since the over exploitation of traditional finfish species, fisheries are now looking at alternate markets such as cephalopods (Pierce*, et al.*, 2008) and given the cost effective method of harvesting, spawning aggregations are generally targeted (Hanlon, 1998).

Additionally, many cephalopod studies collect data in collaboration with fisheries that target only the mature individuals, and rarely include all stages of the life cycle. To adequately assess the environmental effects on the whole population, intensive sampling at regular intervals of all life history stages is required throughout the year (Pecl and Moltschaniwskyj, 2006), especially in the early life stages of cephalopods as small changes in temperature are known to determine the subsequent growth trajectory (Mangold, 1987; Forsythe, 1993; Jackson, 1997; Robin and Denis, 1999; Hatfield, 2000; Martinez*, et al.*, 2000; Pecl, 2004; Pecl and Jackson, 2008). For some species however, it is not possible to assess all life history stages as the location of the population outside the spawning season is generally not known or is highly dispersed (e.g. *Sepioteuthis australis* (Moltschaniwskyj and Steer, 2004)). This study is unique in

that individuals from all stages of life were used to describe how different aspects (such as average weight, sex ratios, somatic and reproductive residuals) of the population changes with the varying environment.

Euprymna tasmanica is a small multiple spawning cephalopod (Steer*, et al.*, 2004) that has a solitary, benthic lifestyle which is unique compared to most squid species (Norman and Lu, 1997). The post-hatching dispersal range for small bottom dwelling species like sepiolid squid is thought to be limited to less than one kilometre (Boletzky, 2003) and unless actively migrating as adults, individuals will have a greater reliance on their life history strategy to survive the varying environment compared to pelagic squid which can migrate hundreds of kilometres (Boyle and Boletzky, 1996). Insight into how the population changes over time will help explain some of the mechanisms that this short-lived species rely on when adapting to the variable environments they inhabit, which is important when trying to disentangle the variation in life history traits. Given its low migration *E. tasmanica* is a good model species to describe the biological processes supporting their flexible life history traits. This chapter aims to use monthly samples of a population over two consecutive years to describe changes in the structure of a population of short lived squid in response to seasonal variations in environmental conditions. Biological differences among individuals with different environmental life histories will be used to explain the differences observed at the population level, specifically the potential influence of water temperature on characteristics such as individual size, reproductive and somatic conditions.

2.2 Materials and Methods

2.2.1 Site location and collection

Euprymna tasmanica were collected from the tidal flats at the northern end of Kelso Beach in the Tamar estuary, Tasmania (-41.10° S, 146.79° E) (Figure 2.1). Sampling occurred monthly from December 2007 to December 2009; collections did not occur in March 2008, January, September, and November 2009 due to poor weather conditions limiting access to the site. Strong tidal currents at the site and the nocturnal activity patterns of *E. tasmanica* restricted collections to an hour either side of the night low tide. Collections were done after sunset by divers on snorkel using dip nets to collect individuals within 0.5 – 2 meters of water. Although collection of animals relies on visually spotting each individual, it was assumed that all animals encountered were seen and collected. It was possible to collect animals as small as 0.02g by this method; therefore it was assumed that the only bias was towards animals that were active during the collection period and those within snorkelling depth. The skin surrounding the eyes of *E. tasmanica* reflects a green iridescent glow under torch light, making it possible to see squid that were partially buried or covered in sand. Once located, squid were gently scooped up into the net and transferred to zip lock bags. Squid were transported live to the Launceston campus of the University of Tasmania in sealed bags of seawater placed on ice in a polystyrene box. Each month 29-41 squid were captured, with the exception of July, September, October, and November 2008, and October 2009 when only 11-19 individuals were collected, due to time limitations imposed by currents. Water temperature measurements were supplied by AbTas, an abalone farm 2.5km south (-41.11° S, 146.81° E) of the collection site, who record temperature continuously at a depth of ~5m depending on the tide.

Figure 2.1: Position of collection site at Kelso, west Tamar, Tasmania (Source: OpenStreetMap, 2012).

2.2.2 Morphometric measurement

Each squid was cold-water euthanasiaed[1] and blotted dry with paper towels and weighed to the nearest 0.01g before dissection. *Euprymna tasmanica* has a rounded bobtail shaped body with no gladius, the absence of a hard structure makes it difficult to obtain accurate length measurements. Therefore, only mantle weight was used as a measure of somatic size. The ovary/testis of each animal weighing >1g were dissected out, weighed to the nearest 0.01g and fixed in 20mL of FAACC (10% formalin, 5% glacial acetic acid and 1.3% calcium chloride) in preparation for histological analysis.

[1] This project was performed with ethic approval (A009492) under the UTAS animal ethics act.

Fixed ovary and testis reproductive tissue was transferred to 70% ethanol 24 hours before taking it through an ascending series of ethanol (80-100%), cleared in xylene, and infiltrated with paraffin wax. Tissue blocks were sectioned at 5µm and stained with Haematoxylin and Eosin, and mounted using DPX resin. To confirm sex and determine stage of maturity, slides of gonad tissue of squid >1g were viewed under 400x magnification. The reproductive tissue of all squid <1g had either not yet formed or was too small to detect macroscopically and were therefore classed as immature. Each individual >1g was assigned one of five (in the case of females) or one of four (in the case of males) reproductive stages based on the microscopic structure of the ovary or testis (Table 2.1). Individuals were either classed as reproductively immature (stage one and two) or reproductively mature (stage three to five).

The statoliths of *Eurypmna tasmanica* are not suitable for determination of age and there is no residual shell (Moltschaniwskyj and Cappo, 2009), therefore alternative methods to estimate age were used. Moltschaniwskyj and Carter (2010) found that for animals of known age held in captivity, the concentration of muscle tissue RNA is strongly related with age (r^2=0.90). Given the absence of any other method to age wild individuals it was assumed that the relationship was the same for wild individuals throughout the year, and estimates of RNA were used to estimate the age of individuals using the following equation:

$$\text{age(d)} = 146.8 - 21.3 \times \frac{\text{RNA (ug)}}{\text{muscle tissue (mg)}}$$

For squid >1g, a section of mantle muscle >0.05g, with the skin removed, was snap frozen using liquid nitrogen and stored in -80°C for RNA analysis (Appendix 1). RNA concentration was measured using dual wavelength absorbance (Ashford and Pain, 1986), both modified for squid tissue.

Table 2.1: Description of the five stages of reproductive maturity for male and female squid based on the microscopic structure of the gonad. Modified from Sauer and Lipinski (1990).

Stage	Male	Female
1	Tubules not clearly differentiated. Large primary spermatocytes	Primary oogonia with no clearly defined cytoplasmic area. Secondary oogonia having a cytoplasm surrounding a well defined nucleus. Possible one or two follicle cells attached.
2(immature)	Primary spermatocytes congregate along inside wall of the tubule	Oocytes contain large germinal vesicle surrounded by an irregular corona. Follicle cells have attached to the oocyte and begun to proliferate on its surface, surrounding the oocyte and changing from squamous to cuboidal cells.
3 (mature)	Both primary and secondary spermatocytes present, some early spermatids.	Follicular epithelium begins to invade the oocyte as follicles of tissue with a high mitotic rate making the end of their maximum penetration into the oocyte in the formation of a syncytium.
4	Primary and smaller secondary spermatocytes present. Plenty of early and mature spermatids towards centre of tubule. Spermatozoa in abundance.	The syncytium formed by the follicle is active in vitellogenesis and the formation of a chorion. The follicular folds are being displaced towards the periphery of the oocyte by the formation of yolk.
5	-	Final degeneration of the follicular syncytium has taken place and the mature oocyte is ready for ovulation.

2.2.3 Determination of reproductive and somatic conditions

Absolute size of the mantle and ovary may not be good indicators of morphological characters as they are often heavily influenced by body size and indices, e.g. gonosomatic indices, are not size independent (Jakob*, et al.*, 1996). Regressions with body mass are used to

remove the influence of total body mass, allowing the comparisons of gonad and muscle mass independently of total body size (Hayes and Shonkwiler, 1996). Standardised residuals from a regression of the morphological character, gonad weight against total body weight and mantle weight against total body weight were used as mass independent measures of the reproductive and mantle tissue, respectively. Residuals are the difference between the actual measured value and the value predicted by the regression equation. To standardise the residuals, each residual was divided by the standard deviation of the predicted values. Individuals with positive residuals have heavier gonads or mantle weight for their total body weight and are defined being in good reproductive or somatic condition. In contrast individuals with negative residuals have lighter gonads or mantle weight for their total body weight and are defined being in poor reproductive or somatic condition. This approach of using standardised residuals to obtain size independent measures of the morphological characters has been successfully used in other cephalopod comparative life history studies (Moltschaniwskyj and Semmens, 2000; Pecl, 2004).

2.2.4 Statistical analysis

To examine the influence of temperature on population structure and individual life history characteristics the data was examined twice, initially by the season of capture and secondly by categories based on water temperature experienced. Based on the month of capture each individual was caught in either Summer (December-February), Autumn (March-April), Winter (June-August) or Spring (September-November). Examining individuals by the season of capture provided an illustration of the change in population structure over time, while sorting individuals by the temperature categories allowed comparisons among groups of individuals with similar temperature histories, as a function of the direction of change rather

than average water temperature. The date of capture and estimated age was used to assign individuals to one of four categories of temperature experience. Individuals were categorised as having spent most of their life in environments of either warm (above 17°C), cooling (17°C - 13°C), cold (below 13°C), or warming (13°C - 17°C) temperatures (Figure 2.2).

To determine how the date of capture and temperature condition influenced the biological processes of size and age, individuals were allocated to one of six sizes; extra small (≤2g), small (2.1-4.0g), medium (4.01-6g), large (6.1-8.0g) larger (8.01-10g), and extra large (>10g) and three age classes (<50 days, 51-100 days, >100 days). Size and age classes were compared among the season of capture using a χ^2 test of independence. Changes in the ratio of mature and immature individuals among the catch dates and temperature categories were examined using a χ^2 test of independence.

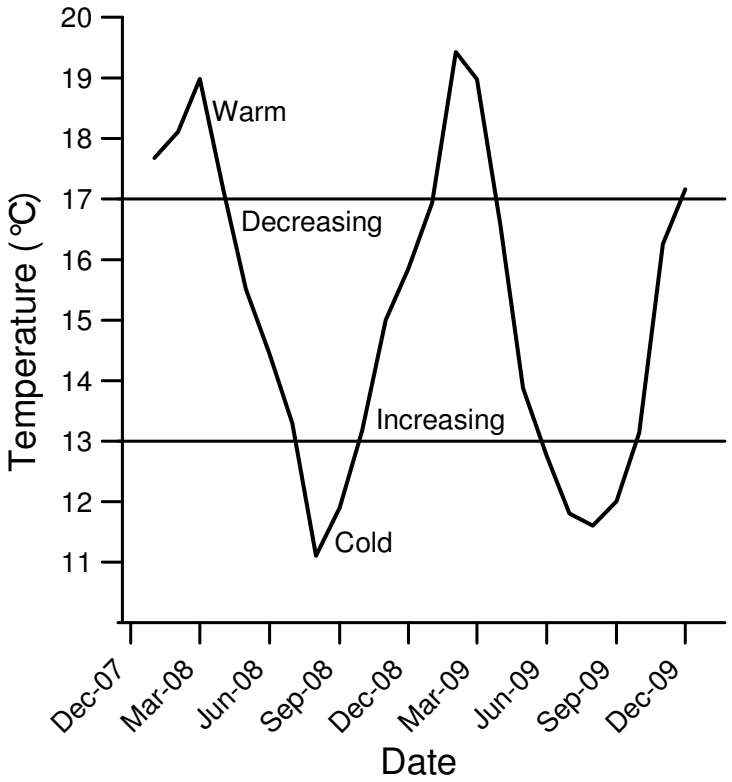

Figure 2.2: Average monthly temperatures (°C) from December 2007 -2009, showing the range of water temperatures in the four temperature categories.

The life history plasticity in cephalopods tends to be sex specific (Pecl*, et al.*, 2004), therefore the frequency distributions of size, age, and maturity were compared among the four temperature categories using a χ^2 test of independence for males and females separately. All individuals < 1g were classified as immature and were used in both male and female analyses. Both sex ratios and ratios of immature and mature individuals in each sex were compared among temperature categories using a χ^2 test of independence. For each sex, estimates of size-at-age were generated using total body weight (g) and estimated age (days). Initial examination of the data showed exponential growth so weight was log10 transformed before using a ANCOVA to compare the relationships among the temperature categories.

A factorial analysis of variance ($P<0.05$) was used to determine if the average weight, standardised reproductive or somatic residuals were affected by temperature category. A Tukey's post hoc test was used to determine where differences occurred.

2.3 Results

2.3.1 Population structure (organised by date of capture)

Euprymna tasmanica ranged in size from 0.2g - 18.50g, with small squid (<4g) being present in every month during the two years. In the laboratory, the estimated weight of newly-hatched *E. tasmanica* was approximately 0.33g (Chapter 3); therefore we were able to sample individuals across life history stages from newly hatched individuals to reproductively mature individuals. Differences in the size frequency among season caught (χ^2 = 170.83, *df 35*, P< 0.001) was predominantly due to the appearance and disappearance of larger animals (Figure 2.3). Animals <4.0g were present in greater numbers than expected during warming and warm seasons (Spring, Summer 2008 and Summer 2009) and fewer than expected in cooling and cold seasons (Autumn, Winter 2008 and Winter 2009) (Figure 2.3). In contrast, individuals in both the intermediate size classes (4.1-6.0g, 6.1-8.0 and 8.1-12.0g) were present in greater numbers than expected during the cooling and cold seasons (Autumn, Winter 2008 and Winter 2009) and fewer in the warming and warm seasons (Spring, Summer 2008 and Summer 2009) (Figure 2.3). Squid >12g were relatively rare in the population, but were relatively more abundant in the winter of 2008 and 2009 (Figure 2.3d).

Ages were estimated for 173 males, 151 females and 187 immature individuals (assuming that the 179 individuals <1g were <50 days). Immature individuals ranged in age from <50 to 97 days (mean = 50.79, ± 0.42 SE), while males ranged 56 to 142 days (mean = 102 ± 1.21 SE) and females from 54 to 133 (mean = 99 ± 1.27 SE). Differences in age frequency among the seasons of capture (χ^2 = 202.31, *df 1*4, P< 0.001) followed a logical pattern with peaks of young individuals (<50 days) being followed by peaks of old individuals (>100 days) around 4-6

months later (Figure 2.4). Young individuals were caught in greater numbers than expected when water temperature was warm (Summer and Spring 2008), while higher than expected frequencies of old squid (>100 days) were caught when water temperature was cool (Autumn, Winter 2008 and Winter 2009) (Figure 2.4c). The frequency of mid-aged squid (50-100 days) had less defined peaks that occurred over a wide range (six months), starting shortly after the peak of young individuals (Figure 2.4).

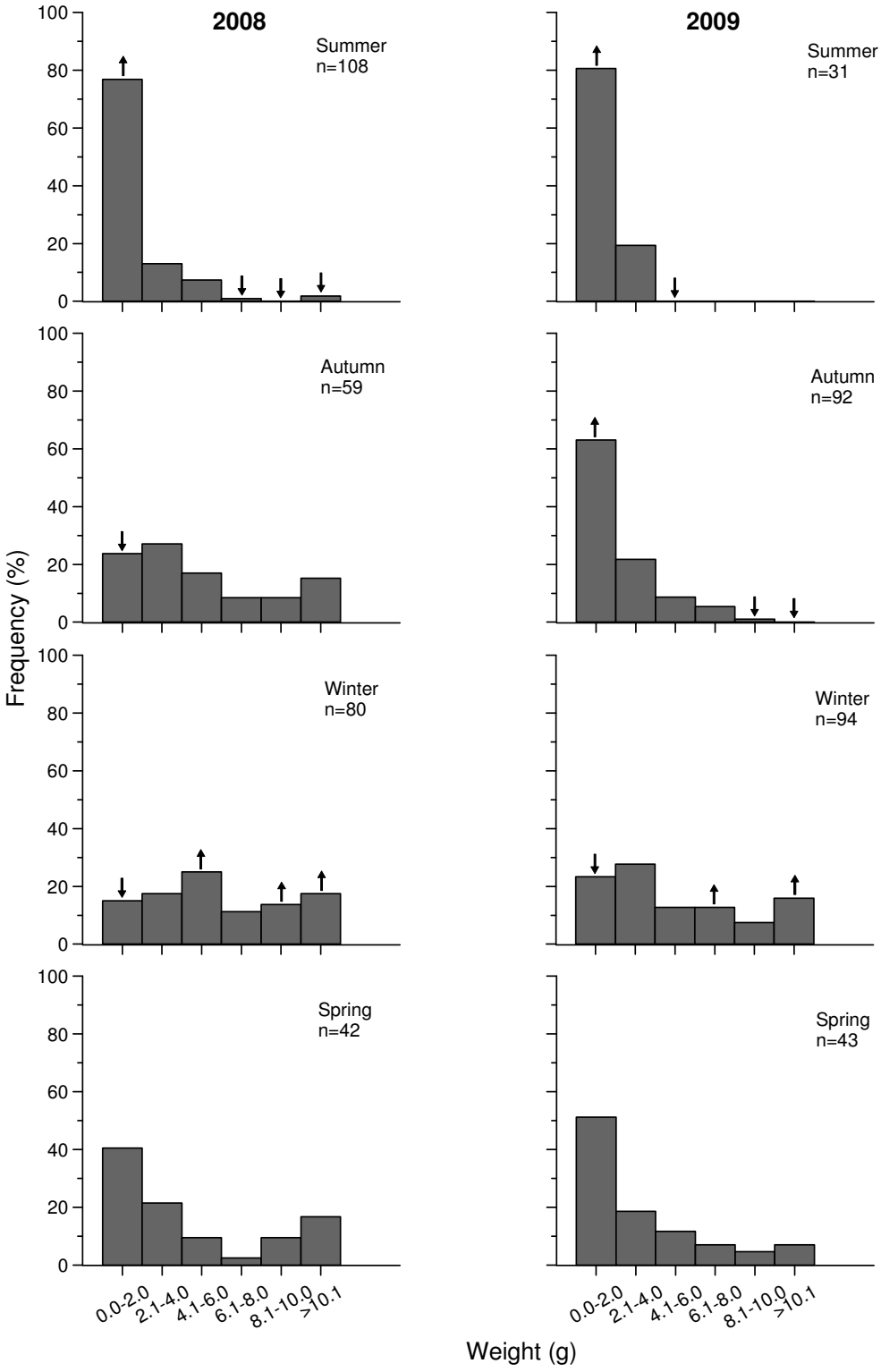

Figure 2.3: Size frequencies of the four seasons of capture, Summer (December-February), Autumn (March-May), Winter (June-August) and Spring (September-November) sorted by weight (g). ↑ and ↓ arrows indicate months that have significantly more or less than expected frequencies, respectively.

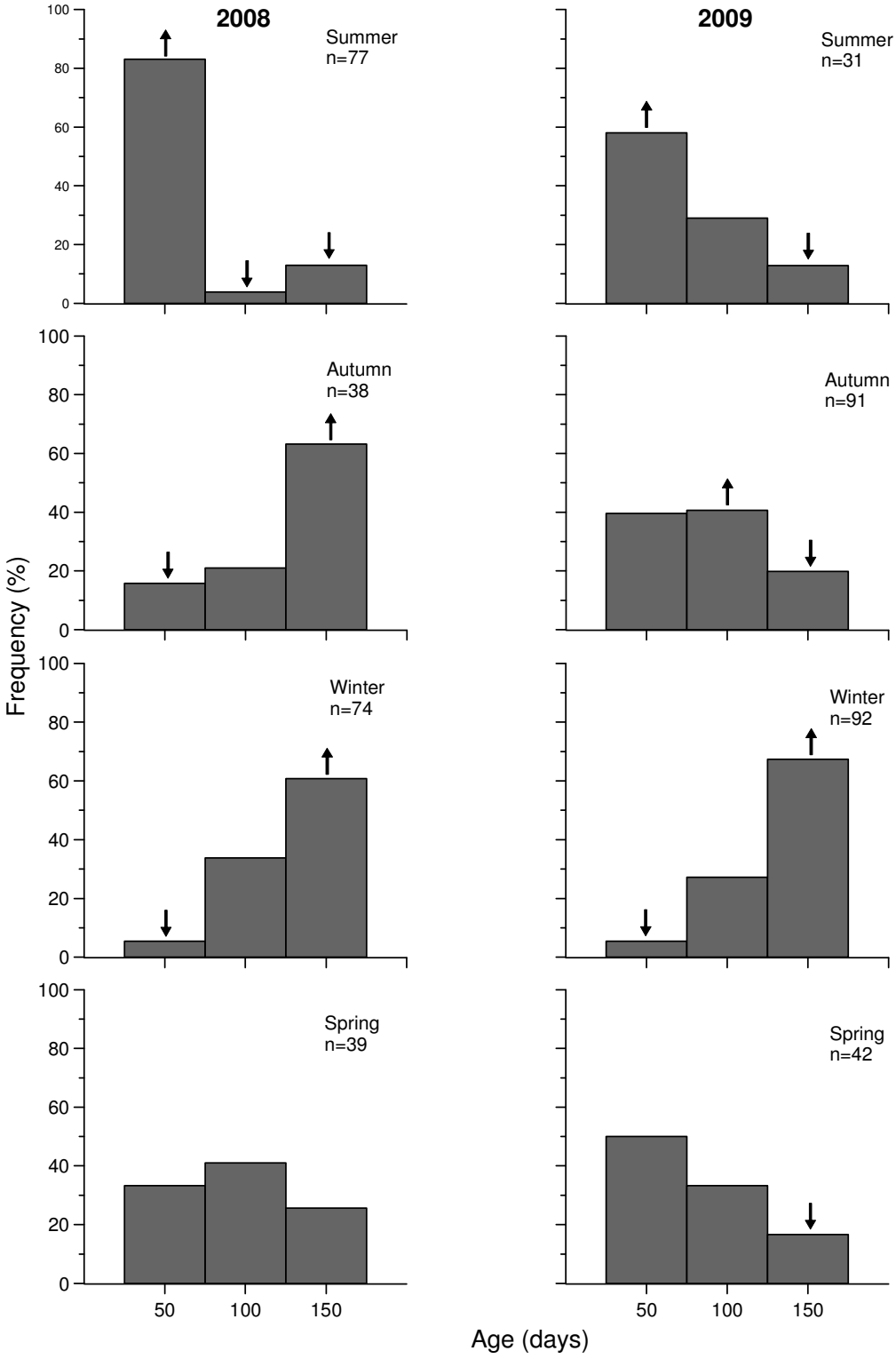

Figure 2.4: Age frequencies of the four seasons of capture, Summer (January-March), Autumn (April-June), Winter (July-September) and Spring (October-December) sorted by age (days). ↑ and ↓ arrows indicate months that have significantly more or less than expected frequencies, respectively.

The pattern of the ratio of immature and mature squid among the months (χ^2 = 80.06, *df* 7, P<0.001) was largely driven by the size frequencies, in that the maturity ratios favoured immature individuals (90-100%) during summer and mature individuals (50-60%) during the winter (Figure 2.5).

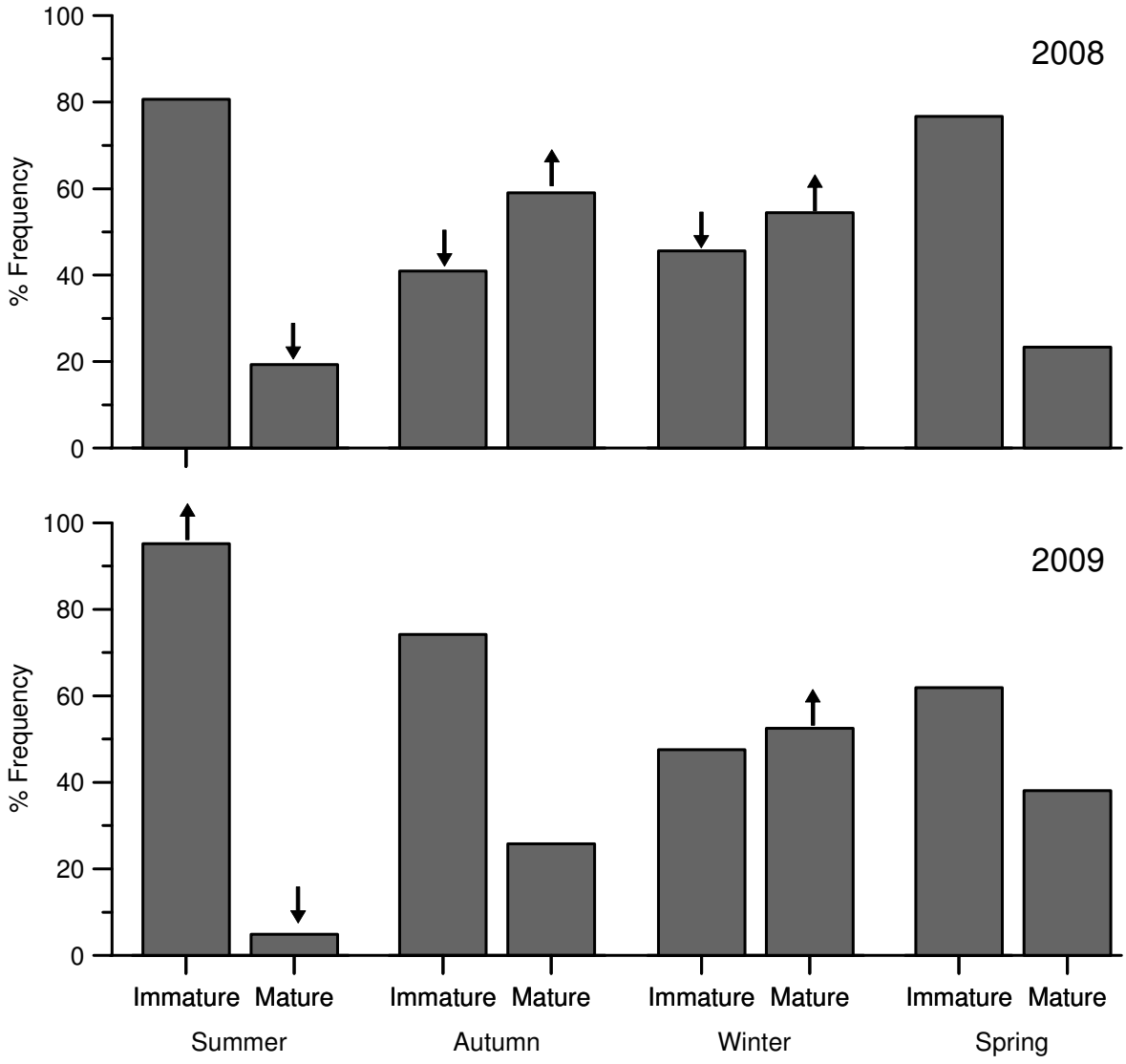

Figure 2.5: Percent frequencies of immature and mature squid sorted by the season of capture. ↑ and ↓ arrows indicate months that have significantly more or less than expected frequencies, respectively.

2.3.2 Individual life histories (organised by temperature condition)

The water temperature individuals experienced during the greater part of their lives significantly affected the weight of immature squid (F=52.06, *df* 3, 318, P<0.001), with squid that experienced cooling and cold water being on average 1.6g and 1.0g heavier than individuals from warming and warm water respectively (Figure 2.6).

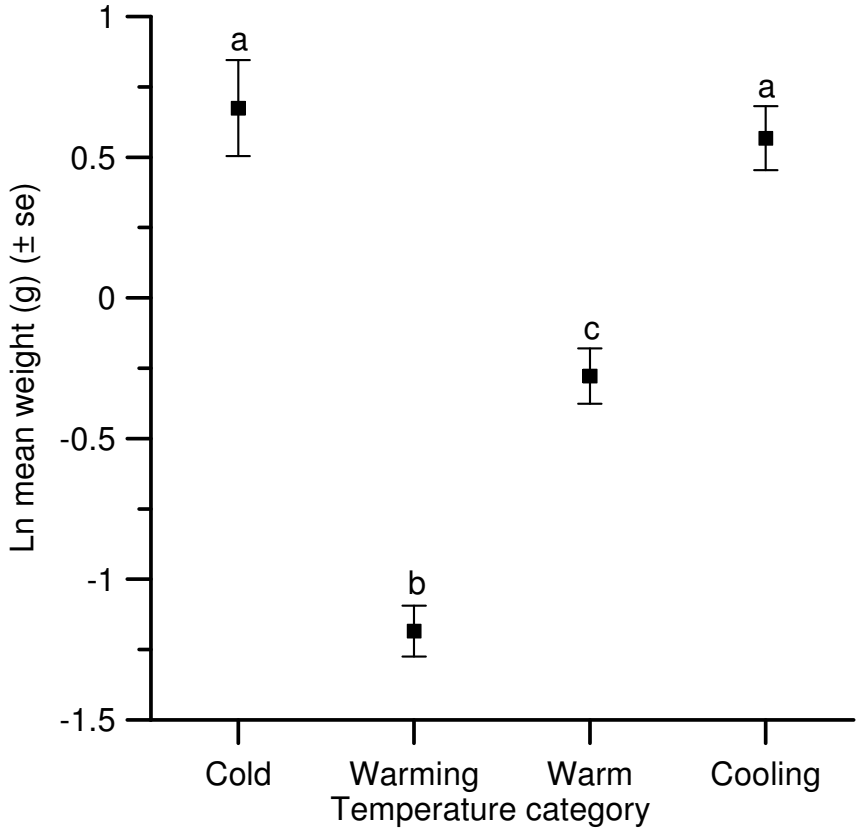

Figure 2.6: Mean weight (Ln) for immature squid of the four temperature conditions. Means with different letters are significantly different from each other.

Water temperature did not significantly contribute in explaining the variation in size-at-age of male squid. However there was a weak significant exponential relationship between weight and estimated age across all males (F=103.02, *df* 1, 174, P<0.001), with males growing on average 0.024g Ln body weight per day (Figure 2.7), only 37% of this variation in size was

explained by age. In contrast, there was a significant interaction between age and temperature on the weight of female squid ($F_{age*temperature\ category}$=3.38, *df* 3,143, P=0.02). The difference in weight among temperature categories was only apparent in old females (>100days), with females in the cold temperature category being twice the size of females in the warming and warm temperature categories (Figure 2.8).

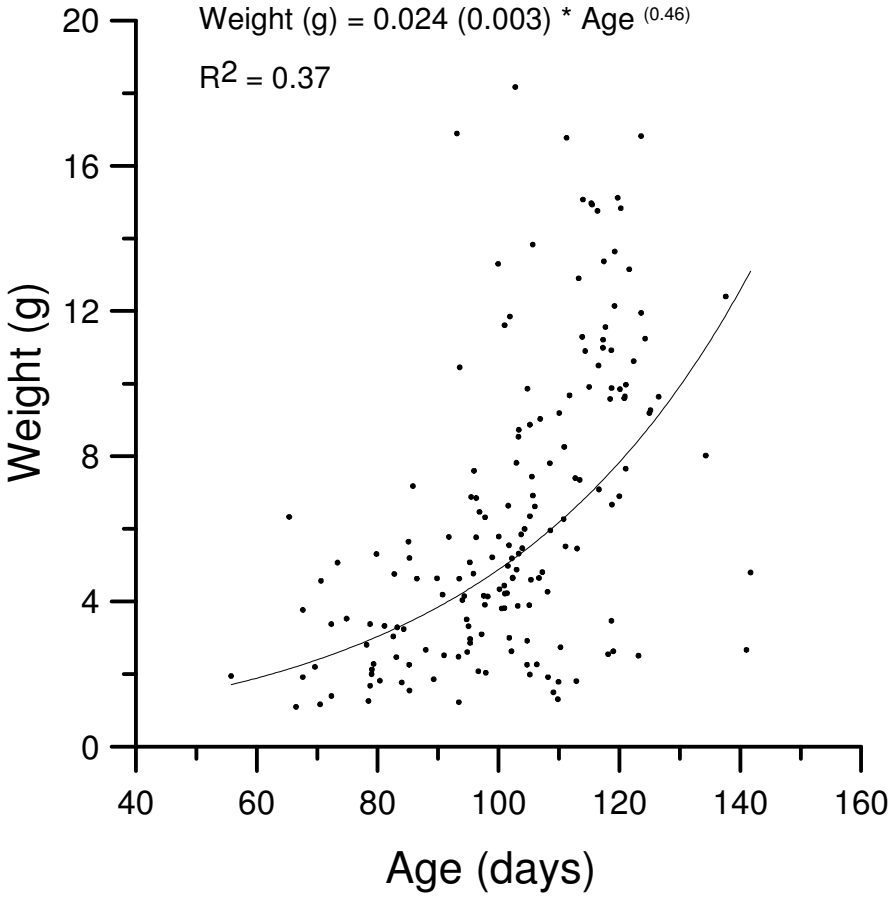

Figure 2.7: Size at age relationship for male squid >50 days old. Numbers in brackets are standard errors.

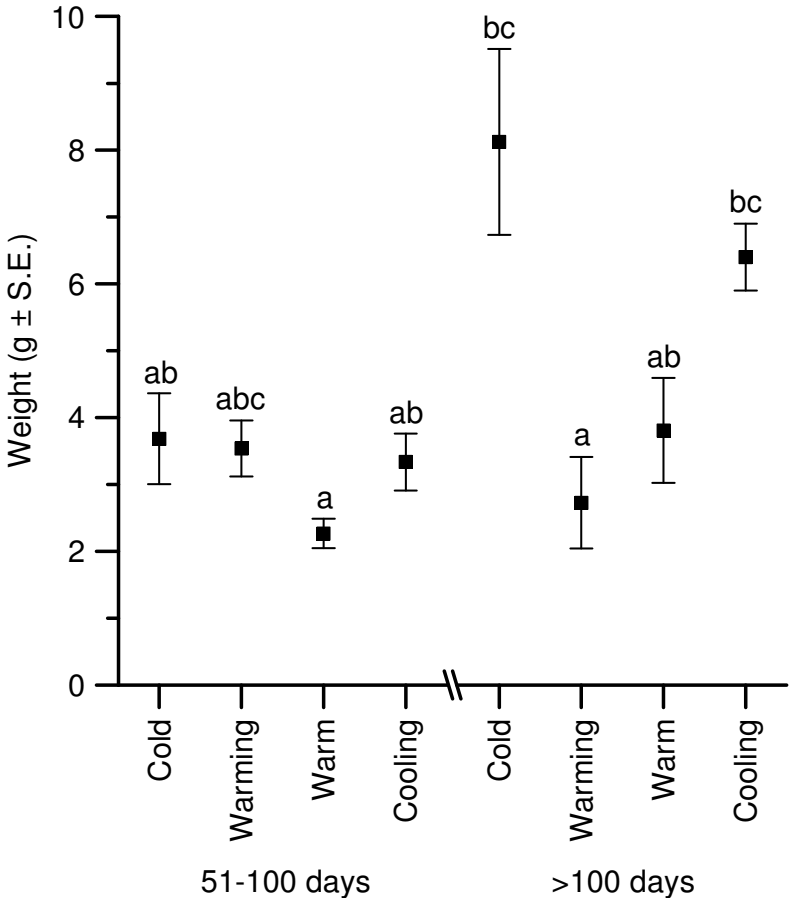

Figure 2.8: Mean weight of females among the four temperature categories in each size class >50g. Means with different letters are significantly different from each other.

The ratio of immature and mature squid differed among the four temperature conditions for both males (χ^2 =69.22, *df* 3, P< 0.001) and females (χ^2 =68.30, *df* 3, P< 0.001). Mature males and female were more frequent in the cooling and cold temperature categories than expected, while there were fewer mature males in the warming temperature category and fewer mature females in the warming and warm temperature categories than expected (Figure 2.9).

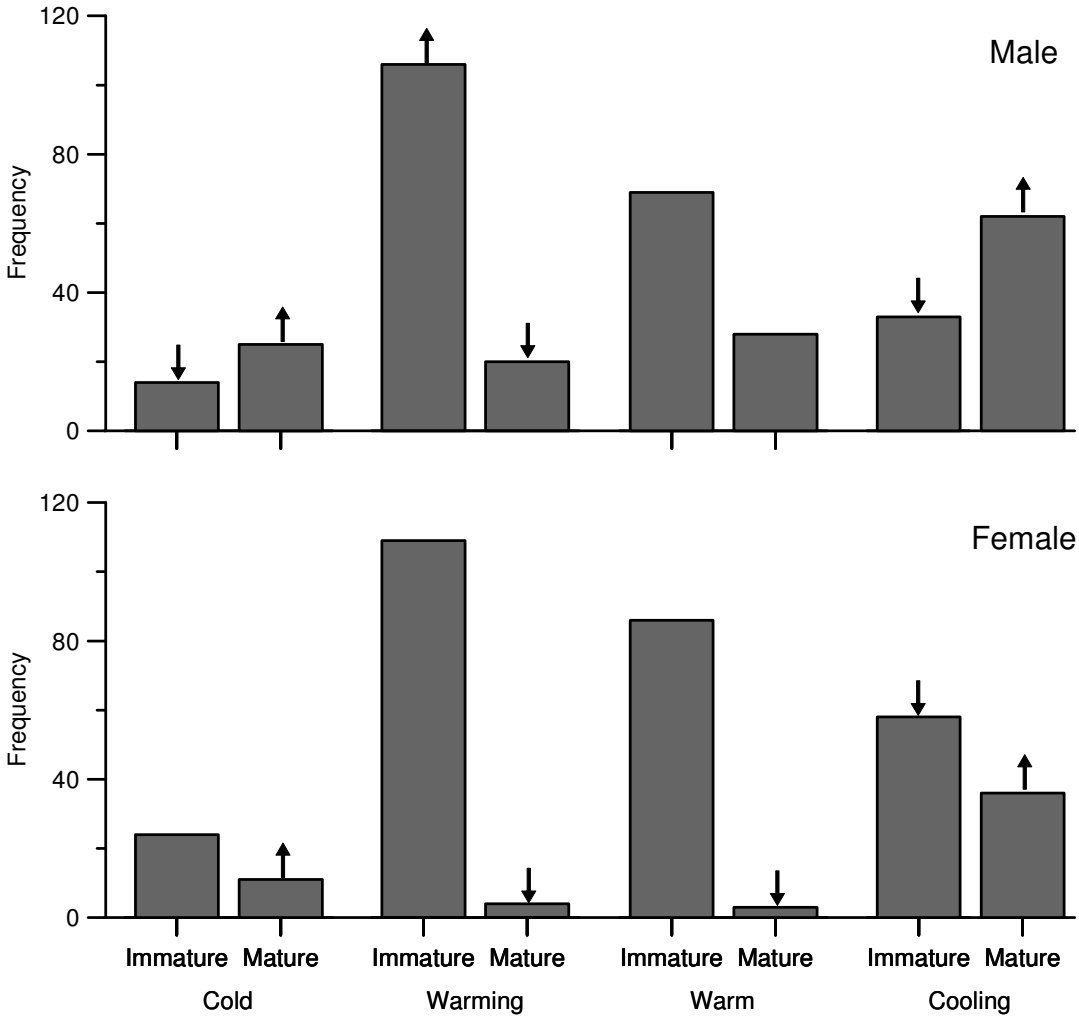

Figure 2.9: Frequency of immature and mature male and female squid in each of the four temperature categories. Arrows indicate categories with more ↑ or fewer ↓ females than expected.

The temperature category squid experienced had similar significant effect on the average reproductive condition of both mature male (F=14.78, *df* 3, 170, P<0.001) and mature female (F=8.05, *df* 3, 147, P=0.001) squid. Males that experienced warming water temperatures, were in poorer reproductive condition compared to males from all other temperature conditions (Figure 2.10a), while females that experienced warming water temperature were in poorer reproductive condition compared to females from cold and cooling temperature conditions (Figure 2.10b).

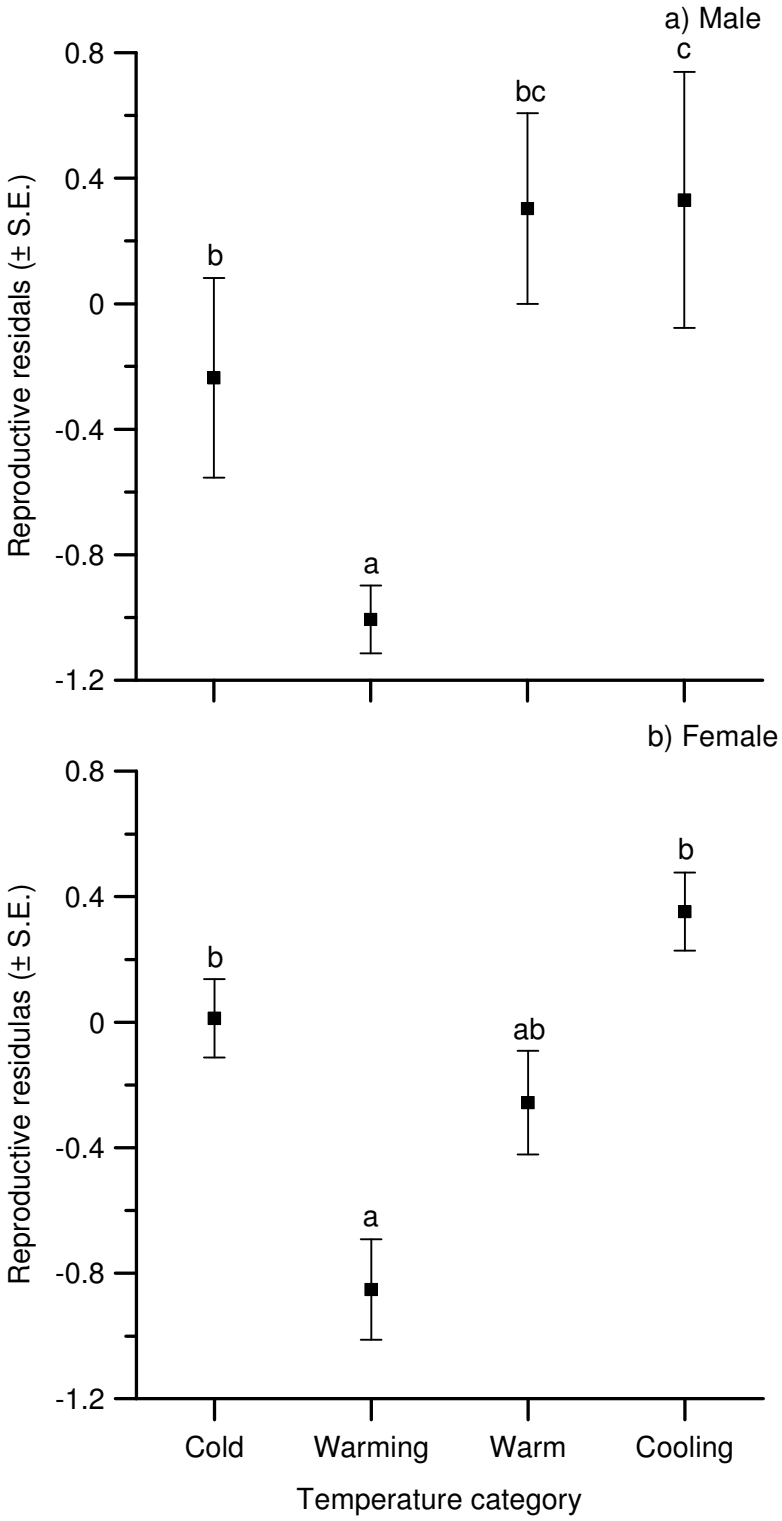

Figure 2.10: Mature male and female reproductive residuals of the four temperature categories. As the post hoc test could not find any significant difference in females it is assumed that the lowest (cold) and highest (cooling) values are significantly different from each other.

The temperature condition squid experienced had significant effects on the somatic condition of both immature ($F=9.87$, *df* 3, 66, $P<0.001$) and female ($F=5.11$, *df* 3, 137 $P=0.002$) squid. Immature individuals that experienced warming water temperature had lighter mantles for their size compared to immature individuals in the cooling and cold temperature categories (Figure 2.11a), while females that experienced cold water had larger mantles for their size compared to individuals from all other temperature categories (Figure 2.11b). The temperature conditions did not significantly contribute in explaining the variation in somatic condition of males.

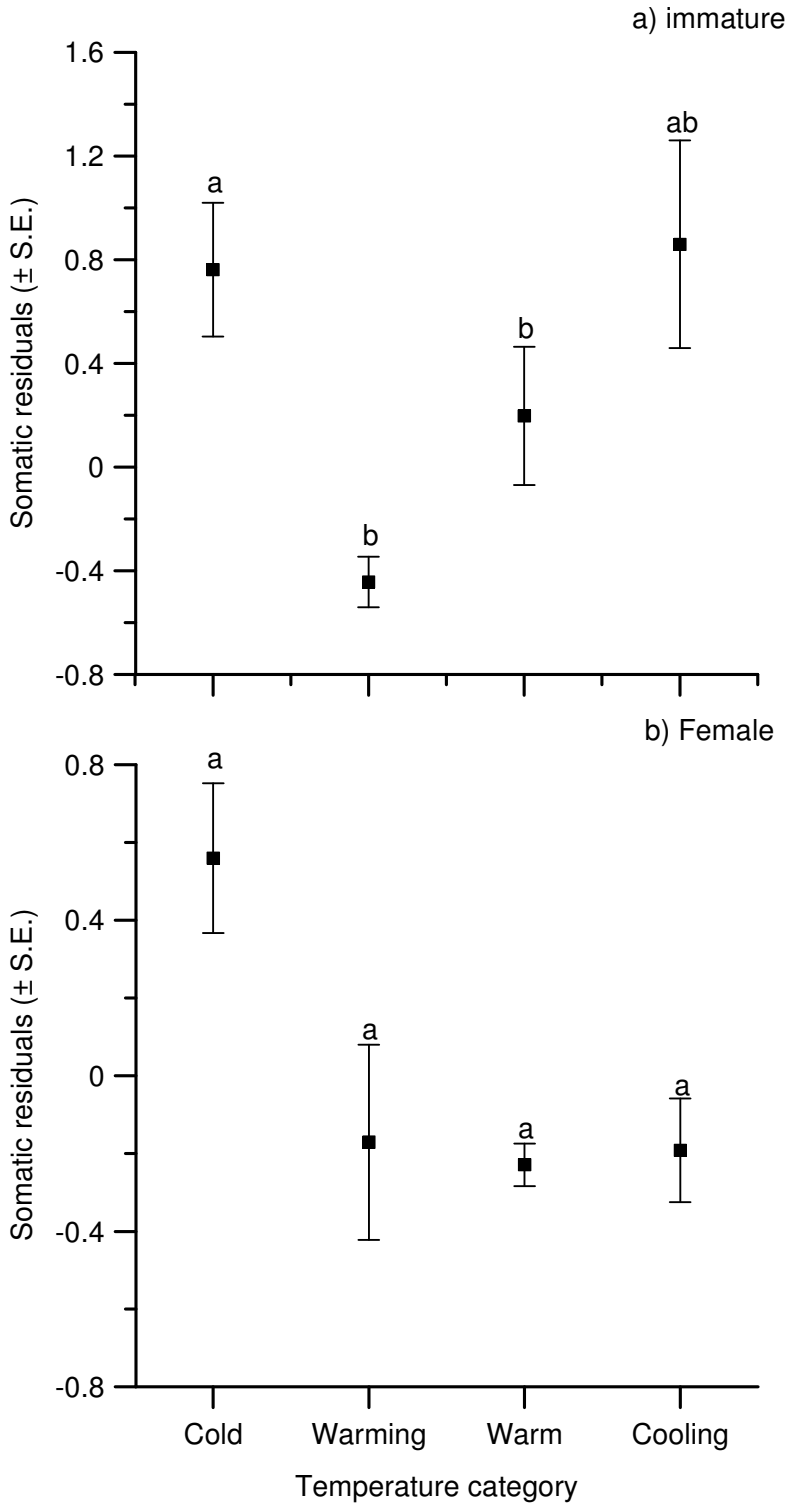

Figure 2.11: Immature and mature female somatic residuals of the four temperature categories. For immature squid means with different letters are significantly different from each other, while in females the post hoc test could not find any significant difference so it is assumed that the lowest (cooling) and highest (cold) values are significantly different from each other.

2.4 Discussion

Water temperature had a strong influence on the population structure of *Euprymna tasmanica*, with fluctuations in size, age and maturity closely following seasonal changes in water temperature. The increased frequency of small, young and immature individuals caught during spring and summer suggests that large hatching events occurred during periods of warming and warm water. In contrast, the presence of old, large, and mature individuals during periods of cold water temperature, suggests that the majority of egg production occurs during winter. As water temperature increased, the relative frequency of old, large and mature individuals significantly decreases and in one instance (December 2008) these individuals are absent from the population. Assuming these individual are not moving into deep water where we did not sample, it is possible that populations of *E. tasmanica* may completely lack mature individuals during periods of warming water temperature. The absence of large, mature individuals post winter suggests that individuals spawning over winter die shortly after. The strategy of laying eggs over winter is common in many temperate cephalopods as it insures that offspring hatch in warming water temperatures allowing for rapid growth (Sauer, et al., 1992; Roberts, 2005; Leporati, et al., 2008). This population structure better suits an aggregative spawning species that spawns synchronously over a defined spawning period of a few months (Figure 2.12a). In captivity *E. tasmanica* were able to live as long as nine months (Chapter 3), and although one annual spawning population is possible (i.e. with a three month egg development time), a single recruitment failure could lead to a collapse in the population. Many tropical squid compensate their short lifespan with asynchronous spawning (Pecl and Jackson, 2008) (Figure 2.12c), however significant differences in the size, age and maturity frequencies among the seasons also suggest that *E. tasmanica* is not asynchronous but has an

extended spawning season over a few weeks to months (Figure 2.12b). Extended spawning seasons over summer are not unusual among the cephalopods (Sauer and Lipinski, 1990; Rocha and Guerra, 1996; Rocha, et al., 2001; Boyle and Rodhouse, 2005) and is possibly one of the most important mechanisms responsible for the species survival. To further explain what is happening between each season the life history of individuals is needed, highlighting the importance of knowing age.

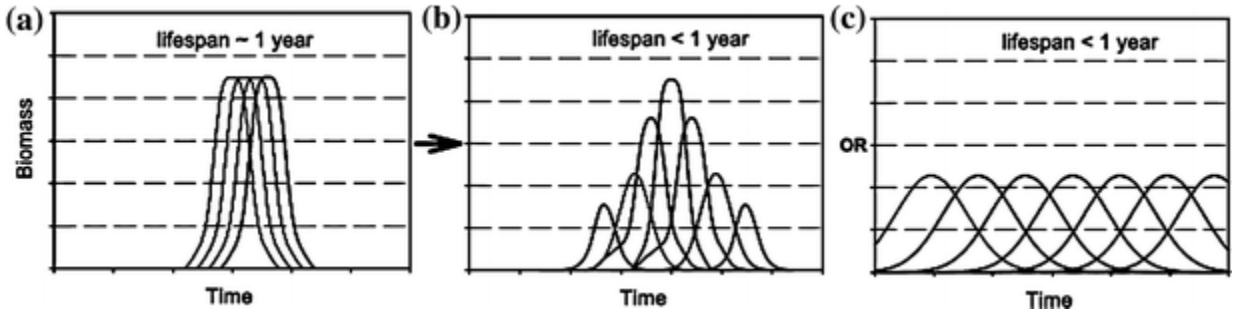

Figure 2.12 Diagrammatic representation of the fluctuation of squid biomass over a 1 year period (a) Annual spawning species that aggregate to spawners with an defined spawning season of a few months, resulting in a sucessive wave of recruitment. (b) As individual life span shortens, breading season extends beyond a few months, although seasonal peaks in biomass production are still evident. (c) Aseasonal, continuous recruitment with no syncronisity in spawning and no peaks in biomass (indicitave of many tropical squid species). This diagram is from Pecl and Jackson (2008).

Each month up to 30 individuals were permanently removed from the sampled population. One problem with destructive sampling is the possibility of introducing significant mortality and reduction in population size, and changing the population dynamics through localised depletion. Female *E. tasmanica* are multiple spawners, laying up to 25-170 eggs per batch (Norman and Reid, 2000). Although sampling from the same local area, this study assumed that the number of animals taken each month was small relative to the potential recruitment, and repetitive sampling had little influence on the size and dynamics of the local population, at least for the duration of this study.

The short life span, fast growth and early maturation of short-lived species exaggerate the difficulties of studying populations (Boyle and Boletzky, 1996), and only after sorting individuals by the temperature category they experienced, rather than the date of capture, the environmental effect on different life history stages became apparent. The environmental effects on growth were sex specific, with weight differences being found among temperature categories of immature individuals and mature females only. The weight of females differed significantly among temperature category, but only in females >100 days old. In the >100 day age class, females that experienced cold and cooling water temperatures were more than twice the size of females that experienced warming water temperature, suggesting that the maximum size is limited by the temperature experienced, and sizes above 4-5 g may only be achievable in cold or cooling water temperatures. Similar growth patterns are common in many squid species where individuals experiencing warm temperatures grow fast, mature early but reach smaller body sizes (Pecl and Jackson, 2008). While growth of females was influenced by water temperature, the relationship between weight and age for males was independent of temperature category, indicating that growth of mature male *E. tasmanica* is not affected by water temperature. This is in contrast to other species of squid where growth generally increases with warmer water temperatures (Hendrickson, 2004; Pecl*, et al.*, 2004). The different factors effecting growth between the sexes in this study may be due to the different physiological demands in reaching maturity; since males have a relatively smaller reproductive investment they may be able to respond to environmental changes more rapidly (Jackson and Domeier, 2003). In many near shore loliginid squid, maturity is considered a function of body weight not age (Pecl, 2001; Jackson, 2004a) and since the physical demands on egg production are greater than sperm production, females would need more time to reach larger sizes before

becoming mature (Ceriola and Jackson, 2010). It is important to note that the behavioural investment associated with reproduction has not been quantified in this study and it is possible that males are making a greater behavioural investment in reproduction by competing with other males (Hanlon and Messenger, 1996; Jantzen and Havenhand, 2003). Additionally, growth varies significantly with size, with smaller immature squid generally having faster growth compared to adults (Houlihan*, et al.*, 1998; Pierce*, et al.*, 1999; Ho*, et al.*, 2004; Semmens and Jackson, 2005; Moltschaniwskyj and Carter, 2010). Without knowing the age for all individuals sampled makes it difficult to comment on growth. In this study age estimates were limited to individuals >1g due to insufficient tissue needed for RNA analysis. Estimation of the age for individuals >1g, using RNA concentration assumes that the relationship between RNA concentration and age derived from laboratory reared animals can be extrapolated into the wild population. The absence of hard structures with a record of growth in *E. tasmanica* limits age estimation, however, it may be possible to tag juveniles using a barium isotope (Pecl*, et al.*, 2010), recapture them as known age adults and determine the RNA concentration; thereby producing a relationship for wild animals. In this study caution was used when interpreting age and growth data.

Differences in somatic condition among the temperature categories closely resembled the differences found in size, in that immature individuals that experienced warming water temperature were both smaller and in poorer somatic conditions compared to immature individuals that experienced cooling and cold water temperatures. Similarly, females that experienced cold water were both larger and in greater somatic condition compared to females from all other temperature categories. The reduced somatic condition of *E. tasmanica* females

that experienced warming water temperatures is unexpected as faster growing squid are generally in better somatic condition, for example, *Sepioteuthis australis* females that hatch in late autumn and winter grow slower and have the poorest somatic condition (Pecl and Moltschaniwskyj, 2006). As accurate length measurements are unattainable for *E. tasmanica* mantle weight measurements were substituted for mantle length measurements, possibly explaining the difference found in this study compared to other species of squid. However the better somatic condition of mature females that experienced cold water could be a result of their longer lifespan (Chapter 3) if the production of somatic tissue continues to the latter stages of their adult life (Rodhouse, 1998).

In mature individuals, comparisons of reproductive condition between temperature categories gave a clear picture of the reproductive investment of these individuals, with both males and females experiencing warmer water being in poorer reproductive condition compared to individuals experiencing cooling water temperatures. Given the temperature range experienced is similar for both warming (13°C-17°C) and cooling (17°C-13°C) temperature categories (Figure 2.2) it appears the direction of change in water temperature has significant effect on reproductive condition. Temperature is considered one of the main factors contributing to the variability in growth, maturation and size-at-maturity especially during the early juvenile phase (Mangold, 1987; Forsythe, 1993; Jackson, 1997; Robin and Denis, 1999; Hatfield, 2000; Martinez*, et al.*, 2000; Pecl, 2004; Pecl and Jackson, 2008). Therefore individuals in the warming temperature category would have experienced cold water as juveniles, and as a consequence matured at a smaller size compared to individuals in the cooling water temperatures that experienced warmer water as juveniles and matured when bigger. Since the

maturity process of many cephalopods is a function of size rather than age (Dimmlich and Hoedt, 1998; Pecl, 2001; Jackson, 2004a), individuals experiencing cooling water temperatures, are larger at sexual maturation and may have a greater capacity to invest energy to reproduction at any point in time. Whether this greater investment into reproduction relates to an increase in total fecundity is not known, as smaller females that can only allocate small amounts of energy to reproduction at any point in time may be partitioning reproductive effort into smaller more numerous egg batches. This dichotomy of batch size vs. batch frequency is common in many cephalopod species (Boyle*, et al.*, 1995; Moltschaniwskyj, 1995; Maxwell and Hanlon, 2000).

Assuming that all immature squid are <50 days old, comparing the average weight of immature squid among temperature categories may provide some insight into the variability of juvenile growth. Immature individuals that experienced cooling and cold water temperatures were heavier than those that experienced warming and warm water temperatures. This difference in total body weight is likely to be a result of faster growth rates, increased hatching size or maturing later with larger body sizes. However, assuming that growth rates of wild *E. tasmanica* is similar to that of lab reared *E. tasmanica* (Chapter 5), and is reduced in cooler water, the large size of immature squid in the cooling and cold temperature categories is not a factor of fast growth but rather a factor of initial hatching size, maturing later at larger sizes or a combination of the two. Water temperature is known to effect hatchling size in some squid species, with smaller hatchlings emerging during warmer waters (e.g. *Loligo vulgaris* (Villanueva, 2000); *Sepioteuthis australis*, (Steer, et al., 2003a) *Loligo opalescens* (Vidal*, et al.*, 2002b)). Although egg size may not be a direct response to water temperature, in species that have no

parental care of their progeny, egg size is positively correlated with maternal size (Rodrigues*, et al.*, 2010), which as previously seen is related to water temperature experienced. Recruitment of *E. tasmanica* is not limited to one specific season but occurs at different frequencies throughout the year. While hatching occurs all year round, individuals that hatched during warm water are generally considered to experience more favourable conditions for growth and survival ((Pecl, 2004; Pecl*, et al.*, 2004), indeed these animals grew faster and matured earlier but their lifespan was also much shorter and for the population to survive more than one year, *E. tasmanica* relies on the longer living, slower growing later maturing, cold water cohort to bridge the gap between summer cohorts. With the ability to change life history characteristics depending on the environment, many squid species are regarded as opportunistic organisms (Jackson and O'Dor, 2001; Rocha*, et al.*, 2001; Boyle and Rodhouse, 2005; Pecl and Moltschaniwskyj, 2006; Pecl and Jackson, 2008), however this study illustrates that this flexible lifestyle is more of a necessity that populations of short-lived squid rely on for their survival.

Chapter 3 Individual life histories – a morphological approach to how the environment shapes an individual

3.1 Introduction

The pattern of growth in most cephalopods has been described as non-asymptotic, in that somatic growth continues throughout their entire live span, even during and after spawning (Jackson, 1994; Jackson and O'Dor, 2001). Growth rates of cephalopods are rapid and highly variable, with differences in size and age at maturation varying even between individuals of the same species (Arkhipkin, 1994; Brodziak and Macy, 1996; Pecl and Moltschaniwskyj, 2006). Given somatic and reproductive growth proceed together over much of the life cycle it is generally not possible to describe each process separately, rather it is likely that the variability in reproductive strategies of a species is related to the variable patterns of growth (Mangold*, et al.*, 1993). Additionally, many cephalopods provide little evidence of any significant trade-off between somatic and reproductive growth, as energy for reproduction is sourced directly from food and not from stored energy (Rodhouse and Hatfield, 1990; Moltschaniwskyj, 1995; Moltschaniwskyj and Semmens, 2000; Rosa*, et al.*, 2004; Rosa*, et al.*, 2005). Early studies by Wodinsky (1977) and O'Dor and Wells (1978) were able to describe a trade-off between somatic and reproductive growth in female octopus by examining somatic growth in the absence of reproduction. In these studies, the authors found that inhibiting growth of female ovaries, by removing the optic nerve, increased somatic growth rates (Wodinsky, 1977; O'Dor and Wells, 1978). However these studies did not consider the external factors influencing the variation in growth.

Many abiotic and biotic factors affect growth rates in cephalopods, including photoperiod (Koueta and Boucaud-Camou, 2003), temperature (Forsythe and Van Heukelem, 1987; Forsythe, 1993; Pecl, 2004), nutrition (Moltschaniwskyj and Martinez, 1998; Jackson and Moltschaniwskyj, 2001b; Vidal, et al., 2002a), and sexual maturation (Wodinsky, 1977; O'Dor and Wells, 1978; Semmens*, et al.*, 2004). Increased temperature generally increases growth rates; however, recent studies have shown that temperatures outside of the physiological range of the species will reduce growth rates (Beukema*, et al.*, 2009). The relationship between ration however is more complex with food quality (Domingues*, et al.*, 2009), feeding frequency (Iglesias*, et al.*, 2006), and ration levels (Moltschaniwskyj and Martinez, 1998; Jackson and Moltschaniwskyj, 2001b) all having significant effects on growth and maturity.

Although whole animal somatic growth is easily measured, it is one of the most complex developments of an organism and is the outcome of a series of biological and physiological processes (Forsythe and Van Heukelem, 1987). In the past, many equations have been used to assess growth in cephalopods, such as von Bertalanffy (Arreguín-Sánchez*, et al.*, 2000) and the Schnute model (Brodziak and Macy, 1996; Jackson and Moltschaniwskyj, 2001b). However when measuring growth, a single equation is not the best method as it does not adequately describe the pattern of growth at each stage of the individuals life, especially the early and late stages (Forsythe and Van Heukelem, 1987). There have been many ways of describing growth in cephalopods (see Moltschaniwskyj (2004) for a review), but when comparing animals of a different size the only useful measurement is the percentage change in size over a given time interval (Forsythe and Van Heukelem, 1987). Ever since the reviews by Forsythe and van

Heukelem (1987) the use of instantaneous relative growth rate (IRGR) has become commonly used to compare growth within and among cephalopods species.

Our best understanding of growth in cephalopods has come from both controlled laboratory experiments (Forsythe and Van Heukelem, 1987; Forsythe*, et al.*, 2001) and through statolith based ageing studies of wild populations (Jackson, 1997). Both approaches have their advantages and disadvantages; estimates of the growth rates of wild individuals rely on the accuracy of age determination techniques, while estimating growth rates from captive animals assumes that growth under laboratory conditions accurately portrays growth in the wild. Furthermore, most of these studies describe growth in the life history stages available to them, e.g. research done in collaboration with cephalopod fisheries that largely capture mature individuals (Arguelles*, et al.*, 2001; Villegas, 2001; Bainy and Haimovici, 2012). To describe the environmental effects on growth and how different characteristics of growth effect maturity and reproduction, the whole life history of an individual is needed, especially in early life where most of the variation of growth occurs (Mangold, 1987; Forsythe, 1993; Jackson, 1997; Robin and Denis, 1999; Hatfield, 2000; Martinez*, et al.*, 2000; Pecl, 2004; Pecl and Jackson, 2008).

Laboratory studies that follow growth throughout an individual's lifespan typically find that cephalopod growth can be described using a bi-phasic model (Forsythe and Van Heukelem, 1987; Forsythe, 1993) due to a shift from a rapid exponential growth phase during the juvenile phase to a slower adult growth phase (Forsythe and Van Heukelem, 1987; Jackson, 2004a; Semmens*, et al.*, 2004). Although the adult growth phase is commonly exponential, some octopus species grow logarithmically (Cortez*, et al.*, 1999; Semmens*, et al.*, 2004) or linearly for example the short-finned squid, *Illex illecebrosus* (Dawe and Beck, 1997). While the

inflection point between both phases has been described in a number of cephalopod species (Forsythe and Van Heukelem, 1987; Jackson, 2004a; Arkhipkin and Roa-Ureta, 2005; Schwarz and Perez, 2010), the biological cause and the significance of the change is unclear. Some studies have suggested that the inflection point may represent a significant change in energy allocation away from somatic growth and directed into reproductive growth (Forsythe and Van Heukelem, 1987; Mangold, 1987; Rodhouse, 1998). If this theory was true then it would be expected that females, given their higher reproductive investment to reproduction, would be smaller than mature males at the same age (Pauly 1998). However this is generally not the case, with females of approximately 70% of cephalopod species attaining larger body sizes than males (Vidal, et al., 2002b).

Despite these uncertainties, somatic and reproductive growth appear to proceed together over much of the life cycle in cephalopods, with growth being an important factor in determining the age and size at maturation (Mangold*, et al.*, 1993). Given the reliance of reproductive processes on growth it is important to study both processes together. The present laboratory based study aimed to explain differences in growth and reproductive output of female *E. tasmanica* that experience different environmental conditions. In particular how temperature and ration effect the pattern of growth, IRGR, longevity, size and age at laying the first egg batch, as well as the differences in the reproductive output such as, batch size, egg size, embryo development time and hatchling survival. By studying how temperature and ration influence growth and reproduction of individuals within the laboratory provided insight into the environmental effects that may influence the life history characteristics of individual in nature and will provide a basis from which field based studies can be modelled.

3.2 Materials and Methods

3.2.1 Collection of wild adults

Mature *Euprymna tasmanica* (10 males and 10 females) were collected from two sites at the mouth of the Tamar estuary, Kelso (-41.00^{o} S, 146.78^{o} E) and Low head, (41.00^{o} S, 146.01^{o} E) in August and September 2008 (Figure 3.1). Animals were collected by divers on either snorkel or SCUBA, visually locating animals and using dip nets to catch individuals. Each squid was gently placed into individual ziplock plastic bags with seawater and within two hours of capture, squid were transported back to the seawater facility on the Launceston campus of the University of Tasmania. On arrival at the facility, the ziplock bags containing the squid were floated in a temperature controlled re-circulating seawater system (15°C) until the water temperature in the plastic bag equilibrated. Each squid was held separately in a 12L floating mesh bottom container within one of two 1,000L raceways, which were part of a 2,500L recirculating seawater system with biofilter, UV sterilizer, protein skimmer, and heater-chiller unit. Each container had a hide (section of a PVC pipe) which was used by the females to attach eggs.

3.2.2 Production of Generation one

Wild caught squid were held at 15^{o}C and fed live mysids (*Tenagomysis tasmaniae*, *Paramesopodosis rufa*, and *Anisomysis mixta australis*) daily to satiation. During September and October of 2008, each female was mated randomly with at least two out of 10 males before egg deposition occurred (Figure 3.1). Mating was evident when the male wraps his arms around the female holding her for up to three hours. For each female for which mating was observed the

date and which male was recorded. After mating, females were removed from the males' container and returned back to her container. All individuals were given at least one day between each mating attempt. As females deposited eggs on the hides, the hides were removed and replaced with new hides to encourage further egg production. Hides with eggs were labelled with the mother's identification and date of egg deposition. Each hide was held in separate 5L floating mesh bottom containers floating in the raceway until hatching occurred.

	Start	End	Days
Breeding trial	27/8/08	18/11/09	309
Generation One	27/8/08	4/12/08	68
Mature adult collection	27/8/08	18/9/08	16
Mating and Egg laying	22/9/08	22/9/08	1
Egg development	23/9/08	4/12/08	50
Generation Two	29/10/08	18/11/09	266
Hatching	29/10/08	4/12/08	25
Acclimation (18°C and 13°C)	3/1/09	5/1/09	1
Mating	1/4/09	22/6/09	58
Egg laying	3/4/09	4/10/09	129
Embryo development	3/4/09	18/11/09	160
Hatching	10/6/09	18/11/09	113

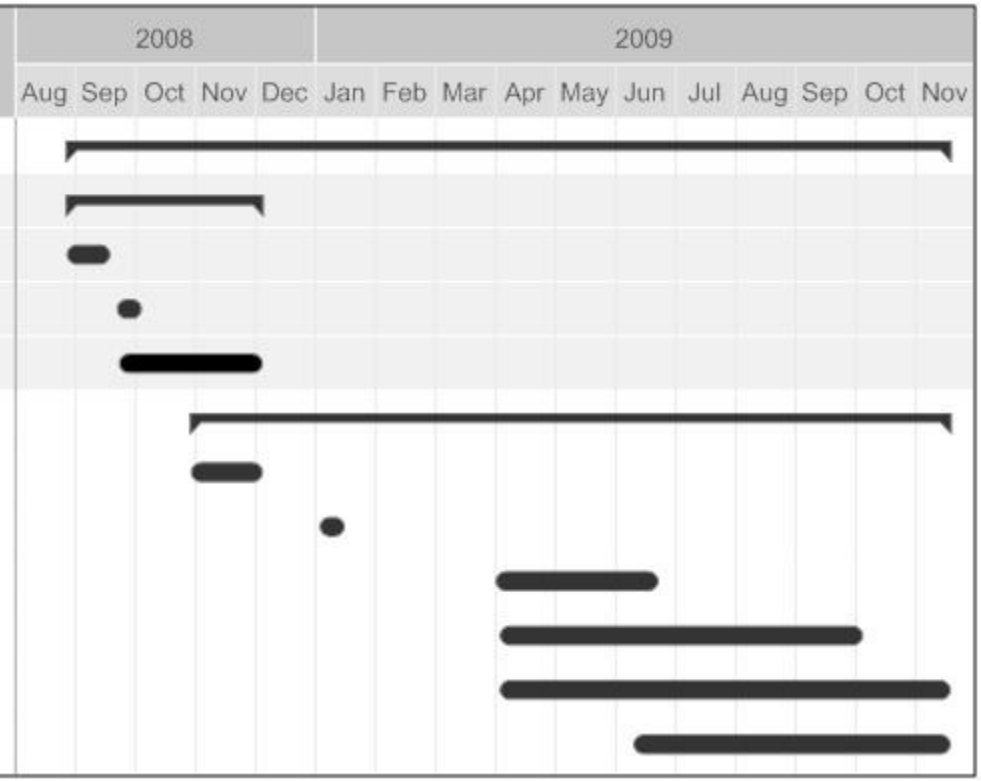

Figure 3.1: The production of two generations of *Euprymna tasmanica* over 2008-2009, including the timing of mating, egg deposition, embryo development and hatching for each generation.

3.2.3 Generation two

Individuals from generation one hatched between October and December 2008 (Figure 3.1). Once hatched, hatchlings were held individually in 0.5L floating mesh bottom containers with a waterproof label to identify each individual squid according to its mother, egg mass, and date hatched. Each label also had a scale bar for size calibrations when measuring growth from photographs. For the first 30-40 days of their lives individuals were held at 15°C and fed to satiation before being randomly allocated to one of four treatments using a random number generator (Figure 3.1). The four treatments included combinations of two temperatures (18°C and 13°C) and two rations (high-fed which were fed to satiation daily and low-fed which were fed to satiation two to three times a week). In some squid species young individuals, despite being hungry, refuse to eat food that is present in the tank for an extended period (Moltschaniwskyj and Jackson, 2000). Therefore, every day any uneaten food was removed before replacing with fresh mysids. Temperatures used were chosen to match summer and winter averages of 2008-2009 from the collection site. Water temperature measurements were supplied by AbTas, an abalone farm 2.5km south (-41.11° S, 146.81° E) of the collection site, who record temperature continuously at a depth of ~5m depending on the tide. Individuals were held in each combination of ration and temperature treatment for four months until sexes could be differentiated macroscopically via the presence or absence of the hectocotylus, at which point males were eliminated from the experiment. Mating of generation two occurred between April-June 2009 (Figure 3.1). To supply each female with one mate a week, 10 mature males were collected from the wild and held individually in 5L floating mesh bottom containers. Males were given one week to acclimatize after which each male was paired and allowed to

mate with one female every second day. To reduce paternal influence on offspring fitness each female was randomly assigned and mated with at least three males. After each mating was observed, males were removed and placed in isolation for a minimum of one day between each mating.

Length is not a good measure of growth in soft bodied animals (Forsythe, 1984) and is dependent on the state of muscle contraction (Cortez*, et al.*, 1999), therefore to measure growth rates of each individual, a photograph of the dorsal view of each individual squid while sitting on the bottom of the container was taken every 2-3 weeks. The identification label was included in every photo enabling the photographs to be calibrated. The dorsal surface area of each individual was determined from each photo using ImageTool v 3.0 (UTHSCSA) and the area converted to an estimated weight using two linear regression equations (depending on the size of the animal) derived from the area of 26 squid of known weight.

If area < $2mm^2$ Weight (g) = 0.6379 x area (cm^2) – 0.059

If area > $2mm^2$ Weight (g) = 1.098 x area (cm^2) – 0.4812

Using the weight estimates from each photo, changes in size over the four months allowed estimates of growth to be calculated for each individual. Once mature, females were mated with haphazardly chosen wild males. As each batch of eggs was deposited, hides were removed, photographed, and replaced with new hides to provide spawning substrate for production of subsequent batches. The number of batches produced, the number of eggs in each batch, total number of eggs laid, egg volume, and hatchling size were recorded and compared among females in the four treatments. To calculate average egg volume, 10 eggs

were measured from the photograph of each batch. *Euprymna tasmanica* lays individual spherical eggs each with its own protective casing. In some cases the protective casing has a flattened surface at the base (point of attachment) and a small peak where the protective casing seals during egg laying (Figure 3.3). To insure that all eggs were measured the same; the smallest width from the photograph was used as the standard diameter when calculating egg volume. The radius (r) was then calculated and used in the following formula to estimate volume:

$$\text{Volume} = 4/3\,\pi r^3$$

3.2.4 Statistical analysis

Instantaneous relative growth rates (IRGR; percent increase in body weight per day) for each female were calculated to allow the comparison of growth rates among the four treatments using the equation:

$$IRGR = b \times 100 = \left(\frac{LnWt_2 - LnWt_1}{t_2 - t_1}\right) \times 100$$

where $\ln Wt_2$ is the natural log of the body mass at time t_2, $\ln Wt_1$ is the natural log of the body mass at time t_1, t_2 is the age at the second measurement and t_1 is the age at the first measurement (Forsythe and Van Heukelem, 1987). If growth is exponential, IRGR values calculated for each growth increase will remain constant and the point at which IRGR values start to decrease can be used to indicate the end of an exponential growth phase (Hatfield*, et al.*, 2001). For each individual, decreases in IRGR values were used to calculate the age and weight of inflection between growth phases, where a dramatic decrease in IRGR values indicates a change in growth rate (Figure 3.2). To describe the growth of each individual,

exponential, linear and logarithmic growth curves were fitted to data sets of each identified growth phase and the greatest r^2 value was used to indicate the curve with the best fit. Although growth was observed post spawning, large decreases in weight post spawning made estimating the final stages of growth difficult. Therefore, when analysing the IRGR's only data prior to first batch deposition was used. A two-way factorial analysis of variance was used to determine if the treatments explained the variability in growth rates, longevity, and the age and weight of the inflection between the two phases of growth.

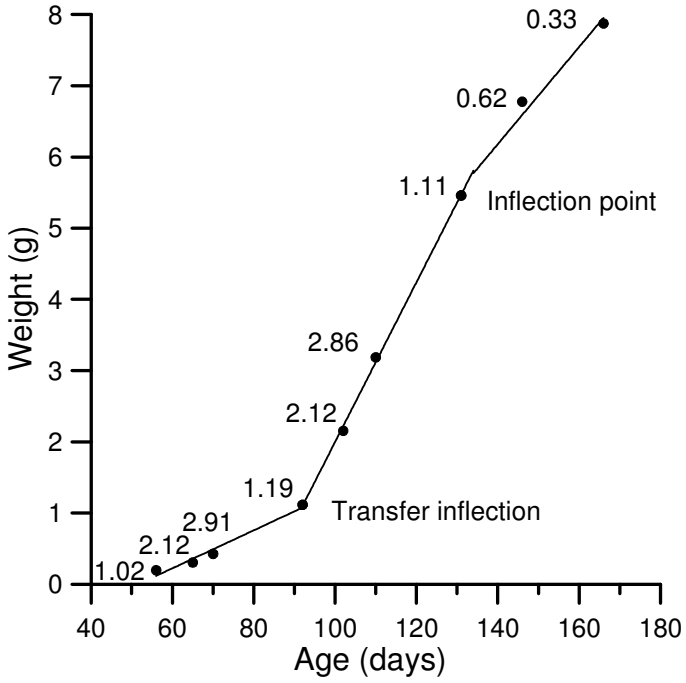

Figure 3.2 The growth of a female squid showing the IRGR values (above data points) and the transfer inflection and inflection points.

A factorial analysis of variances was used to determine if the treatments had an effect on the age (days) and weight (g) at which females produced their first batch of eggs. As the majority of females only laid up to 2 batches, to eliminate any differences between multiple batches produced by females, only a female's first egg batch was used when comparing batch

size, average egg size and the embryo development time among treatments. A factorial analysis of variances was used to determine if the treatments had an effect on maternal longevity, batch size (eggs), egg volume (mm^3), embryo development (days between laying and hatching), and hatchling size. Regression analysis was used to determine if a relationship exists between egg size and hatchling size.

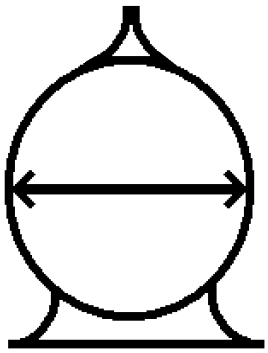

Figure 3.3: A diagram of an egg showing the peak and widened base. All measurements of diameters were taken from the shortest width as shown by the arrow.

3.3 Results

3.3.1 The influence of temperature and ration on the growth trajectory

The growth of 46 female *Euprymna tasmanica* was tracked from the day they were transferred to each treatment until death; 12 females experienced low temperature/low ration (LT/LR), 15 low temperature/high ration (LT/HR), nine high temperature/low ration (HT/LR), and 10 high temperature/high ration (HT/HR). Declines in the IRGR values indicated a three-phase growth model with two inflection points at which changes in growth rates occurred (Figure 3.4). The mean weight and age for the first inflection was 1.4g (se = 0.55) and 61 (se = 6.55) days respectively and there was no evidence that any treatment or treatment combinations explained any variation in age ($F_{temperature*ration}$=0.32, *df* 1, 46, P=0.577) or size ($F_{temperature*ration}$=0.92, *df* 1, 46, P=0.34). This first inflection point generally occurred 9 days after transferring juveniles to their respective treatments and is likely an artefact of the controlled experiment rather than a 'true' inflection. Therefore, to simplify the comparisons between this study and the literature, this inflection will be called the 'transfer inflection' and the second inflection point between the early and late growth phases will be referred to as the 'inflection point'.

The pattern of growth was highly variable among females, but could be adequately described with two linear models for both the early (before inflection) and late (after inflection) growth phases, with the r^2 values for linear fits ranging between 0.74-0.98 and 0.70-0.99, respectively (Figure 3.4). Temperature had a significant effect on growth rate of females during the early growth phase (F=29.09, *df* 1, 46, P<0.001), with squid held in 18°C growing on average

47.34% faster than those held in 13°C (Figure 3.4). The effect of temperature was independent of ration ($F_{temperature*ration}$ =0.005, *df* 1, 46, P=0.94), and ration had no significant effect on the growth rate of female squid during the early phase of growth (F=1.96, *df* 1, 46, P=0.17), with the mean growth of individuals held in high and low rations being 0.043g/day (se=0.002).

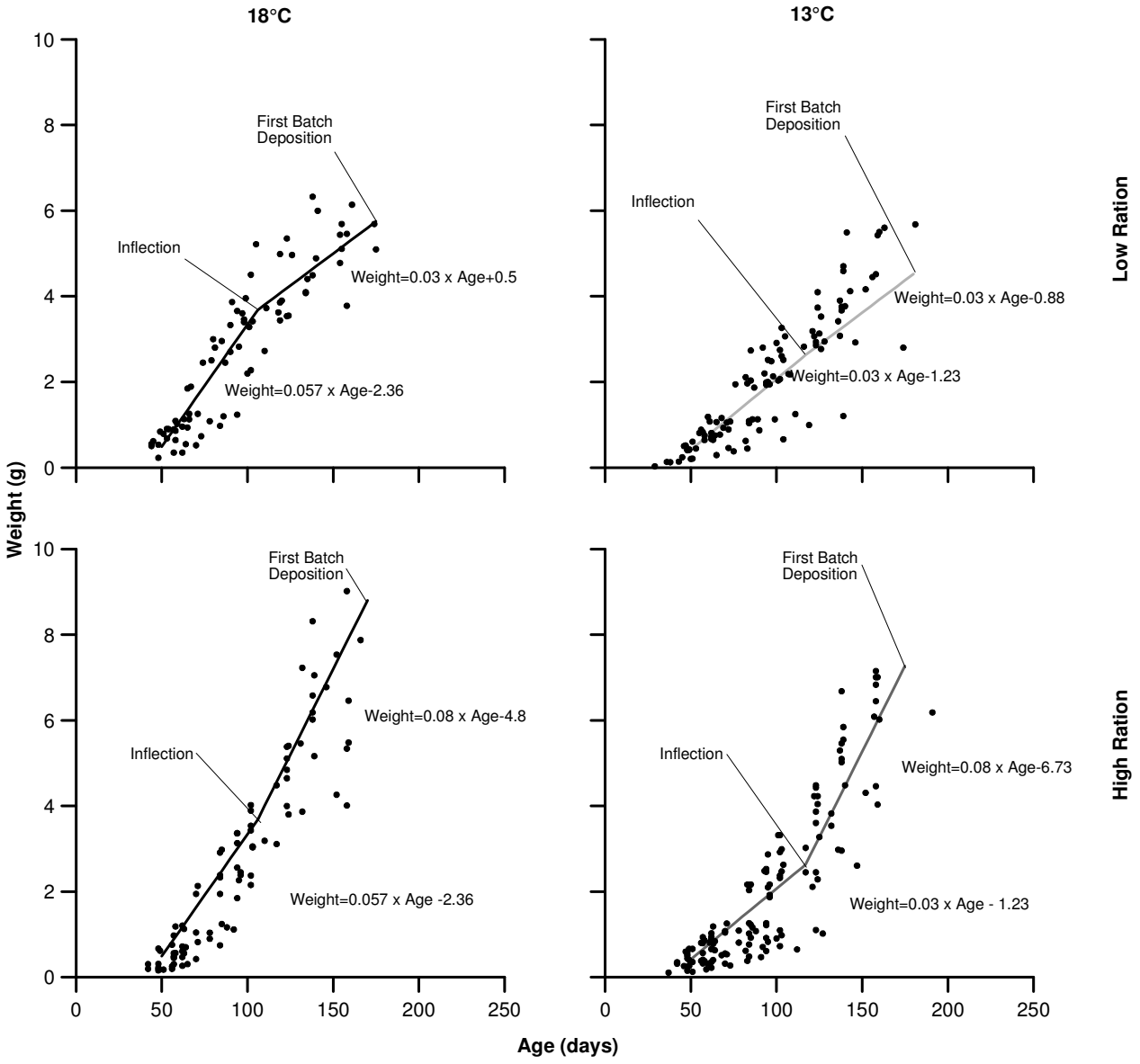

Figure 3.4: Average growth trajectories for each temperature and ration treatment, showing the inflection point, start of batch deposition and the change in growth. Each trajectory is plotted over the combined age and weight measurements taken from each female.

Variation in the growth rate during the late growth phase was significantly affected by ration (F=41.44, *df* 1, 46, P<0.001), with females fed more food growing 62.50% faster than females fed less (Figure 3.4). The effect of ration was independent of temperature ($F_{temperature*ration}$ =4.06 *df* 1, 46, P=0.054), and temperature had no significant effect of growth during the late phase of growth in females (F=0.12, *df* 1, 46, P=0.73).

The weight of females at the inflection point ranged between 0.65g and 5.22g, and this variation was explained by water temperature (Table 3.1). Females held at 18°C were on average 24% heavier at inflection than females held in 13°C (Table 3.1), an effect independent of ration ($F_{temperature*ration}$=0.14, *df* 1, 46, P=0.72). Ration had no significant effect on the weight of inflection (* No significant difference between rations), with the mean weight for females fed high or low ration being 3.16 (se = 0.16). The age of females at inflection ranged between 78 and 140 and was also explained by water temperature (Table 3.1), with females held at 13°C being on average 11 days older at inflection than females held in 18°C (Table 3.1). Similarly to weight, this effect was independent of ration ($F_{temperature*ration}$=1.67, *df* 1, 46, P=0.20). Ration alone also had no significant effect on the age of inflection (* No significant difference between rations) with the mean weight for females fed high or low ration being 113 days (se = 2.12).

Table 3.1: The average growth rate before and after inflection, weight at inflection, age of first batch deposition, longevity, egg volume and embryo development time for female squid held in 13°C and 18°C. Numbers in brackets are standard errors.

	13°C	18°C	Significance
Weight at inflection (g)	2.80 (±0.22)	3.67 (±0.19)	F=7.12, *df* 1, 46, P<0.01
Age at inflection (days)	117.41 (±2.93)	106.74 (±2.5)	F=7.12, *df* 1, 46, P=0.01
Age at first batch (days)	181.90 (±3.83)	165.64 (±16.08)	F=10.81, *df* 1, 30, P=0.003
Weight at first batch (g)	6.60 (±0.22)	5.71 (±0.30)	F=5.11 ,*df* ,1 ,30 P=0.031
Longevity (days)	204.84 (±6.95)	177.10 (±5.57)	F=13.10, *df* 1, 41, P<0.001
Total eggs produced (eggs)	121.10 (±14.1)	121.80 (±29.38)	F=0.05 *df* 1. 36. P=0.82*
Average egg volume (mm^3)	60.60 (±0.50)	32.90 (±0.33)	F=16.90, *df* 1, 30, P<0.001
Developmental time (days)	88.11 (±0.69)	53.13 (±1.50)	F=0.99, *df* 1, 33, P=0.76

* No significant difference between rations

Table 3.2: The average growth rate before and after inflection, weight at inflection, age of first batch deposition, longevity, egg volume and embryo development time for female squid fed low and high rations. Numbers in brackets are standard errors.

	Low ration	High ration	Significance
Weight at inflection (g)	3.39 (±0.16)	2.97 (±0.25)	F=1.70, *df* 1, 46, P=0.20 *
Age at inflection (days)	114.71 (±3.31)	111.56 (±2.78)	F=0.20, *df* 1, 46, P=0.66 *
Age at first batch (days)	181.29 (±5.16)	169.12 (±3.11)	F=6.54, *df* 1, 30, P=0.016
Weight at first batch (g)	5.82 (±0.23)	6.64 (±0.80)	F=4.06, *df* 1, 30 P=0.53 *
Longevity (days)	208.51 (±7.52)	180.85 (±5.76)	F= 11.63, *df* 1, 41, P<0.001
Total eggs produced (eggs)	78.39 (±9.87)	164.39 (±23.46)	F=11.59 *df* 1. 36. P=0.002
Average egg volume (mm^3)	40.90 (±0.47)	57.50 (±0.58)	F=4.28, *df* 1, 30, P= 0.047
Developmental time (days)	76.72 (±4.87)	85.64 (±1.12)	F=2.69, *df* 1, 31, P=0.11

* No significant difference between rations

3.3.2 The influence of temperature and ration on female reproductive output

More that 70% of the 46 females deposited one or more batches during their life; nine out of 12 individuals in LT/LR, 13 out of 15 in LT/HR, eight of 11 in HT/LR, and six out of nine in HT/HR. While one female produced a maximum of 9 batches, this was a rare occurrence and only 23% of all the females produced more than 2 batches. The number of batches each females laid was not a function of temperature (F=2.21, *df* 1, 35, P=0.15), ration (F=2.52, *df* 1, 35, P=0.12), or an interaction between the two ($F_{temperature*ration}$ =0.01, *df* 1, 35, P=0.94). The average age at which females deposited their first batch of eggs was a function of ration (Table 3.2) and temperature (Table 3.1), but not an interaction between the two ($F_{temperature*ration}$ =0.14, *df* 1, 30, P=0.71). Females fed to satiation deposited eggs on average 12 days earlier than females fed less (Table 3.2), while those held in warm temperatures deposited their first batch of eggs on average 16 days earlier than females held in cool temperature (Table 3.1). The weight of the female at laying the first batch of eggs was a function of temperature (Table 3.1), with females held in cool temperature being on average 0.89g heavier when depositing their first batch (Table 3.1). The effect of water temperature was independent of ration ($F_{temperature*ration}$ =0.005, *df*, 1, 30 P=0.94), and ration had no significant effect on female weight at first batch deposition (* No significant difference between rations), with the mean weight of female in both rations being 6.23g (se = 0.19).

The size of each batch ranged from 8-159 eggs and the influence of ration on batch size depended upon the water temperature ($F_{temperature*ration}$=6.10 *df* 1, 30, P=0.019). Squid in the combination of high temperature and high ration produced on average 128 eggs/batch; approximately double the number of eggs in all other treatments (Figure 3.5). The average egg

size within a females first batch was significantly affected by both water temperature (Table 3.1) and ration (Table 3.2) but not an interaction between the two ($F_{temperature*ration}$ =0.105, *df* 1, 30, P= 0.75). The average volume of eggs deposited by females held in cold water was double the volume of eggs from females held in warm water (Table 3.1). Similarly, eggs deposited by females fed to satiation were approximately 25% bigger than eggs deposited by females on a low ration (* No significant difference between rations). Ration significantly influenced the total number of eggs produced by each female (Table 3.2), with females fed on the high ration producing over twice as many eggs compared to females fed a low ration (Table 3.2).

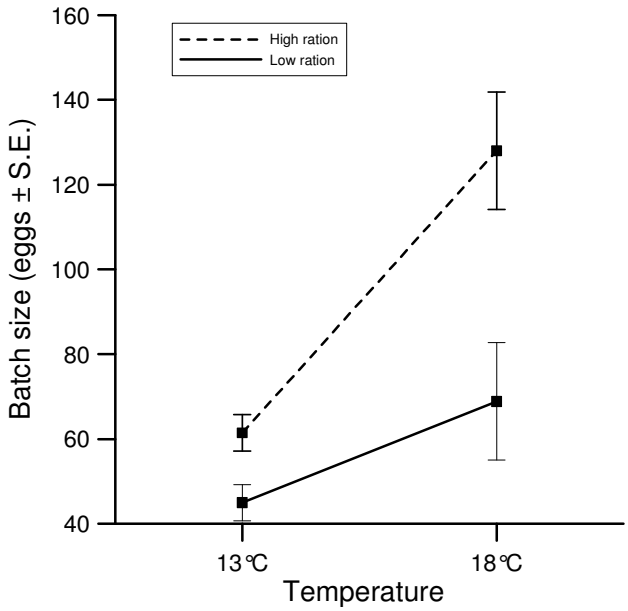

Figure 3.5: The average batch size between treatments.

3.3.3 The influence of temperature of female longevity

The longevity of females ranged between 154-282 days and was significantly affected by both temperature (Table 3.1) and ration (Table 3.2), but no significant interaction was found

between water temperature and ration ($F_{temperature*ration}$ =0.67, *df* 1, 37, P=0.42). Squid lived on average 28 days longer when held in 13°C and when fed less food (Table 3.1 & Table 3.2).

3.3.4 The influence of temperature and maternal ration on embryo development and hatchling success

The hatching success of the second generation was poor during the study, with approximately 13% of the total eggs hatching. Of the 597 successfully hatched eggs, 234 were from females' first batches. Successful development was significantly dependant on temperature (χ^2=182.85, *df*, 3, P<0.001), with warmer temperatures producing fewer hatchlings than expected regardless of the ration (Figure 3.6). When comparing the development time of embryos, both water temperature (F=390.57, *df* 1, 233, P<0.001) and the mothers ration (F=4.936, *df* 1, 233, P= 0.027) significantly affected the development time of the embryos, while the interaction between temperature and ration was not significant (F=0.39, *df* 1, 230, P= 0.54). Embryos from warm water hatched on average 39 days sooner than embryos in cold water (Table 3.1), and embryos of mothers that were fed on low rations hatched nine days earlier than eggs from mother fed a high ration (* No significant difference between rations). The size of hatchlings was not affected by the temperature the embryos were held in (Table 3.1), the ration the mothers were fed on (Table 3.2), or an interaction of both ration and temperature (F=1.58, *df* 1, 33, P= 0.217). The average hatchling size was 0.33g (se = 0.006). There was also no significant relationship between the volume of the egg and hatchling size (F=0.38, *df* 1, 35, P=0.54). The average hatchling size was 0.66g (se = 0.01).

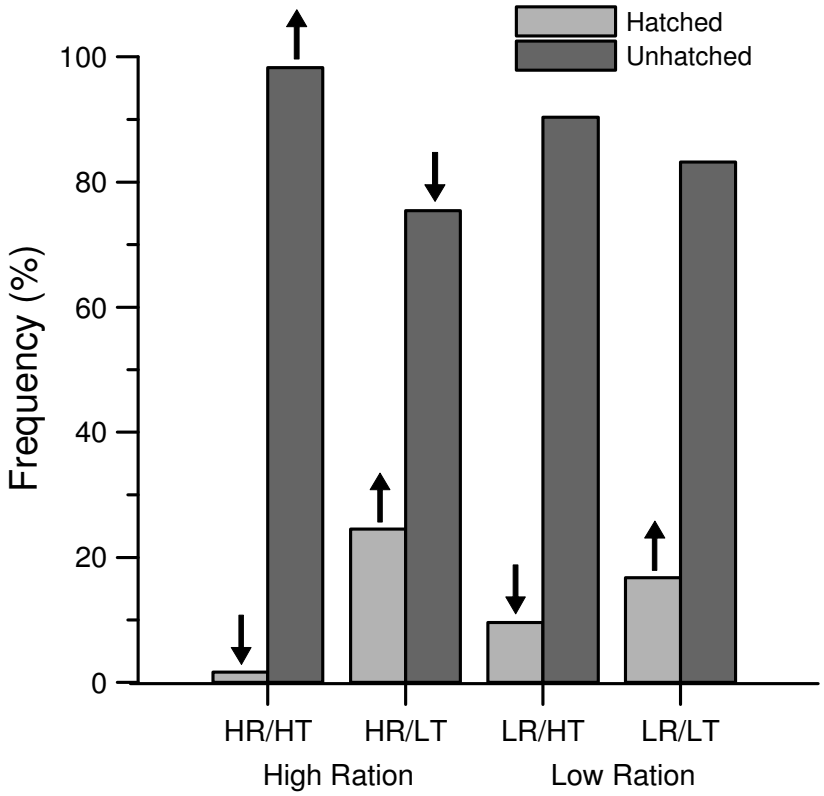

Figure 3.6: The hatching success of first batch of eggs among the four treatments, arrows indicate where more or less juveniles are expected.

3.4 Discussion

In female *Euprymna tasmanica* both growth and reproductive processes were influenced by water temperature and ration, which is comparable to many species of cephalopods, with increases in temperature and ration generally increasing growth rates and decreasing the size and age at maturity (Mangold, 1987; Boyle, 1990; Collins*, et al.*, 1995; Jackson*, et al.*, 1997; Raya*, et al.*, 1999; Jackson and Moltschaniwskyj, 2001a; Hatfield and Cadrin, 2002; Jackson and Moltschaniwskyj, 2002; Hendrickson, 2004). In this study, both water temperature and ration, although having similar effects on growth and reproduction, were generally independent of each other. This was clearly displayed in the growth rate among treatments with temperature influencing growth during the early stage of growth, while ration influenced growth in the late phase.

3.4.1 Two-Phase Growth

Two clear phases of growth were present in *E. tasmanica* and although being highly variable between individuals, growth in both the early and late phase was best described by linear models. Growth in most cephalopods is generally found to be exponential during the early (Forsythe and Van Heukelem, 1987) and late phase (Forsythe, 1993; Brodziak and Macy, 1996; Hatfield, 2000), however a few studies have described logarithmic (Cortez*, et al.*, 1999; Semmens*, et al.*, 2004) and linear (Jackson and Choat, 1992; Dawe and Beck, 1997) growth patterns during the late phase. Variability in the patterns of growth is also common among individuals of the same species (Hatfield*, et al.*, 2001). This large variability in the patterns of growth among and between species is possibly just a feature of the plasticity demonstrated by cephalopods (Boyle and Boletzky 1996).

Regardless of the variability, differences in the early growth rates of female *E. tasmanica* were largely attributed to temperature, which is a common trait in many cephalopod species with temperature having a significant effect on growth during the early stages of life (Mangold, 1987; Martinez*, et al.*, 2000). Individuals that experienced warmer water temperatures grew faster and reached the inflection point at a younger age and smaller size compared to individuals that experienced colder water. During the late phase of growth, between the inflection point and first batch deposition, females fed high rations had higher growth rates. For females in cold water, this resulted in a relatively smooth transition between early and late growth phases, while in warm-water females the change was more visible, especially when fed a low ration (Figure 3.4). In cephalopods a significant decrease in growth post inflection is often attributed to a change in resource allocation away from growth (Forsythe and Van Heukelem, 1987; Mangold, 1987; Rodhouse, 1998). However a review by Pauly (1998) suggests that this change in growth is likely attributed to metabolic constraints that limit the size at which an individual can grow exponentially. As growth after the inflection point, was attributed to ration and not temperature, squid that had access to larger resources of food were able to maintain a higher growth rate compared those fed a lower ration. This suggests that after inflection, if food is limited, somatic growth is compromised, which in turn may influence the reproductive output as larger individuals are generally more fecund (Roff, 1981). While these characteristics may seem favourable, large body size generally comes with slower growth and longer developmental time (Roff, 1981). Therefore the overall advantage will depend on the relative changes in the fecundity verses the probability of survival, as longer developmental time is likely to decrease survival (Roff, 1986).

3.4.2 Lifespan

The lifespan of *Euprymna tasmanica* is considered to be relatively short (around five to eight months (Sinn and Moltschaniwskyj, 2005)); however, during this study individuals lived for up to nine months, with an average longevity of six to seven months depending on temperature and ration. Assessing the abundance in food in the wild is difficult and usually assumed not to limit growth and longevity, therefore, temperature is generally reported to be the main factor influencing longevity, with cohorts of the same species varying with changes in seasonal temperature (Forsythe and Hanlon, 1988; Arkhipkin*, et al.*, 2004; Hendrickson, 2004). This study however showed that increases in both temperature and ration shortened the lifespan of female *E. tasmanica* by approximately one month. In some species, reproduction is known to cease in low fed adults (Shanley and Kirkwood, 2000), suggesting that the increased longevity may be a consequence of a trade-off between adult survival and reproduction (Metcalfe and Monaghan, 2001). While reproduction did not appear to cease in low fed *E. tasmanica* a reduction was evident, with low ration females producing both smaller and fewer eggs per batch.

Whether the increase in lifespan is due to the increased size of cephalopods in cold water or just a factor of slower metabolism is unknown. A review into the growth difference among cephalopods has indicated that the difference in size among cephalopod species can be an indicator of the lifespan, with larger cephalopod species generally living longer than smaller ones (Forsythe, 1984; Wood and O'Dor, 2000). Despite the obvious biological differences among species, variability in lifespan is also a factor of geographical area (e.g. temperature vs. tropical; see Jackson (2004a) for a review), with tropical species generally having a shorter

lifespan than temperature species. This effect has been observed between the widely dispersed populations of *Illex coindetii,* where temperate populations (Gonzalez and Guerra, 1996) live six months longer than populations from the tropics (Arkhipkin, 1996). Although cooler temperatures slow metabolism and extend the lifespan of many animals (Brett, 1979), this phenomenon is very important in short lived species experiencing cooler environments as they cannot spawn over multiple years. However, a small increase in the lifespan of a female due to cooler temperatures may increase her chance of producing progeny more successfully, because unlike females with a shorter lifespan, she will be able to produce multiple batches that are deposited over an extended spawning period.

3.4.3 Reproductive biology

Temperature and ration have significant effects on longevity and size-at-maturation in cephalopods, which in turn influence the reproductive characteristics (Mangold, 1987; Forsythe and Hanlon, 1988; Semmens and Moltschaniwskyj, 2000; Forsythe*, et al.*, 2001; Jackson and Moltschaniwskyj, 2001b). In the current study temperature and ration both had significant effects on all aspects of the reproductive biology, however apart from the batch size the influence on the two factors were independent of one another. In *Euprymna tasmanica,* an elevation of 5°C water temperature during the entire lifespan decreased the age at first egg deposition by 16 days, weight by 0.89g, and halved the average egg size, while more food decreased the age at first egg deposition by 12 days and increased average egg size by approximately 25%. Only in the case of batch size was the effect of water temperature dependent on how much the females were eating, with individuals experiencing a combination of high temperature and ration producing average batch sizes of around 128 eggs, which was

approximately twice the number of the other treatments. These results are common for many multiple spawning squid (Kuipers*, et al.*, 2008; Pecl and Jackson, 2008) and depending on the location and season individuals inhabit, spawning strategies will vary greatly (Boyle*, et al.*, 1995; Arkhipkin*, et al.*, 2000; Pecl, 2001). This ability for an organism to respond to its surroundings can be critical for its success.

3.4.4 Offspring

The relatively small number of embryos that survived to hatching in this study is not thought to be a true representation of the hatching success of *E. tasmanica*, but may be due to laboratory conditions reducing survival (Hanlon*, et al.*, 1997; Vidal, et al., 2002a). Water quality is important for cephalopods, especially during embryo development and early life (Vidal, et al., 2002b). Water conditions in closed re-circulating systems can deteriorate over long periods of time, and although water quality was maintained and new saltwater regularly added, nutrient levels were not measured. Despite the poor hatching success among all four treatments, it was evident that eggs laid in 18°C had the poorest hatching success irrespective of maternal ration. This is in contrast with Steer et al., (2004) who found that hatching success of *E. tasmanica* is a factor of ration and not temperature. In their study they found that females fed less food consistently laid smaller eggs with lower lipid resources which was consistent with high temperature females in our study, possible indicating that hatching success increases with egg size rather than treatment.

Increased egg size has been found to be strongly correlated with female size in many cephalopods, with larger females in a species generally producing larger eggs (Boletzky, 1983;

Laptikhovsky*, et al.*, 2003; Rodrigues*, et al.*, 2011). This trend however is less obvious is species that lay small eggs relative to maternal size (Laptikhovsky*, et al.*, 2003; Laptikhovsky*, et al.*, 2008). Although having quite large eggs (>10% mantle length) there was no relationship between maternal size and offspring size or fecundity of *E. tasmanica,* suggesting that the differences in egg and batch size are likely to be responses of external factors rather than an inherent trait of maternal body size. Similar results have been found in a study comparing five polar and deep sea sepiolids (Laptikhovsky*, et al.*, 2008), and given that temperature and ration explain some of the variability in egg and batch size it is possible that the environmental factors mothers experience strongly influences the reproductive strategy adopted. Additionally, it has been suggested that the reproductive output may also depend on the stability of the environment in which offspring will hatch (Nigmatullin and Laptikhovsky, 1994) (e.g. mothers that lay eggs in environments with high instability may increase fecundity at a cost of decreased egg size).

Variation in hatchling size has also been seen to be a factor of temperature and ration in cephalopods (Laptikhovsky and Nigmatullin, 1993; Pecl, 2004; Ceriola and Jackson, 2010; Collin and Salazar), with warmer temperatures reducing development time resulting in smaller hatchlings. Hatching size is thought to be a good measure of hatchling competency and potential survival (Pecl, 2004) with larger hatchlings being able to avoid predation and starvation due to higher swimming abilities and prey capture (Steer*, et al.*, 2003b). Maternal ration has been found to influence the hatching size of *E. tasmanica*, with females fed a higher ration having significantly larger hatchlings (Steer*, et al.*, 2004). In the present study, why no difference in size among hatchlings was found is unknown, but is likely to be linked to the poor

hatching success previously discussed. Regardless of poor hatching success, embryo development time was reduced by both increased temperature and lower maternal rations. As the rate of yolk absorption is lower in colder temperatures (Vidal, et al., 2002a) a slower embryo development time of eggs held in cold water is expected. However, as the embryo development time is also influenced by maternal ration it appears that the embryo development time does not solely rely on factors imposed by temperature but on the resources available to the mother.

The results from the present study have shown that trade-offs between growth, reproduction, longevity and fecundity continually occur throughout the life of this short lived squid. For ease of interpretation these trade-offs can be classified by two broad categories; those relating to the environment that the juveniles experience and those relating to the phenotype of the spawning mothers (Parker and Begon, 1986). In *E. tasmanica*, juveniles held in optimum temperatures or with an abundance of food appear to make the most of growth early in life, and when approaching maximum size they can afford to allocate a larger percentage of resources to reproduction. While fast growth and high reproductive investment both seem advantageous, organisms often are seen to grow at lower rate than they are physically capable of (Arendt, 1997). In this study, individuals held in cold temperatures and had reduced access to food did not achieve the same growth rates and as a result were forced to start allocating energy to reproduction at smaller sizes. Because they had not yet reached their maximum size they continued to allocate energy to growth alongside reproduction. Allocating less energy to reproduction when food is limited allows individuals to survive to breed again when food resources increases (Roff, 1992). In addition to the increased survival

for the mother, batches laid during periods of limited food availability also reduce the competition between siblings; ensuring greater proportions of hatchling survival (Roff, 1981). Results from this study have shown that *E. tasmanica* has the ability to balance energy requirements for reproduction and survival that optimize fitness in a given environment. This ability to switch between life history strategies is especially important for *E. tasmanica* as populations occupy habitats where prey availability is spatially patchy and temporally unpredictable (Steer*, et al.*, 2004).

Chapter 4 - Growth mechanisms - An individual's strategy to maximize fitness in a given environment

4.1 Introduction

In ecology, there is increasing evidence that environmental factors effecting individuals have important consequences to the structure of populations and communities making it necessary to integrate the processes occurring at an individual level to those operating at the population level (Gimenez, 2010). At an individual level, the processes of growth display considerable variation in response to environmental conditions. This variation in growth is referred to as an individual's life history "plasticity" (Weatherley, 1990; Boyle and Boletzky, 1996; Pecl, 2001; Jackson, 2004b). Plastic responses to environmental conditions, especially in the larval stage, affect size at metamorphosis and juvenile growth and ultimately determine survival and reproductive schedules (Gimenez, 2010). Therefore, being able to describe the environmental influences on the processes of growth at different life stages is a critical feature in determining fitness at both the individual and population levels. Growth can be measured in many ways, from a whole-animal level, which measures changes of parameters such as body length and weight, to analytical measures assessing the relative growth of organs, tissues, and body constituents such as concentrations of protein, lipid and water (Weatherley, 1990). Despite the many approaches of measuring growth, growth is essentially a series of processes that change the relative size or number of different cell types, and any measurement in the change of growth is a reflection of the change in rate or form of cellular growth. The advantage of studying growth analytically, such as the biological organization of muscle fibres, is that it

provides us with an insight into the processes of growth behind the changes seen at the whole-animal level (Moltschaniwskyj, 2004).

In both fish and squid, muscle tissue comprises approximately 90% of an individual's body mass (Moltschaniwskyj, 1994), and because of this, studying the growth of an individual requires knowledge of the mechanisms effecting the increase in muscle tissue (Weatherley, 1987; Moltschaniwskyj, 1994; Pecl and Moltschaniwskyj, 1997; Semmens and Moltschaniwskyj, 2000). Muscle tissue growth occurs by two processes, from the enlargement of existing muscle fibres (hypertrophy) to the production of new fibres (hyperplasia) (Weatherley and Gill, 1985). In fish, hyperplasia ceases as individuals approach their final body size and any further growth is limited by both the number of fibres at that time and the physiological constraints of fibre size (Weatherley and Gill, 1985; Weatherley, 1990). Growth in cephalopods, however, is remarkably different to their teleost counterparts in that growth rates are very rapid and many species show no asymptote in body size (Jackson, 1994; Jackson and O'Dor, 2001). The size frequency distribution of muscle fibres in individuals of different ages and size revealed that non-asymptotic growth occurs due to continuous generation of new muscle fibre throughout their life (Moltschaniwskyj, 1994; Preuss*, et al.*, 1997; Pecl and Moltschaniwskyj, 1999). As a result, body size is not limited by the number of fibres produced (Moltschaniwskyj, 2004). Studies of the histological structure of muscle tissue have provided insight into how growth changes in response to different levels of food and water temperature (Moltschaniwskyj and Martinez, 1998), but few have examined how growth at the muscle tissue level differs among wild cephalopods growing at different rates (Pecl and Moltschaniwskyj, 1999; Semmens and Moltschaniwskyj, 2000; Ho*, et al.*, 2004). Studying the processes of growth at lower levels of

biological organization in fast and slow growing individuals has allowed us to understand the plasticity of growth in squid (Semmens and Moltschaniwskyj, 2000).

Squid mantle tissue consists of two muscle types; circular muscle fibres separated into rectangular blocks by thin regions of radial muscle fibres (Figure 4.1) (Ward and Wainwright, 1972). The radial muscle fibres extend from the outer to the inner surface of the mantle and operate to 'thin' the mantle during inhalation after the power stroke of the circular muscles (Packard and Trueman, 1974), however, whether this is their sole function is not known (Bone*, et al.*, 1981). The majority of the muscle mass is made up of circular muscle fibres which consists of three distinct zones within the mantle (Gosline and Demont, 1985), a centrally located zone of mitochondria-poor (CMP) fibres, which is sandwiched between two layers of superficially located, mitochondria-rich (SMR) fibres (Bone*, et al.*, 1981; Mommsen*, et al.*, 1981; Preuss*, et al.*, 1997). The SMR fibre seems to be an important source of slow hovering locomotory power in hatchlings, but becomes decreasingly important as squid grow (Preuss*, et al.*, 1997). In recently hatched squid, the ratio between CMP and SMR fibres is 1:1, but as individuals grow the proportion of CMP fibres greatly increases and in some species can make up to 94-96% of the mantle tissue in mature individuals (Thompson*, et al.*, 2008). This disproportional increase of CMP fibres is believed to be responsible for the fast and powerful mantle contractions, required for the rapid accelerations during jet propulsion in adult squid (Bone*, et al.*, 1981; Mommsen*, et al.*, 1981; Gosline and Demont, 1985; Bartol, 2001). Given the larger increase in CMP fibres compared to SMR fibres, it is believed the increase of CMP fibres is responsible for the majority of muscle growth in the mantle muscle tissue of cephalopods (Moltschaniwskyj, 1994; Pecl and Moltschaniwskyj, 1997; Thompson*, et al.*, 2008).

Many factors affect the dynamics and rates of growth in cephalopods, e.g. food intake (Chapter 3, Koueta and Boucaud-Camou, 1999), temperature (Chapter 3, Weatherley*, et al.*, 1980; Moltschaniwskyj and Martinez, 1998) and maturation (Jackson and Mladenov, 1994; Moltschaniwskyj, 1995; Brodziak and Macy, 1996; Moltschaniwskyj and Martinez, 1998); however this tends to be species specific. In some squid species a breakdown of mantle muscle occurs at the onset of maturation, e.g. *Moroteuthis ingens* (Jackson*, et al.*, 2004), while in other species maturation occurs at no apparent cost to somatic growth at the whole animal level (Hatfield*, et al.*, 1992; Ho*, et al.*, 2004). To date, many studies have used laboratory based experiments to examine growth in muscle tissue, and although these were useful in explaining the process behind the non-asymptotic growth, the patterns of growth in captive cephalopods do not necessarily reflect those of wild animals (Pecl and Moltschaniwskyj, 1999). The few studies focusing on muscle growth in wild animals have provided a description of the processes in muscle tissue growth in association with whole-animal growth and reproduction (Moltschaniwskyj, 1997; Pecl and Moltschaniwskyj, 1997; Martinez and Moltschaniwskyj, 1999; Semmens and Moltschaniwskyj, 2000; Ho*, et al.*, 2004). However little is known about the influence of external factors, such as temperature, on the growth processes at the level of the muscle tissue growth in wild squid.

In addition to the environmental influences, absolute size is an important factor in the growth of an individual, with larger individuals usually having an 'athletic' edge over their smaller siblings (Meekan and Fortier, 1996), possibly due to an increased ability to capture prey and avoid predation (Steer, et al., 2003b). However, when looking at the effect of temperature on the muscle tissue dynamics it is hard to determine if the characteristics displayed are factors

of the environment or are characteristics of different sized squid. Therefore, when measuring the environmental effects on the fibre dynamics it is important to consider other factors such as maturity, sex and size. This chapter aims to use differences in fibre production and growth among individuals that have experienced different temperature conditions to obtain insights into the processes that are ultimately expressed as rates of growth, body size, and somatic and reproductive condition. Assessing changes in the structural organisation of mantle muscle tissue among temperature conditions demonstrates how temperature experience alters and influences muscle fibre growth. While including factors such as weight provides additional understanding into how the current status of an individual is influenced by past growth.

4.2 Materials and Methods

4.2.1 Collection of wild animals and morphometric measurement

Details of the location and dates of collection of wild animals are provided in Chapter 2.2.1 and the morphometric measurements taken from each individual in Chapter 2.2.2 .

4.2.2 Tissue preparation

All squid <1g were not dissected but after being weighed, were placed directly into 20ml of FAACC (10% formalin, 5% glacial acetic acid and 1.3% calcium chloride) for muscle tissue analysis. Fixed mantle tissue was transferred to 70% ethanol 24 hours before processing. Mantle tissue from each individual was dehydrated in a graded ethanol series (80-100%), cleared in xylene, infiltrated and set in paraffin wax. Tissue blocks were sectioned at 5µm along the longitudinal axis so that circular muscle fibres were cut transversely. Sections were stained with Haematoxylin and Eosin and mounted under DPX.

To assess the differential growth of muscle tissue in different areas of the mantle, the mantle was divided into three 'regions' along the longitudinal axis, anteriorly near the lip of the mantle, exactly half way along the mantle (middle) and posteriorly close to the terminal end of the mantle (Figure 4.1). From each region, an image was captured at 400x magnification using a microscope mounted camera 'Jenoptik Prog Res C14' and Adobe Photoshop CE v8.0. Each image contained 4-19 muscle blocks, the widths of all blocks were measured to the nearest 1 µm using a straight-line perpendicular to the radial fibres. The position of the straight line along each block was random, using a random number table and a transparent 30cm ruler placed on the screen. The fibre density of each block was estimated by counting each circular fibre that

intercepted the line of each muscle block. To calculate the fibre size, 20 mitochondria-poor muscle fibres were randomly selected using a random number table and a transparent 30x30cm grid placed on the screen and measured to the nearest 1 µm. The block width, fibre frequency and fibre size were all measured using ImageTool (©UTHSCSA 1996-2002, v3.0).

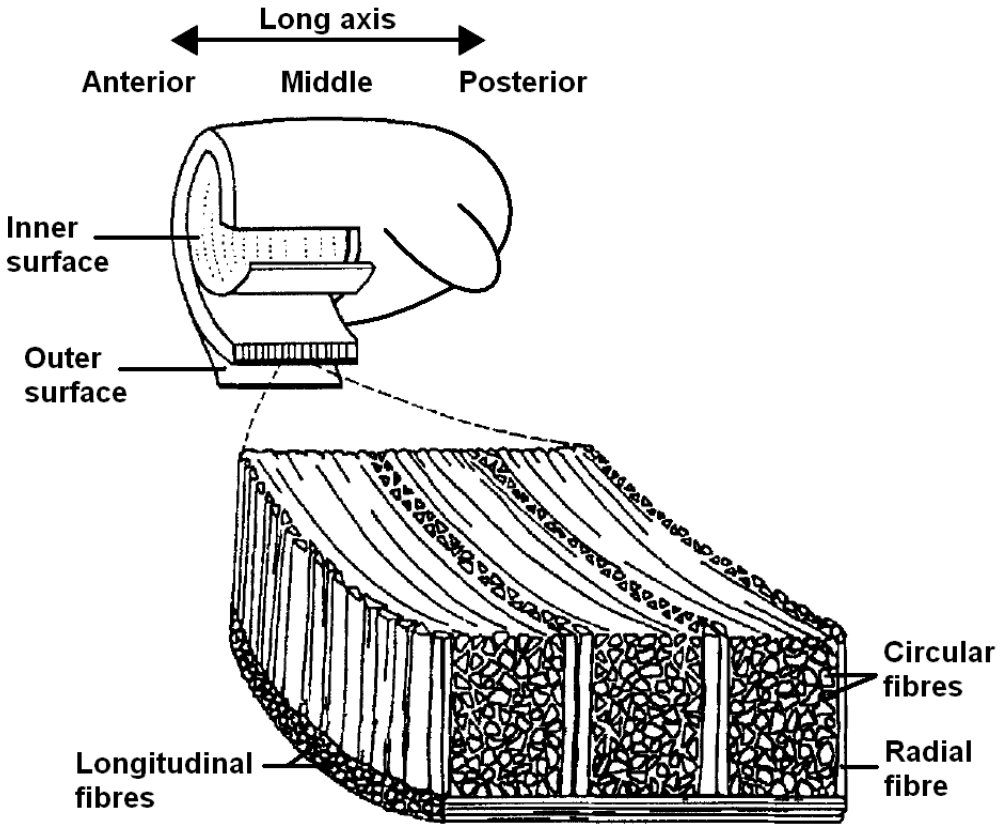

Figure 4.1: Diagram of squid mantle muscle showing the orientation of muscle fibres within the mantle muscle - modified from (Pecl and Moltschaniwskyj, 1997).

To determine when each individual hatched and hence the temperature conditions they have experienced, it is necessary to use the date of capture and age of the individual. However, age determination of *Euprymna tasmanica* is problematic as none of the hard structures are suitable for age determination (Moltschaniwskyj and Cappo, 2009). Therefore, estimates of age were obtained from the concentration of RNA in the muscle tissue, which is strongly correlated

with age in captive animals (Moltschaniwskyj and Carter, 2010). It was assumed that this relationship was the same for wild individuals throughout the year, and estimates of RNA were used to estimate the age of individuals using the following equation:

$$\text{age (d)} = 146.8 - 21.2 \times \text{RNA (µg)} \div \text{muscle tissue (mg)}$$

$$(r2 = 0.90)$$

For squid >1g a section of mantle muscle >0.05g, with the skin removed, was snap frozen using liquid nitrogen and stored in -80°C for RNA analysis. RNA concentration was measured using dual wavelength absorbance (Ashford and Pain, 1986), modified for squid tissue. All squid <1g had insufficient mantle tissue to estimate RNA and were classed as being <50 days old.

4.2.3 Data Analysis

Individuals were organized into categories by the water temperature conditions they experienced by subtracting 50% of the estimated age from the date of capture. This allowed comparisons among groups of individuals that had experienced similar temperature conditions, as a function of the direction of change in water temperature rather than average water temperature. The date when individuals were at 50% of their estimated age was used to assign individuals to one of four temperature regimes. Individuals were classed as having spent most of their life in one of four temperature groups; warm water (> 17°C; January-March), cooling water temperatures (17°C - 13°C; April-June), cold water (< 13°C; July-September), or warming water temperatures (13°C - 17°C; October-November) (refer to Chapter 2, Figure 2.3).

Size frequency distributions of muscle fibres provide a clear picture of the process of growth occurring in the muscle tissue. Muscle block and fibre width data were pooled for

individuals for each combination of temperature category and mantle region and were compared using a three-way log-linear analysis. This allowed a test of the hypothesis that the observed size frequencies distributions were independent of mantle region (anterior, middle, or posterior) and water temperature category (cold, warming, warm, or cooling).

The width of muscle blocks in squid varies with individual size (Moltschaniwskyj, 1994; Pecl and Moltschaniwskyj, 1997; Semmens and Moltschaniwskyj, 2000), sex (Pecl, 2004), and mantle region (Pecl and Moltschaniwskyj, 1997). Therefore, a three-way analysis of covariance was used to determine if an interaction between temperature condition, mantle region, and sex explained variation in average block width, average fibre density, and average fibre diameter of each individual. The covariate, total body weight, was included to remove any differences due to body size. If the covariate was significant, pairwise comparisons of the adjusted means using a sequential Bonferroni correction allowed the determination of significant differences (Field, 2009). If the covariate was not significant, a Tukeys post hoc test allowed the determination of significant differences, in the instances where the post hoc test did not identify a significant difference it was assumed that at least the lowest and highest values were significantly different from each other. All assumptions for ANCOVA were checked before analysis, and the natural log for the covariate and the dependent variable were used when appropriate.

4.3 Results

4.3.1 Block width

The muscle block widths of *E. tasmanica* ranged between 5.73-69.79µm; the size frequency distribution of muscle block widths differed among the different regions of the mantle, but the difference depended upon the temperature conditions experienced ($\chi^2_{\text{mantle region*temperature category}}$ =81.83, *df* 24, P<0.001). To break down this interaction, separate chi-square tests between size distributions of block width and temperature categories were performed for each mantle region separately. The size frequency distribution of block widths differed significantly among the four temperature categories in all regions of the mantle; anterior (χ^2=278.53, *df* 12, P<0.001), middle (χ^2=692.48, *df* 12, P<0.001) and posterior (χ^2=697.71, *df* 12, P<0.001). Regardless of the water temperatures experienced by an individual the anterior region of animals was consistently dominated by small blocks (<20 µm), however in cooling and cold water temperatures fewer than expected <20 µm block widths were present (Figure 4.2). In contrast, more than expected <1.2 µm fibres were present when temperatures were warming or warm. At the middle and posterior regions, small block widths remained the most frequent when temperatures were warming or warm, but became least frequent when temperatures were cooling and cold (Figure 4.2). In the individuals that experienced cooling and cold water temperatures large muscle blocks (35µm) were the most frequent in the middle and posterior regions (Figure 4.2).

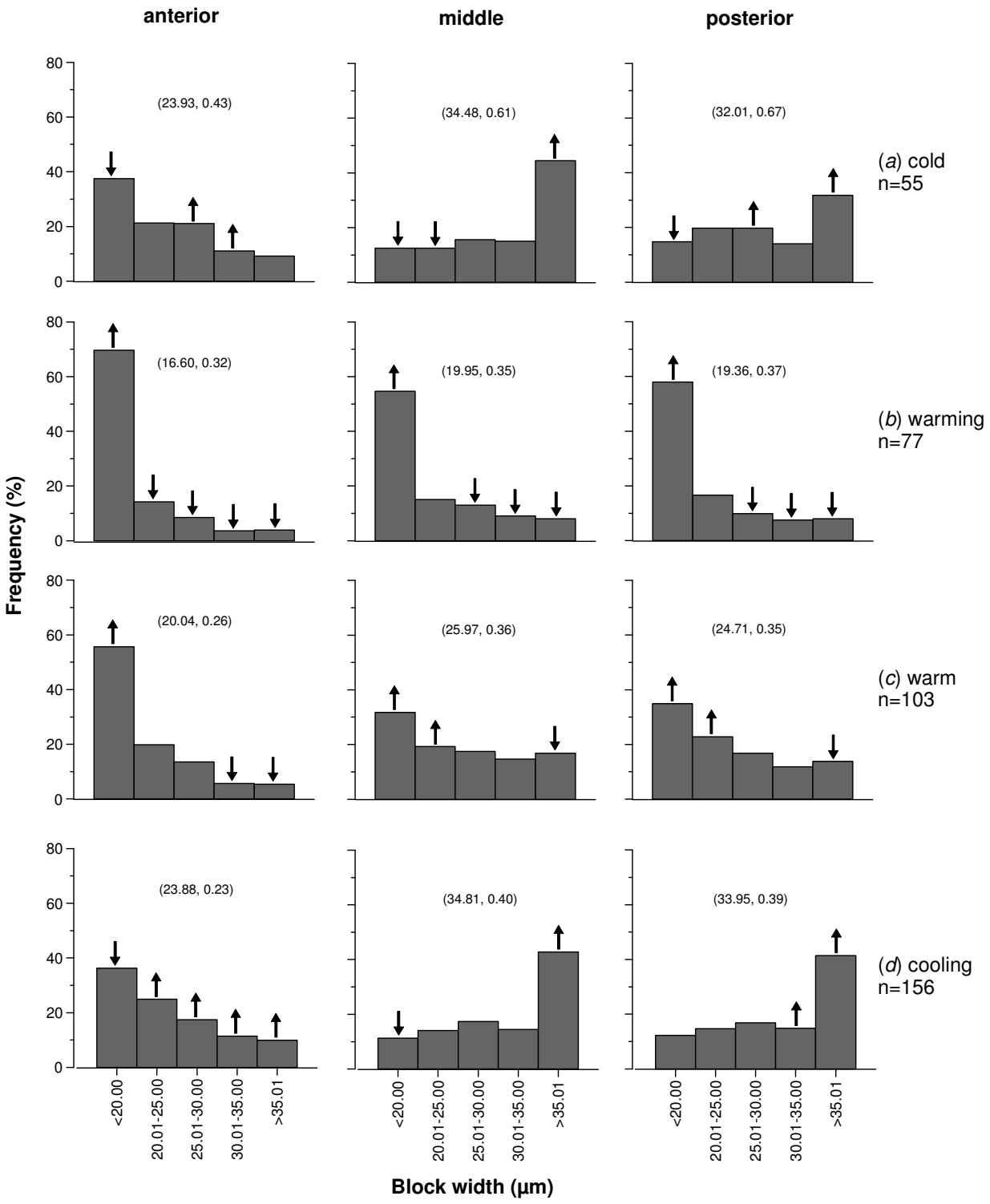

Figure 4.2: Size frequency distribution of block widths in the anterior, middle, and posterior region of the mantle for the four temperature categories. Arrows indicate size classes with more (↑) or less (↓) than expected frequencies. Values in brackets are the mean block width and standard error for each temperature period, respectively.

There was a significant linear relationship between total body weight and the average block width (F=2033.47, *df* 1, 462, P<0.001), with 81% of the variation in block width being explained by total body weight (Figure 4.3). Differences in the average block width (adjusted for total body weight) among the different temperature conditions depended on the gender group ($F_{temperature\ category*sex}$=3.16, *df* 6, 179, P=0.006) but not the mantle region or an interaction between the two. Immature individuals that had experienced warming temperatures had smaller muscle blocks than immature individuals that had experienced cooling temperatures (Figure 4.4). Immature individuals that had experienced warming temperatures had larger muscle blocks than immature individuals that had experienced cold temperatures (Figure 4.4). In the case of mature males and females there appeared to be no effect of temperature history on the average size of muscle blocks (Figure 4.4).

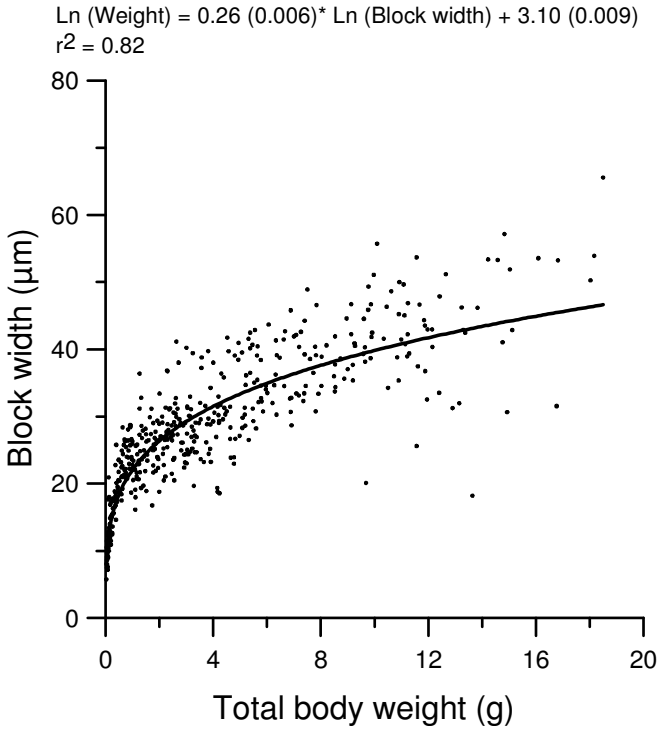

Figure 4.3: Relationship between weight and block width. Numbers in brackets are standard errors.

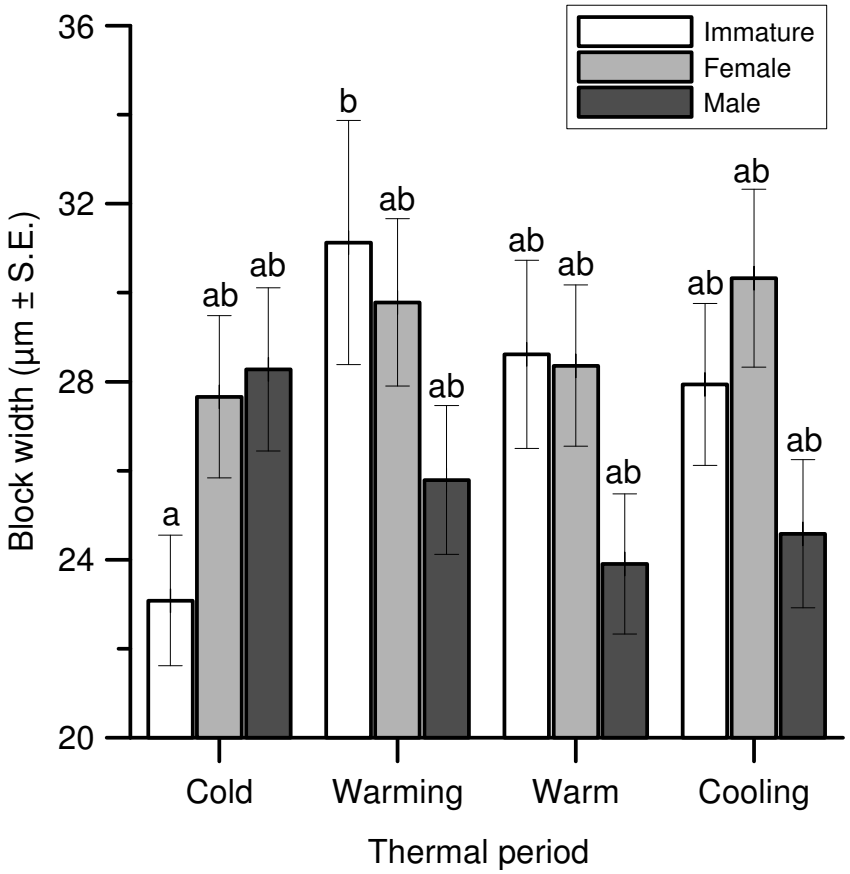

Figure 4.4: Mean block width ± standard error (adjusted for the covariate, weight) from immature, male and female squid for each temperature period. Means with different letters are significantly different from one another.

4.3.2 Fibre diameter

The fibre diameter of *E. tasmanica* ranged between 0.81-3.42 µm, with the size frequency distribution of fibre diameters varying among the temperature conditions experienced by animals and the different regions of the mantle ($\chi^2_{\text{mantle region*temperature category}}$=185.05, *df* 24, P<0.001). To break down this effect, separate chi-square tests on the size distribution of fibre diameter and temperature category were performed separately for each mantle region. The size frequency distribution of fibre diameter differed significantly among the

four temperature categories in all regions of the mantle; anterior (χ^2=185.24, *df* 12, P<0.001) middle (χ^2=54.16, *df* 12, P<0.001) and posterior (χ^2=71.50, *df* 12, P<0.001). The anterior region of all animals regardless of water temperature was consistently dominated by small fibres (<1.2 µm), however individuals that experienced warming and warm temperatures had fewer small fibres than expected (<1.2 µm), while individuals that experienced cooling and cold temperatures had more small fibres than expected (Figure 4.5). This trend was reversed in the posterior region, with individuals that experienced warming temperatures having more than expected small fibres, while individuals that experienced cooling temperatures had less than expected small fibres (Figure 4.5).

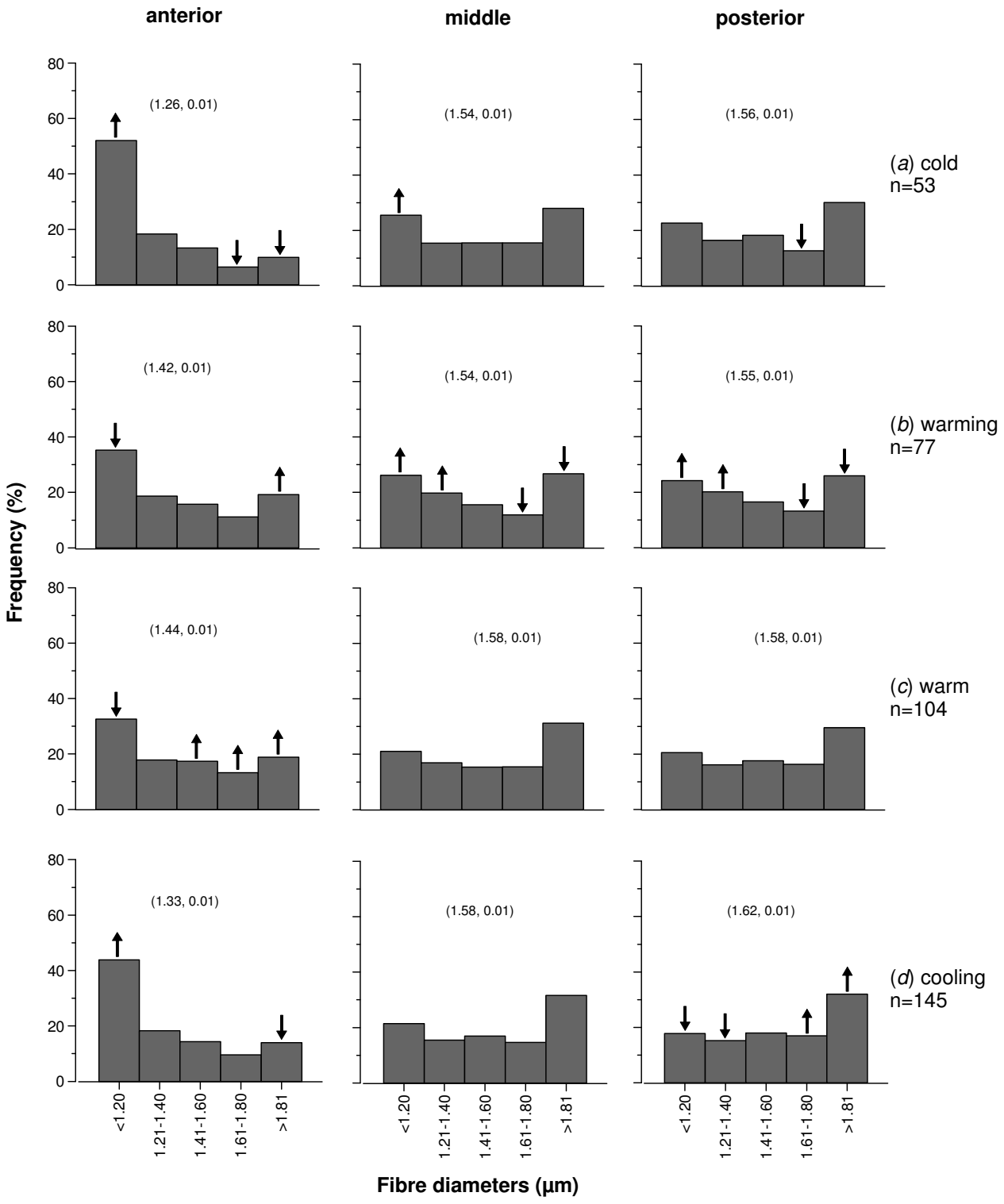

Figure 4.5: Size frequency distribution of fibre diameters in the anterior, middle, and posterior region of the mantle for the four temperature categories. Arrows indicate size classes with more (↑) or less (↓) than expected frequencies. Values in brackets are the mean block width and standard error for each temperature period, respectively.

There was no relationship between the total body weight and the average fibre diameter. Changes in the average fibre diameter depended on the temperature condition ($F=3.41$, *df* 3, 179, $P=0.02$) and mantle region ($F=12.62$, *df* 2 179, $P<0.001$) but not the gender group or an interaction between the factors. The fibre diameter of individuals that experienced warming water temperatures was approximately 0.2µm larger than the muscle fibres of individuals that experienced cold temperatures (Figure 4.6). In the case of fibre region, the average size of fibres in the anterior region was approximately 0.2µm smaller than the fibres in the middle and posterior region (Figure 4.7).

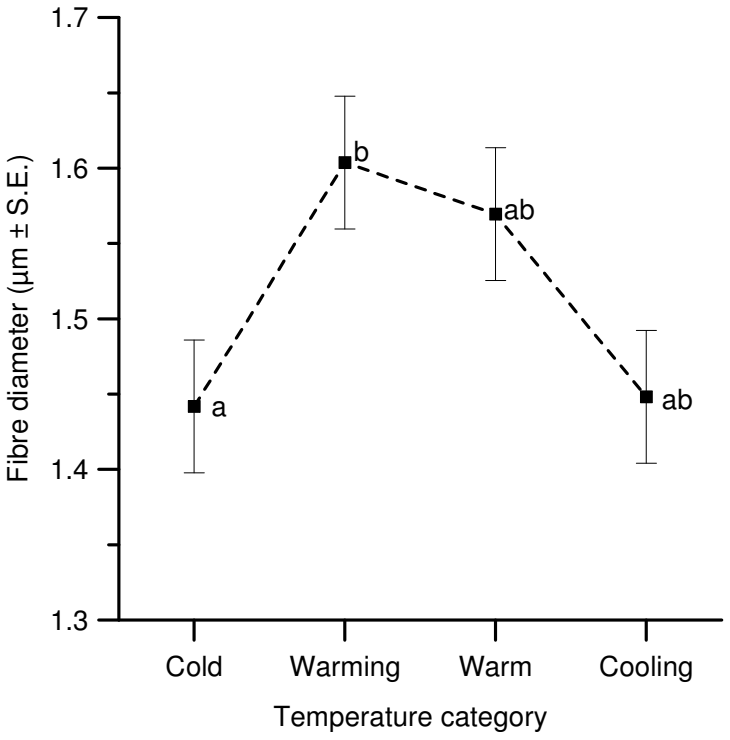

Figure 4.6: Mean fibre diameter ± standard error for each temperature category. Means with different letters are significantly different from one another.

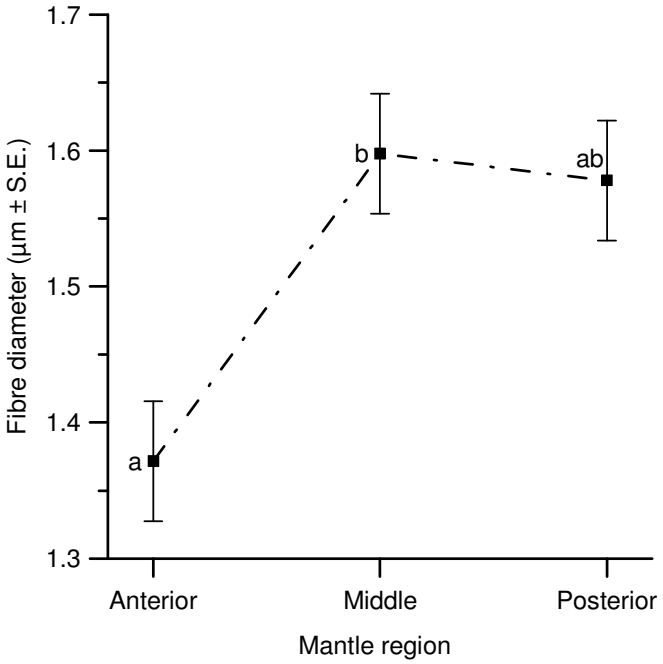

Figure 4.7: Mean fibre diameter ± standard error for each mantle region. Means with different letters are significantly different from one another.

4.3.3 Fibre density

The fibre density of *E. tasmanica* ranged between 2-18 fibres/block. The average fibre density of individuals was significantly related to total body weight (F=2271.00, *df* 1, 463, P<0.001), with 83% of the variance in fibre density being explained by total body weight (Figure 4.8). Differences in the average fibre density (adjusted for total body weight) among the different temperature conditions depended on the mantle region ($F_{\text{temperature category*mantle region}}$=2.27, *df* 6, 179, P=0.04) but not the gender group or an interaction between the two. The fibre density among fibre regions differed depending on the water temperature individuals experienced. For individuals that experienced warm water the density of fibres in the anterior region was less than the density in the middle region, while for individuals that experienced cooling and cold water conditions the average fibre density in the anterior region was less than

the density in the middle and posterior regions (Figure 4.9). The density for individuals experiencing warming water temperature was similar between all mantle regions (Figure 4.9).

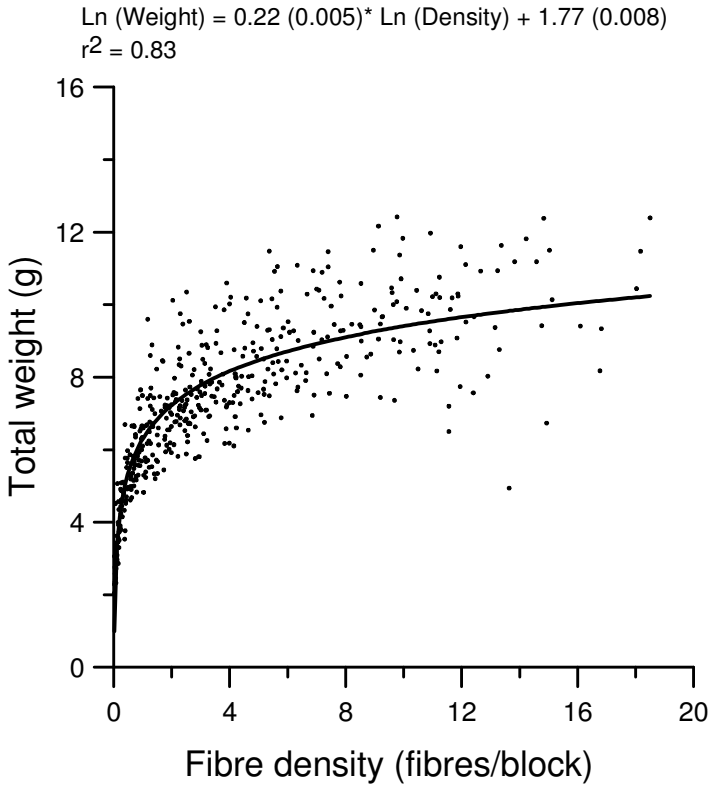

Figure 4.8 Relationship between weight and fibre density. Numbers in brackets are standard errors.

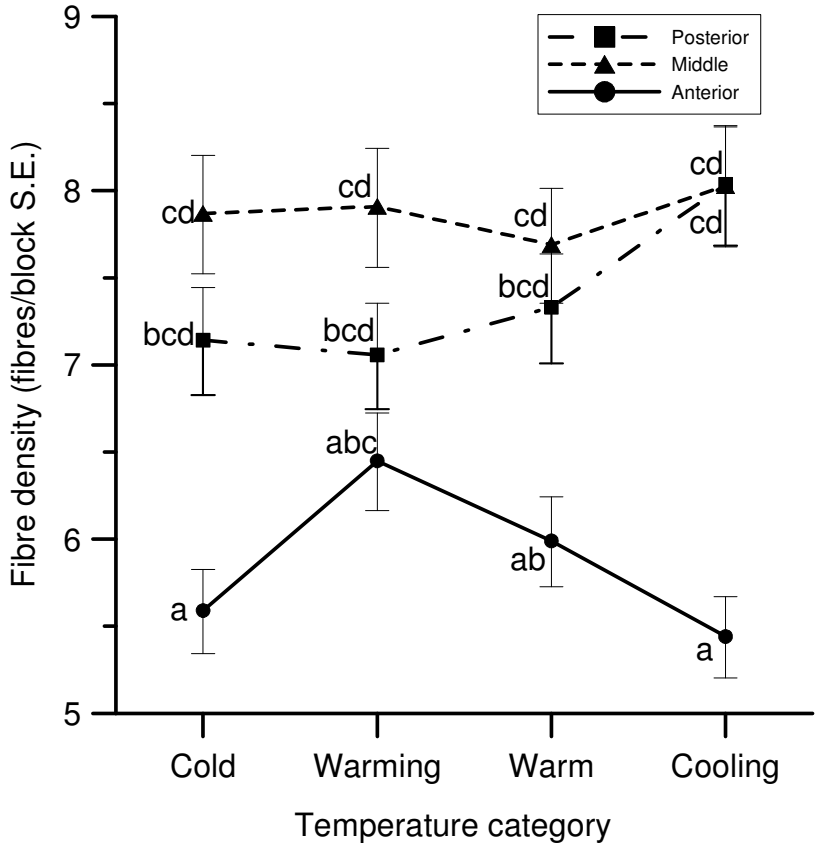

Figure 4.9: Mean fibre density ± standard error (adjusted for the covariate, weight) from the anterior, middle and posterior regions for each temperature category. Means with different letters are significantly different from one another.

4.4 Discussion

Muscle fibres of *Euprymna tasmanica* occurred at all sizes throughout the mantle, suggesting that growth takes place through the generation of new fibres (hyperplasia) and the increase in size of existing fibres (hypertrophy), which is a phenomenon common to cephalopods (Moltschaniwskyj, 1994; Preuss*, et al.*, 1997; Pecl and Moltschaniwskyj, 1999). In *E. tasmanica* both fibre density and block width appeared to be closely related, with both processes increasing with total body weight. This suggests that hyperplasia is the main mechanism responsible for the increase in block width and hence total body growth. These results are similar to that of the pale octopus, *Octopus pallidus* (Semmens*, et al.*, 2004), and is consistent with the hypothesis of Forsythe and van Heukelem (Preuss*, et al.*, 1997) who suggest that due to the small size of muscle fibres in squid, increases in body mass will require higher rates of hyperplasia compared to hypertrophy.

Additionally, there was no relationship between fibre size and total body size, suggesting that the increase in total body weight was not a response to an increase of hypertrophy. These results are similar to the fibre growth found in squid *Sepioteuthis australis* (Semmens and Moltschaniwskyj, 2000) and cuttlefish *Sepia elliptica* (Martinez and Moltschaniwskyj, 1999), in that as body size increases the relative rates of fibre growth remains constant, but are in contrast to many other cephalopod species where hypertrophy is considered as the main driver fuelling their rapid growth (Forsythe and Van Heukelem, 1987; Moltschaniwskyj, 1994; Pecl and Moltschaniwskyj, 1997; Ho*, et al.*, 2004). However these initial observations are made with no consideration of an individual's life history, and were not a true reflection of the processes of growth in nature.

Like many cephalopods, the growth rate and body weight of *E. tasmanica* is strongly influenced by water temperature, with individuals experiencing cold water growing slower, living longer and achieving greater body sizes (Chapter 3). While many studies have documented the effect of temperature on cephalopod growth (Mangold, 1987; Hatfield, 2000; Martinez, *et al.*, 2000; Villanueva, 2000; Forsythe, *et al.*, 2001), this effect has always been linked to the juvenile growth phase. When comparing the block width among gender groups block width was generally larger in warmer water, however these effects were only found in immature individuals. Similarly fibre diameters were larger in individuals from warm water however these effects were regardless of the gender group (Table 4.1). As block width is strongly linked to fibre density, it is likely this influence of temperature on hyperplasia is responsible for the variability in juvenile growth that is common to cephalopods (Mangold, 1987; Martinez, *et al.*, 2000; Pecl, 2004; Pecl and Jackson, 2008). Interestingly, during the adult growth phase, the process in which growth continued was strongly determined by the temperature individuals experienced.

In *E. tasmanica* the average muscle block width of individuals that grew in warming water was generally smaller. However as individuals caught in warmer water are generally lighter (Chapter 2), it is possible that block width is a function of body weight rather than temperature. In this study, both block width and fibre size increased logarithmically with increasing body weight. However, after controlling for total body weight, no differences in the mean block width were found among temperature categories. This supports the hypothesis that the high fibre density and large muscle blocks found in cold water individuals is likely a factor of their increased size, due to their longer lifespan, rather than a direct response to water

temperature. In contrast, little difference was found in the block width among mantle regions of squid from warm water, suggesting that fibre recruitment may not always be a continual process but may cease when a certain body size is reached, which is common in many fish species (Weatherley and Gill, 1985; Weatherley, 1990). In the anterior region of the mantle the mechanisms of growth are different from the rest of the mantle, in that, factors influencing the formation of new blocks may not necessarily influence the growth of existing blocks.

In cold water individuals it appears that continual fibre recruitment is an important process of growth, as the fibre density of individuals that experienced cold water individuals was higher in the middle and posterior regions compared to the anterior region. While it may be possible that individuals that experienced warm water did not live long enough for significant difference between the mantle regions to be observed, the reduction of fibre recruitment together with the increased fibre size highlights a change in the relative contribution of the two muscle growth processes. In *Sepioteuthis australis*, small muscle fibres were also less prevalent in faster growing individuals (Ho*, et al.*, 2004), suggesting that the growth of fibres may be more important than hyperplasia in generating faster lifetime growth rates.

Table 4.1: Changes in the average fibre density (fibres/block), fibre width (um) and block width (um) in response to water temperature and total body weight in the anterior and middle/posterior region of the mantle.

Water temperature	Warming/Warm	Cooling/Cold
Average density	Little difference between mantle regions	Smaller fibre in the anterior region
Average fibre size	Large	Small
Average block width (immature individuals)	Wide	Narrow

Furthermore, each growth process is likely to have significantly different energetic demands. For example, by keeping fibre size low the efficiency of cellular processes is maximised in cold water squid and continued hyperplasia may allow greater energetic advantages (Semmens*, et al.*, 2011), which is likely to have cascading effects on future growth and reproductive output. By investing less into fibre growth these individuals were able to attain extended life spans and although growth rate was reduced, individuals were able to continually produce batches of eggs at little or no additional cost to growth. Consequently, while the processes of growth in cephalopods is generally not interrupted by the allocation of energy to reproduction (Ho*, et al.*, 2004), the increased energetic demand of a hypertrophic dominated growth did reduce the lifespan of individuals experiencing warm water (Chapter 3) and although the relative growth rate was increased, the time adults had to spawn was significantly reduced. While *E. tasmanica* individuals hatch all year round, the combination of each growth process results in a peak of reproductive investment during spring and summer. This peak is achieved by both the slow hyperplastic dominated growth of individuals that hatch during winter and the rapid hypertrophic dominated growth of individuals that hatch during summer. While each strategy has its own advantages and disadvantages, the final outcome of each life-history strategy attempts to maximises the survival of their offspring by synchronising the time of reproductive investment with the majority of individuals in the population and producing offspring during periods of warming water temperature.

Chapter 5 – Growth and reproduction – A biochemical approach in assessing the trade-off between growth and reproduction

5.1 Introduction

In many adult cephalopod species substantial somatic growth occurs at a time that energy is also being allocated to reproductive growth, with reproductive growth appearing to occur with little or no cost to somatic growth (Mangold*, et al.*, 1993). This results in non-asymptotic growth, where somatic growth continues at a constant rate throughout most, if not all, of an individual's life (Jackson, 1994; Jackson and O'Dor, 2001). While there may be no direct trade-off between somatic and reproductive growth, it is likely that the variability in reproductive growth is largely dependent on somatic growth (Mangold*, et al.*, 1993). A common feature in life history models of animals is the trade-off between juvenile developmental time and size at reproductive maturity (Roff, 1992; Stearns, 1992). In such models, while achieving a large body size may come with advantages, such as greater fecundity, it also comes with the disadvantages of longer juvenile phase when predation risks associated with smaller body size may be greater (Nylin and Gotthard, 1998). For example, cephalopods that experience cooler water temperatures, although having slower growth, generally attain larger adult sizes because of a longer life span (Wood and O'Dor, 2000; Jackson and Moltschaniwskyj, 2001b). Since fecundity typically increases with body size (Boyle*, et al.*, 1995), slower growing cephalopods that achieve larger adult body sizes may benefit from increased reproductive output. In contrast, fast growth decreases the size and age at maturity in many

cephalopods (Forsythe and Hanlon, 1988; Forsythe, 1993), which reduces the chance of being preyed upon before reaching maturity. This trade-off between reproduction and longevity is the central feature of the life history characteristics of an individual (Stearns, 1992).

Although variation in individual growth rates, to a certain degree, is limited by the physiological constraints of the species, there is compelling evidence to suggest that many organisms possess certain plasticity in their life history characteristics, which allow them to change their growth rate in situations when they need to do so (Nylin and Gotthard, 1998). This is particularly evident in the life history characteristics of cephalopods, which is characterised by their extreme flexibility and rapid response to small changes in environmental conditions (Forsythe and Van Heukelem, 1987; Forsythe, 1993; O'Dor, 1998). For example, changes in water temperature as little as 1^{o}C can significantly impact the growth in cephalopods (Forsythe and Hanlon, 1988). Due to the rapid response in growth to even the smallest changes in environmental conditions, cephalopods are often described as opportunistic organisms (Jackson and O'Dor, 2001; Pecl and Moltschaniwskyj, 2006; Pecl and Jackson, 2008), allowing populations to expand rapidly when environmental conditions are favourable, but lead to rapid declines when conditions are not (Rocha*, et al.*, 2001; Boyle and Rodhouse, 2005).

The ability of cephalopods to rapidly respond to the environment is believed to be based on their efficient feeding and protein based metabolism, which converts food directly into growth rather than storage (Jackson and O'Dor, 2001). In teleost species, the storage of energy to a degree acts as buffer against environmental variability, with individuals of some teleosts species being able to use stored energy for reproduction when food is limited (Kerrigan, 1996; Van Dijk*, et al.*, 2005). However, given somatic and reproductive growth in cephalopods is

sourced directly from food, individuals will have a higher reliance on their surrounding environment at any point in time (Hatfield*, et al.*, 1992; Moltschaniwskyj, 1995). To understand the processes that allow cephalopods to survive and reproduce regardless of the environment requires studies into the life history strategy in terms of resource allocation between reproduction and growth (Heino and Kaitala, 1999).

Measuring changes in size through time, i.e. growth, in the laboratory is relatively straight forward, and although observing growth under specific experimental conditions can be valuable in understanding the processes influencing life history characteristics, growth in the laboratory is generally a poor representation of growth in nature (Pecl and Moltschaniwskyj, 1999). In contrast, because it is difficult to track the same individual through time, measuring the growth of an individual in the field is not straight forward and requires an estimation of age or knowledge of an elapsed time period (Campana, 2001). While many methods have been used to estimate the age of an organism (e.g. tag and recapture, radio chemical dating and length frequency analyses), in most cases age is estimated by counting periodic growth increments of calcified structures (Campana, 2001).

In some cephalopod species, individual growth estimates are calculated from daily increments of calcified structures such as the statolith (Jackson and Choat, 1992), stylets (Doubleday*, et al.*, 2006; Leporati*, et al.*, 2008), and cuttlebone (Bettencourt and Guerra, 2001). However application of these methods is species specific and is not possible for all cephalopod species. For example, in sepiolid squid the crystalline structure of statoliths lacks visible increment structure and there is no other hard structure that can be used to reliably estimate age (Moltschaniwskyj and Cappo, 2009). It is therefore becoming increasingly necessary to find

alternative methods to studying growth that are accurate and universal to all cephalopods species. One alternative approach that may be applicable to cephalopods is the use of biochemical techniques to estimate instantaneous growth rates. The use of many biochemical techniques to measure correlations or indices of instantaneous growth are well established in fish and are usually expressed as ratios of nucleic acids e.g. RNA:DNA, RNA:Protein or RNA:fresh weight (Dutta, 1994; Ferron and Leggett, 1994; Chícharo and Chícharo, 2008).

The rationale of using biochemical techniques for growth assumes that changes in body mass are a function of protein synthesis and degradation. However, rates of protein synthesis are difficult to quantify directly. Since protein synthesis is a function of ribosome activity, and ribosomes contain the majority of RNA in the tissue, estimates of protein synthesis can be expressed as a ratio of protein concentration to RNA (Houlihan*, et al.*, 1993). The use of biochemical indices as measures of instantaneous growth of cephalopods has been supported by a number of laboratory studies. For example, growth rates were correlated to RNA:DNA in *Sepia officinalis* (Clarke*, et al.*, 1989; Melzner*, et al.*, 2005) and *Loligo opalescens* (Vidal*, et al.*, 2006); and RNA:protein in *Octopus vulgaris* and *Eledone cirrhosa* (Houlihan*, et al.*, 1990; Houlihan*, et al.*, 1998).

This study aims to identify the sources of variability in the instantaneous growth of a short-lived squid species, *Euprymna tasmanica*. Variation in growth rates is attributed to a range of biotic and abiotic processes experienced throughout the life history of an individual, which in turn may determine the reproductive strategy adopted (McGrath and Jackson, 2002; Jackson*, et al.*, 2004). Through comparisons of reproductive tissue and total body weight, this

study also provides insight into some of the relationships between reproductive and somatic growth, and in particular if a trade off between each process occurs.

5.2 Materials and Methods

5.2.1 Collection methods

Details of the location and dates of collection of wild animals are provided in Chapter 2.2.1 and the morphometric measurements taken from each individual in Chapter 2.2.2 .

5.2.2 Reproductive assessment

During dissection, gonad tissue was weighed, fixed, and stored in 20ml of FAACC (10% formalin, 5% glacial acetic acid and 1.3% calcium chloride). Tissue was transferred to 70% ethanol for 24 h before being taken through a series of ethanol (80-100%), xylene, and paraffin wax. Tissue blocks were sectioned at 5µm using a Heidelberg Microtome HM 340, stained with Haematoxylin and Eosin using a "Linistainer GLX Mod. 243603" and mounted on glass slides using DPX resin. Squid >1g were identified as either immature or mature by viewing slides of ovary and testis tissue under 400x magnification.

5.2.3 Biochemical analysis

A section of mantle muscle greater than 0.05g with no skin, was wrapped in foil and snap frozen using liquid nitrogen and stored in -80°C for RNA analysis. A section of the frozen mantle tissue (0.05 g – 0.10 g) was weighed then homogenized in 3ml of 0.2 M PCA in plastic polypropylene test tubes using an IKA Homogenizer. Tissue was homogenized in short periods of less than 10 sec to ensure that the tissue did not overheat. Between periods of homogenization, tissue stuck in the homogenizer blades were removed using fine tipped forceps and placed back in the PCA. The homogenized tissue was centrifuged at 5,500 rpm at 4 °C for 10 min, the supernatant discarded, and the pellet washed with 2 mL of 0.2 M PCA, vortex

mixed and centrifuged twice before the RNA concentration was determined. The pellet was re-suspended in 5 mL of 0.3 M NaOH, vortex mixed and incubated in a 37°C bath for 1 h. Every 10 min the tissue was vortex mixed during incubation. After one hour the tubes were cooled for 5 min. Samples of 3.9 ml were transferred to new polypropylene test-tubes, the remaining sample was kept for protein analysis. To each tube 0.867 ml of 20%PCA was added and centrifuged at 5500 rpm at 4°C for 10 minutes. The supernatant was immediately used for RNA analysis. A spectrophotometer was calibrated to zero using a RNA blank (39mL of 0.3M NaOH and 8.67 ml of 20% PCA). Each sample was then read at an absorbance of 260 nm and 232 nm. If any absorbance readings were more than 1.0 nm the sample was diluted by half using distilled water and reread, later new absorbance readings were multiplied by two. The following calculations were used to find how much RNA was in each original sample:

$$\text{RNA (ug/ml)} = (32.0 \times \text{Absorbance 260um}) - (6.11 \times \text{Aborabance 232nm})$$

$$\text{RNA in initial sample} = \text{RNA (ug/ml)} \times 4.767 \text{ x } \frac{5}{3.9} = \text{RNA (ug/ml} \times 6.112)$$

$$\text{Divide by sample weight to get RNA}(\mu g)/\text{sample (mg)}$$

To estimate the amount of protein in each sample six volumes of BSA (10mg of bovine serum albumin dissolved in 0.05 M NaOH) were used to construct a standard curve. From the saved supernatant 50 µl was added to 0.95 ml of distilled water in a glass test tube. One ml of alkaline copper reagent, which is made up of 70ml of (2g NaOH, 10g Na2CO3, 0.1g C4H4O6K2), 10ml of (0.05CuSO4 in 10ml of distilled water) and 20ml of distilled water was added to each sample and left to stand for 10 minutes. Four ml of Folin Phenol reagent (1:28 Folin and Coicalteau's phenol reagent in distilled water) was forcibly and rapidly added to each sample.

Test tubes were then placed in a 55°C heat block for 5 minutes. After cooling to room temperature each sample was read at 740um using a spectrophotometer. For each assay, samples were run in duplicate and repeated if more than 10% between the duplicates was found. The following calculations were used to find how much protein was in each original sample:

$$\text{Calculate protein concentration (ug/sample) from standard curve}$$

$$\text{Mantle protein (mg/g)} = \text{protein concentration (ug/sample)} \times \frac{100}{\text{tissue weight (mg)}}$$

Absolute size of the gonad and reproductive indices (e.g. gonosomatic indices) may not be good indicators of morphological characters as they are often heavily influenced by body size (Jakob*, et al.*, 1996) Regressions with body mass as an independent variable are used to remove the influence of total body mass, allowing the comparisons of gonad mass independently of total body size (Hayes and Shonkwiler, 1996). Residuals from a regression of the morphological character, gonad weight against total body weight, were used as a mass independent measure of the reproductive tissue respectively. Residuals are the difference between the sample and the estimated function value; therefore individuals with large positive residuals have heavy organs for their size, while individuals with large negative residuals have lighter organs for their size. The use of size independent measures of somatic and reproductive conditions have been successfully used in cephalopods (Moltschaniwskyj and Semmens, 2000; Pecl*, et al.*, 2004).

5.2.4 Data analysis

As age of wild *Euprymna tasmanica* is estimated from RNA concentration (Chapter 2), and small squid did not have adequate tissue to estimate RNA, total weight was used instead of

age as a factor to determine the size when individuals start to allocate energy to reproduction and the size-at-maturity. For each combination of temperature category and sex, a scatter plot of the reproductive tissue and total body weight was used to analyse the relationship between somatic and reproductive growth. A visual inspection of the reproductive-somatic plots for each individual revealed that there were two phases of reproductive growth, both linear, but with a definite inflection point. A segmented piecewise regression was used to determine the weight at the inflection for each individual, and to estimate the size at which individuals started allocating energy to reproduction. To determine if water temperature had an effect on the rate of reproductive investment both before and after inflection, an ANCOVA, with weight as the covariate, was used to compare the reproductive tissue between each temperature category. All assumptions for ANCOVA were checked before analysis, and the natural log for the covariate and the dependent variable were used when appropriate.

For each sex and temperature category, a logistic regression, employing a 0.05 criterion of statistical significance, was used to determine if weight affected the probability of being mature. All immature individuals where the sex was not known were used in each of the male and female analyses. A logistic regression estimates the probability of being mature by fitting data to a logistic curve (logit) (Field, 2009) and requires some overlap in weight of immature and mature individuals. In the temperature categories where there was a complete separation in the size of immature and mature individuals, the weight of the lightest mature individual was defined as the weight at maturity. For all other temperature categories the slope and the odds ratio from the logistic regression equation were used to calculate the weight at 50% probability:

$$\text{odds ratio} = \frac{\text{probability}}{1 - \text{probability}} = \frac{0.5}{1 - 0.5} = 1$$

$$\ln(\text{odds ratio}) = \text{intercept} + \text{slope} \times \text{weight}$$

$$\text{weight at } 50\% \text{ probability} = \frac{(\ln(1) - \text{ intercept})}{\text{slope}}$$

RNA concentration, protein concentration, and RNA:protein ratio's are all assessments of recent growth (Houlihan*, et al.*, 1998), therefore individuals were organized into seasons based on the date of capture. This allowed comparisons of recent growth among groups of individuals that experienced similar temperature conditions. Individuals were classed as being caught in one of four seasons depending on the month of capture; summer (January-March), autumn (April-June), winter (July-September), or spring (October-November). A two-way analysis of covariance was used to determine if inter-annual differences in the RNA concentration, protein concentration, and RNA:protein ratio existed among the seasons individuals were caught. Where significant, total body weight was included as the covariate to remove any differences in size between the seasons. The covariate, total body weight, was included to account for any differences as a function of body size. If the covariate was significant, pairwise comparisons of the adjusted means using a sequential Bonferroni correction allowed the determination of significant differences. All assumptions for ANCOVA were checked before analysis, and the natural log for the covariate and the dependent variable were used when appropriate.

To determine the effects of recent growth rate a multiple regression analysis using stepwise elimination method was used to determine if RNA concentration, protein

concentration, or the RNA:protein ratio was related to sex, total body weight, recent (2 week) water temperature average, somatic and reproductive condition, and reproductive investment (reproductive growth relative to somatic growth). All assumptions for a linear regression were checked before analysis, including the test for multicollinearity between the predictor variables. In a multiple regression the standardised β-values (β), which are measured in standard deviation units rather than the units of each variable, were used to measure the level of importance that each factor had in controlling the variable (Field, 2009). This is useful in providing insight when comparing the importance of predictors in the model.

5.3 Results

5.3.1 Allocation of energy to reproductive tissue

For each sex and temperature category, the relationship between reproductive tissue and total body weight occurred in two phases, with reproductive growth increasing slowly in small individuals and more rapidly in bigger individuals (Figure 5.1 & Figure 5.2). The weight at which individuals started allocating energy to reproduction differed among the temperature categories for each sex. Males that experienced warming water temperatures began reproductive growth at the smallest size of 1.63g, while males that experienced warm, cooling and cold water started at 2.39g, 2.44g and 2.75g respectively. Females started allocating energy to reproduction at much greater sizes compared to males, with females that experienced warm water allocating energy to reproduction at the smallest size of 2.95g. Females that experienced cooling cold and warming water started allocating energy to reproduction at 4.94g, 4.81g and 3.24g, respectively.

Males in the different temperature categories did not differ significantly in their relative rates of reproductive growth either before or after inflection in reproductive growth (Figure 5.1). For females, the pattern of growth was similar among temperature categories before inflection, however after inflection significant differences in the rates of reproductive growth were found among the temperature categories ($F=7.41$, *df*, 3, 61, $P<0.001$). The rate at which reproductive tissue increased with total body weight was slowest for females that experienced warm water and fastest in female that experienced warming water temperature, with an increase of 0.09g and 0.30g for every 1 g of total body weight, respectively (Figure 5.2).

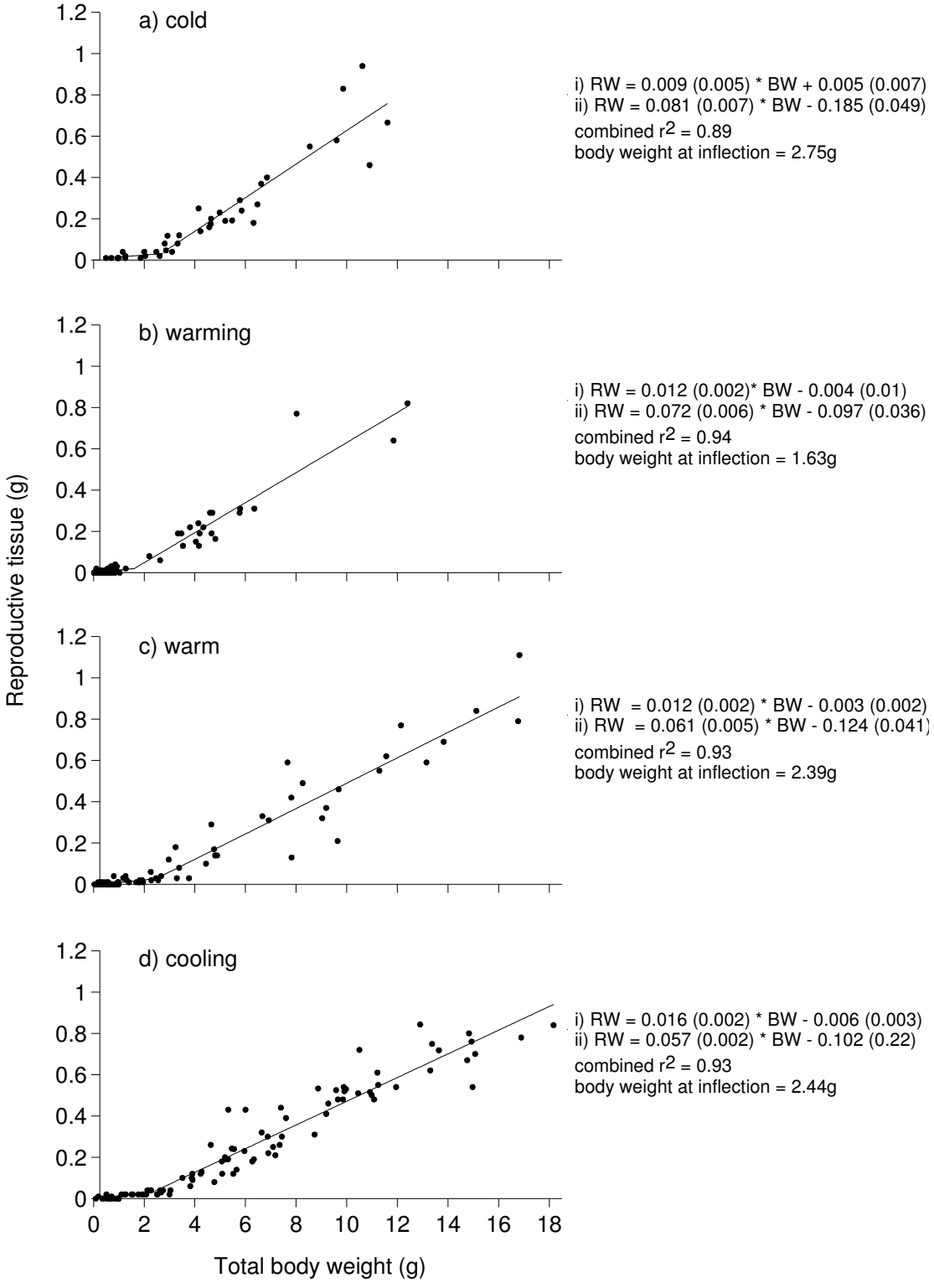

Figure 5.1: The relationship between reproductive tissue weight (g) (RW) and total body weight (g) (BW) for male squid, both before (i) and after (ii) the inflection point when individuals started allocating energy to reproduction in the four temperature categories. Numbers in brackets are standard errors.

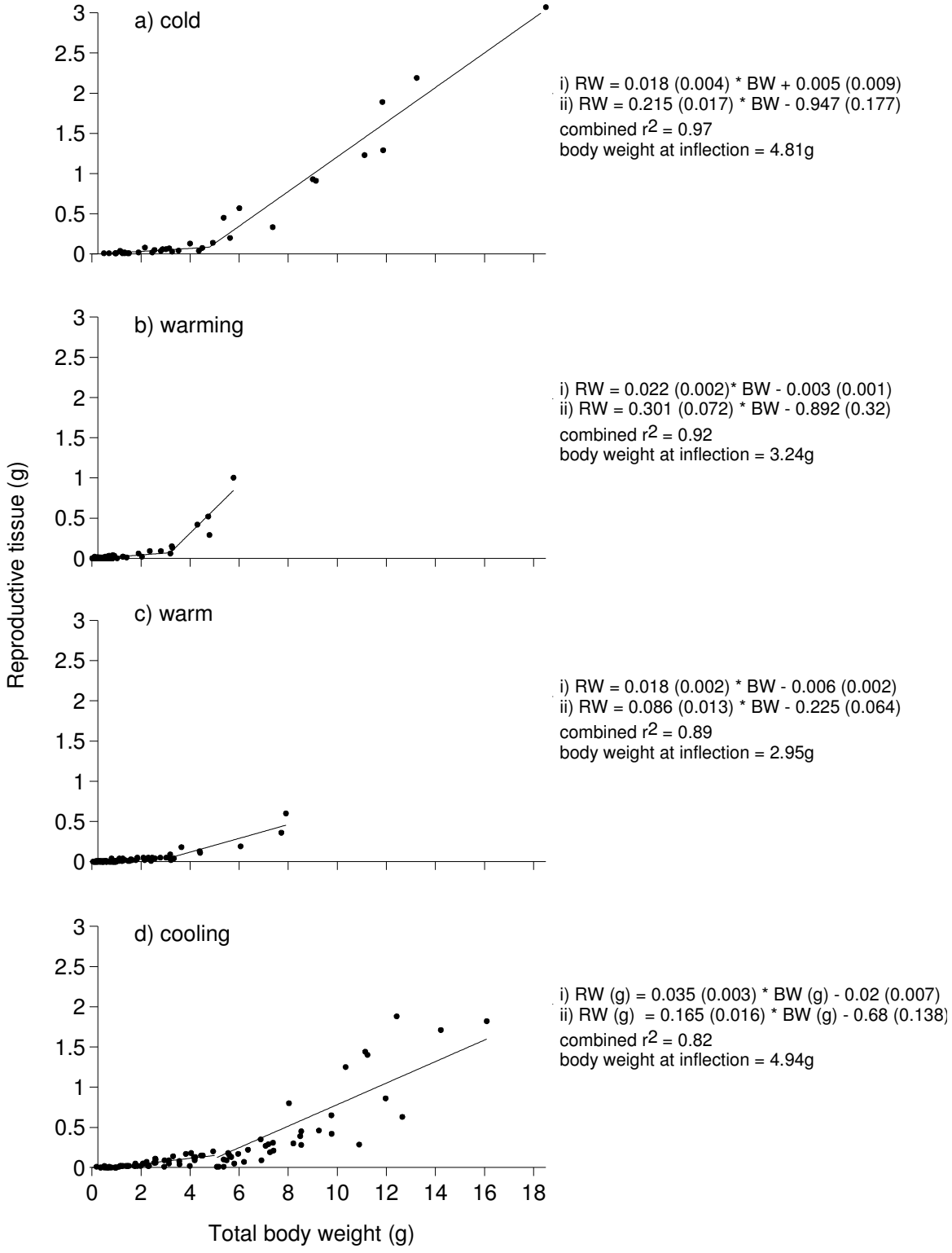

Figure 5.2: The relationship between reproductive tissue weight (g) (RW) and total body weight (g) (BW) for female squid, both before (i) and after (ii) the inflection point when individuals started allocating energy to reproduction in the four temperature categories. Numbers in brackets are standard errors.

5.3.2 Size-at-maturity

The probability of being mature was significantly affected by the weight of both males and females who experienced 'cooling and cold' and 'warming and warm' water temperatures (Table 5.1). The weight at which males and females became mature depended on temperature, with individuals of both sexes that experienced warming water temperature maturing at a smaller size, while individuals that experienced cooling water temperatures became mature when heavier (Table 5.1). The weight of maturity was also sex specific, with males achieving maturity at approximately half the weight of females (Table 5.1).

5.3.3 Growth assessments

RNA concentration

The concentration of RNA in the mantle muscle of *Euprymna tasmanica* ranged from 0.59-4.71um/mg and was significantly related to total body weight and recent water temperature ($F=89.02$, *df* 2, 309, $P<0.001$). The concentration of RNA decreased rapidly with increasing body weight, but increased slightly with warming water temperature (Table 5.2). Together, body weight and water temperature explained 37% of the variation in mantle muscle RNA concentration; however the β values suggest that body weight had a greater contribution. Sex, reproductive allocation and the residuals from the somatic, reproductive and digestive tissues did not make a significant contribution in explaining variation in RNA concentration.

Table 5.1: The logistic regression coefficient, odds ratio and the weight at 50% probability for each sex and temperature category

	Slope (se)	Intercept (se)	Walds χ2	Odds ratio	Weight at maturity (50% probability)
males cold	3.39 (1.49)	-9.38 (4.23)	χ2=5.27, *df* 1, 59, P=0.022	29.62	2.77
males warming	-	-	-	-	2.20*
males warm	5.99 (3.09)	-17.14 (8.76)	χ2=3.76, *df* 1, 101 P=0.05	29.62	3.36
males cooling	3.63 (1.34)	-11.46 (4.13)	χ2=7.37, *df* 1, 95, P=0.007	37.66	3.16
females cold	1.6 (0.57)	-7.38 (2.54)	χ2=8.00, *df* 1, 50 P=0.005	4.94	4.61
females warming	-	-	-	-	4.30*
females warm	-	-	-	-	6.06*
females cooling	0.92 (0.18)	-4.56 (0.88)	χ2=25.13, *df* 1, 97, P<0.001	2.51	5.96

* The probability of being mature could not be estimated due to the complete separation in the weight of immature and mature individuals, for these temperature categories the weight of the lightest mature individual was used as the weight at maturity.

Table 5.2: Multiple regression showing the relationship (in the absence of sex, reproductive allocation, somatic residuals reproductive residuals and digestive residuals) between the RNA concentration and the recent temperature experienced and Ln (weight).

Step 1	*B* (se)	β
Intercept	3.01 (0.08)	
Ln (weight)	-0.597 (0.05)	-0.58
Step 2		
Intercept	2.34(0.23)	
Ln (Weight)	-0.56 (0.05)	-0.54
Ln (temperature from the past 2 weeks)	0.77 (0.21)	0.17

Note: r^2 = 0.34 for step 1,Δr^2 = 0.37 for step 2 (P<0.001).

Inter-annual variation

Differences in the concentration of RNA (adjusted for body weight) in individuals caught in the different seasons depended on year ($F_{season\ caught*sample\ year}$=5.10, *df* 3, 315, P=0.002). During 2008 there was little seasonal difference in the average concentration of RNA, while the concentration of RNA for individuals caught in the spring and summer of 2009 were on average almost 1ug/mg greater than in individuals caught in the winter of 2009 (Figure 5.3).

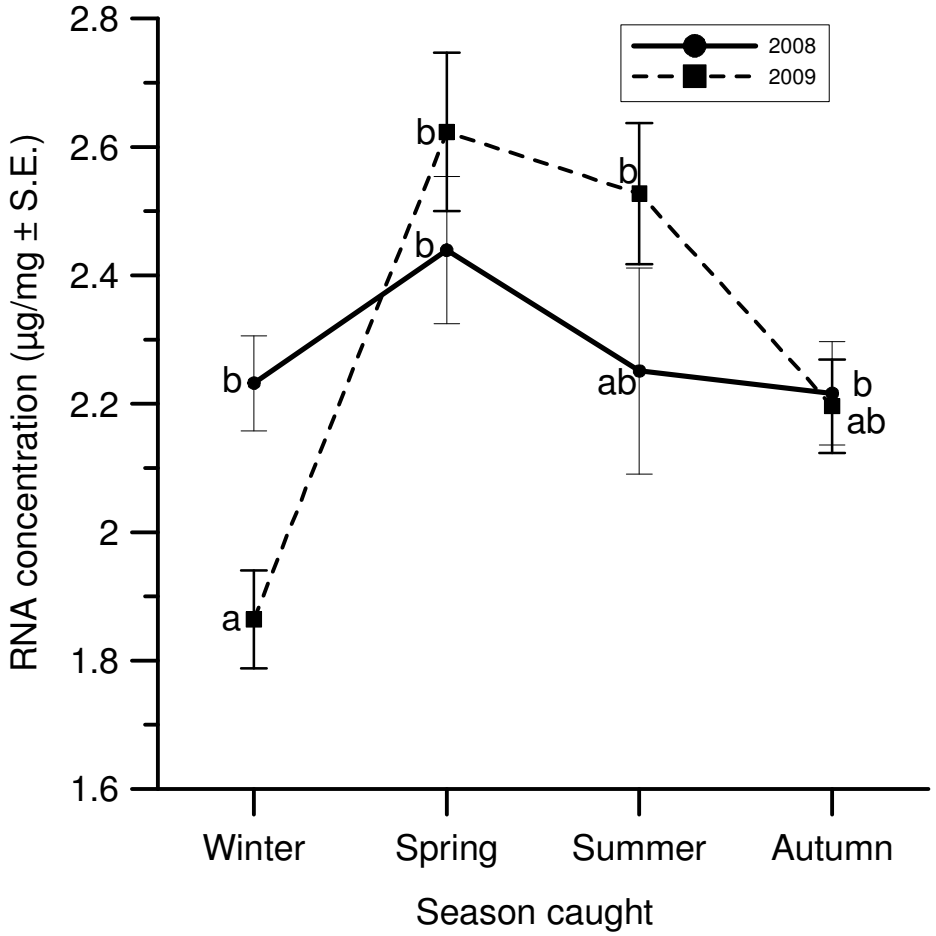

Figure 5.3: Mean RNA concentration (adjusted for weight) of individuals caught in each season of 2008 and 2009. Letters signify where means are significantly different from each other.

Protein

The concentration of protein in the mantle muscle of *E. tasmanica* ranged from 80.34-244.82ug/mg, and was significantly related to body weight and recent water temperature (F=5.61, *df* 2, 309, P=0.004). The concentration of protein increased with both increasing body weight and warming water temperature (Table 5.3). Together, somatic condition and water temperature explained only 4% of the variation in protein concentration. From the β values, both body weight and recent water temperature have a comparable degree of importance in determining the concentration of protein. Sex, reproductive allocation, and the residuals from

the somatic, reproductive, and digestive tissues did not make a significant contribution in explaining variation in RNA concentration.

Table 5.3: Multiple regression showing the relationship (in the absence of sex, reproductive allocation, somatic residuals reproductive residuals and digestive residuals) between the protein concentration and the recent temperature experienced and somatic residuals.

Step 1	*B* (se)	β
Intercept	74.62 (20.92)	
Ln (weight)	20.74 (7.89)	0.15*
Step 2		
Intercept	59.14 (22.13)	*
Ln (Weight)	24.46 (8.06)	0.18*
Ln (temperature from the past 2 weeks)	3.80 (1.85)	0.12**

Note: r^2 = 0.02 for step 1, Δ r^2 = 0.04 for step 2 (P<0.001). *P <0.01. **P<0.05.

RNA:protein

The RNA:protein ratio, a potential measure for the capacity of growth (Houlihan*, et al.*, 1993), ranged from 0.56-3.26, and was significantly related to sex and total body weight (F=129.49, *df* 2, 309, P<0.001). The RNA:protein ratio decreased with increasing body weight and was greater in males compared to females (Table 5.4). Together body weight and sex explained 46% of the variation in RNA:protein; however the β values suggest that body weight had a greater contribution. The recent temperature and the residuals from the gonad, mantle and digestive gland did not help explain differences found in the RNA:protein ratio among individuals. There were significant differences in the relationship between RNA:protein and total body weight for each sex (F=7.21, *df* 1 323, P=0.008), with both males (F=119.84, *df* 1, 172, P<0.001) and females (F=144.67, *df* 1, 151, P<0.001) displayed a significant negative logarithmic

curve (Figure 5.4). In larger individuals (>10g) there was a distinct pattern around the fitted line that was sex specific, with larger females being more likely to have smaller RNA:protein ratios for their weight and males being more likely to have greater RNA:protein ratios for their weight (Figure 5.4).

Table 5.4: Multiple regression showing the relationship (in the absence of recent water temperature, reproductive allocation, somatic residuals reproductive residuals and digestive residuals) between the protein concentration and the sex and weight of individuals.

Step 1	*B* (se)	β
Intercept	2.45 (0.05)	
Ln (weight)	-0.50 (0.03)	-0.67
Step 2		
Intercept	2.63 (0.09)	
Ln (Weight)	-5.12 (0.03)	-0.69
Sex	-0.11 (0.05)	-0.11*

Note: r^2 = 0.45 for step 1, Δ r^2 = 0.46 for step 2 (P<0.001). *P<0.05.

Inter-annual variation

The season and year individuals were caught in did not significantly contribute in explaining the variation of protein concentration (F=0.344 *df* 3, 333, P=0.74).

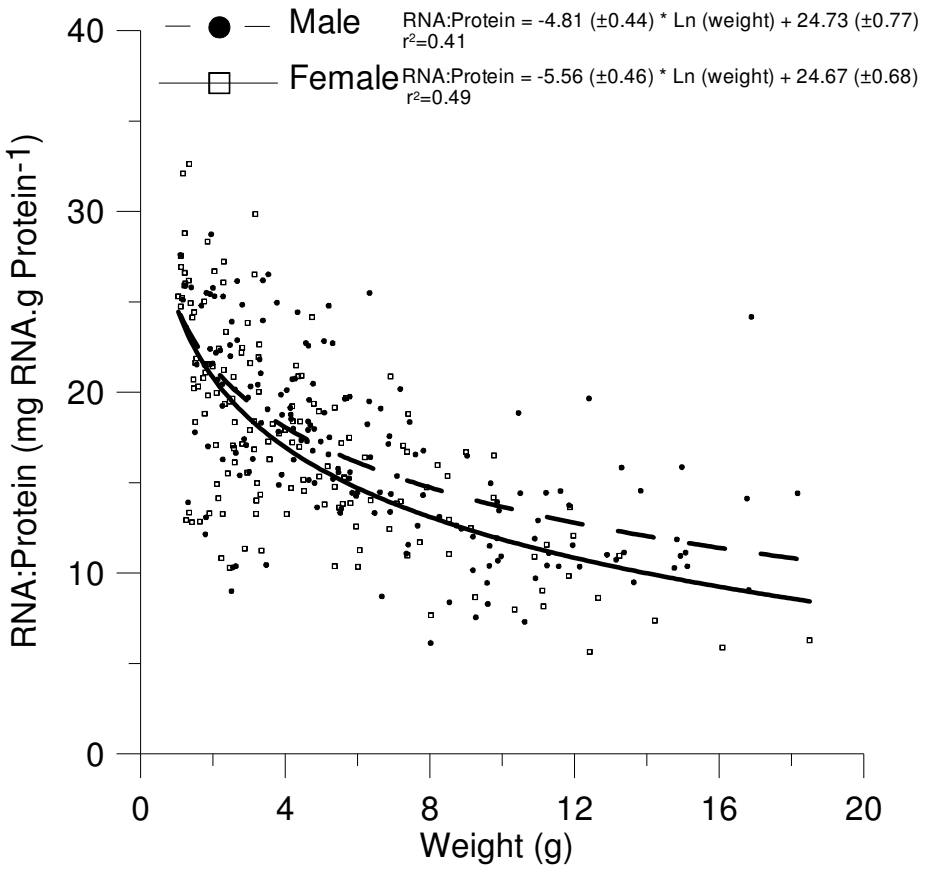

Figure 5.4: Relationship between RNA:protein ratio and weight

5.4 Discussion

5.4.1 Variation in growth

Biochemical measurements from the mantle tissue showed that recent growth in *Euprymna tasmanica* was highly variable, and although the variability was partly explained by water temperature, there was little seasonal effect on instantaneous growth. Body size was more influential in explaining the variation of growth of *E. tasmanica*, with concentrations of RNA decreasing with increasing body weight. Since the majority of RNA is found in ribosome's, which are building blocks for proteins (Pierce*, et al.*, 1999), small individuals with greater concentrations of RNA will have higher rates of protein synthesis that fuel relatively high instantaneous growth rates. This negative relationship between body size and growth rate is a common occurrence in cephalopods e.g. *Loligo forbesi* (Pierce*, et al.*, 1999); *Eledone cirrhosa* (Houlihan*, et al.*, 1998); *Sepioteuthis australis* (Ho*, et al.*, 2004) *Euprymna tasmanica* (Moltschaniwskyj and Carter, 2010); *Moroteuthis ingens* (Semmens and Jackson, 2005) and fish (Houlihan*, et al.*, 1986) and is thought to be associated to the relative proportion of body the occupied by the intestine (Weatherley, 1987). For example, as the body grows not only can the fractural rates of protein synthesis of individual tissue decline, but the relative proportions of the tissue in the body will change with a relative increase in slow synthesising white muscle (Houlihan, 1991). This change is of particular importance in cephalopods that have mantles contributing up to 70% of the total body weight (Moltschaniwskyj and Martinez, 1998).

Although less influential than body size, temperature had a significant effect on the instantaneous growth rate; individuals that experienced warmer water had greater

concentrations of RNA and faster instantaneous growth rates. This positive relationship between growth and water temperature is not only common in cephalopods (Moltschaniwskyj and Martinez, 1998; Pierce*, et al.*, 1999; Domingues*, et al.*, 2006), but many aquatic species (see Houlihan, 1991 for a review). In this study individuals caught in summer were generally smaller (Chapter 2) making it difficult to determine if the increase in RNA concentration was a response to body size or from experiencing warmer water temperatures. However, when the effects of total body weight were accounted for, the season of capture was still a significant factor influencing growth rate, with the concentration of RNA from individuals caught in 2008 being significantly greater in spring and summer compared to winter. These results are comparable to *Loligo forbesi* caught in Scotland where the RNA concentration in the mantle tissue of mature males was greater in summer than in winter (Pierce*, et al.*, 1999). During 2009 no significant difference in RNA concentration was found among each seasons of this study and given the average seasonal temperatures were similar between 2008 and 2009 (Chapter 2), it is likely that water temperature is not the sole environmental factor effecting RNA concentration.

Other environmental parameters such as food availability, food quality (Pierce*, et al.*, 1999; Vidal*, et al.*, 2006) and even photoperiod (Vidal*, et al.*, 2006) may all impact on the RNA concentration in cephalopods. While all animals from this study were collected during the dark, the processes that control food availability in the wild are influenced by several biotic and abiotic parameters (Vidal*, et al.*, 2006) and are therefore complex and difficult to measure. While recent feeding activity has a direct effect on fish metabolism (Carter*, et al.*, 1993; McCarthy*, et al.*, 1994), there is little evidence from manipulative experiments that biochemical

parameters are correlated with recent feeding history in cephalopods (Castro*, et al.*, 1992; Houlihan*, et al.*, 1998; Moltschaniwskyj and Jackson, 2000).

In addition to the environmental parameters, weight, sex, reproductive and somatic condition can also influence the biochemical levels in cephalopods (Ho*, et al.*, 2004; Rosa*, et al.*, 2005). While these parameters made little or no contribution in explaining the variation in protein concentration, 46% of the variation in RNA:protein was explained by sex and weight. Although a large percentage of variation is still unaccounted for, this was the strongest relationship recorded in this study. In a laboratory study, body weight explained 84% of the variation in RNA:protein in reared *E. tasmanica* (Moltschaniwskyj and Carter, 2010). However some caution is needed when comparing results of cephalopods held in captivity as growth processes can be modified in captivity (Pecl and Moltschaniwskyj, 1999). A possible explanation for the difference between the two studies was the size of individuals used, with Moltschaniwskyj and Carter (2010) using smaller individuals from 0.02-7.2g, while individuals in this study ranged from 1.4-18.05g. Despite these differences, both studies confirm that the capacity for growth decreases with individual size, as evidenced by the decrease in RNA:protein ratio with total body mass.

A similar pattern between gender group was also found in both studies, with larger females having smaller RNA:protein values for their size compared to males. This suggests that as individuals grow, the capacity for growth in female *E. tasmanica* decreases at a faster rate than that of males, which is similar to wild *Loligo forbesi*, where the RNA content of males was higher than females (Pierce*, et al.*, 1999). The size at which *E. tasmanica* starts to allocate energy to reproduction was also sex specific, with males starting to allocate energy to

reproduction at approximately half the body size as females. The sexual difference in body size at the onset of maturation is thought to be a factor of the different reproductive demands between males and females, with females needing to invest in larger body sizes due to the increased physical and physiological constraints of egg production compared to sperm production (Rodhouse and Hatfield, 1992; Ceriola and Jackson, 2010).

5.4.2 Reproductive growth

No relationship was found between the reproductive condition of *E. tasmanica* and any of the biochemical parameter measured, suggesting that instantaneous growth did not change in response to reproductive growth. This results in the non-asymptotic growth common to many cephalopod species (Jackson, 1994; Jackson and O'Dor, 2001), where reproductive growth appears to occur with little or no cost to somatic growth (Mangold*, et al.*, 1993). While no significant trade-off was found, it appears that both reproductive and somatic growth are linked through their relationship with body size. As with instantaneous growth, the significant relationship between reproductive weight and total body weight indicates that maturation in *E. tasmanica* is dependent on body size. These results are comparable to other species of cephalopods where the process of maturation is a function of body size rather than age (Boyle*, et al.*, 1995; Jackson*, et al.*, 1997; Dimmlich and Hoedt, 1998; Pecl, 2001; Pecl and Jackson, 2008; Ceriola and Jackson, 2010).

There were several seasonal differences in the size at the onset of maturation, with individuals in warmer water starting to allocate energy to reproduction at smaller sizes compared to those in cold water. Similar results were found between summer and winter populations of female *Sepioteuthis australis* caught in Tasmania, with summer caught females

maturing at smaller sizes (Pecl, 2001). After the onset of maturation, the reproductive growth in relation to body weight was sex specific. In males any difference in reproductive condition was a factor of the body size and males that started allocating energy to reproduction at smaller sizes reach maturity sooner. The reproductive growth of females was more complex with the variation in reproductive condition being influenced by both the weight at the onset of maturation and water temperature. As a result, females that experienced warm water started allocating energy to maturity at smaller sizes compared to female from warming water. However, as the relative rate of reproductive growth was greater in females that experienced warming water, these females were able to reach maturity sooner at the smaller sizes (Figure 5.2). In some cephalopod species, females that mature early reach larger adult sizes and perhaps achieve greater reproductive success due to the positive relationship between body size and fecundity (Pierce*, et al.*, 2008). However despite the fast growth and small size at the onset of maturation, the maximum reproductive weight of females that experienced warming and warm water temperature was less than half that of females that experienced cooling and cold water, possibly due to shorter life spans. Similarly, populations of *Illex illecebrosus* display variations in growth, maturity and lifespan depending on their latitude, with individuals that experience warmer water growing faster, maturing earlier, but living shorter than their northern counterparts (Coelho and O'Dor, 1993; Hendrickson, 2004). Therefore, depending on their environment, females will either grow rapidly and have a reduced reproductive output, or spend longer as juveniles and ultimately have a greater reproductive output, which in essence is the most common feature observed in the life history trade-off of all living animals (Roff, 1992; Stearns, 1992).

While seasonal differences in size and growth rates were evident in the population sampled (Chapter 2 and 4), the high level of unexplained variance in this study suggest that the use of biochemical parameters in *E. tasmanica* may not be as suitable for estimating growth as found in teleosts species. Although the use of biochemical parameters were successful in predicting recent growth in some laboratory studies of cephalopods (Clarke*, et al.*, 1989; Houlihan*, et al.*, 1990; Houlihan*, et al.*, 1998), results from this study suggests that the same methods may not provide the same level of confidence in estimating growth or recent feeding in wild populations. This is most likely due to the differences between the two types of studies, with laboratory experiments being designed with a specific hypothesis in mind, and generally have an isolated and controlled nature, resulting in less complex growth models compared to those generated for wild populations (Semmens*, et al.*, 2004). However, the similar patterns of biochemical parameters between this study and previous laboratory studies highlight the importance of laboratory studies, and although they may not represent the true environment wild squid experience, they are an integral part in confirming specific factors effecting growth.

Before such a tool can be used for estimating growth in wild populations, further research including other environmental variables is needed. However when including additional variables, analyses become highly complex and require large data sets and as suggested by Ho., et al (2004) it may be necessary to use individual based models to understand population based variability in growth and reproduction. Additionally, while the processes of growth in cephalopods may be considerably different to that in fish, new, more effective methods, such as the use of multiple biochemical indices may be gained from applying recent advances in fish growth estimates (Weber*, et al.*, 2003) to cephalopods.

Chapter 6 General Discussion

6.1 Synthesis

Through a combination of the two year population assessment and manipulated laboratory experiments, this study was successful in describing the reproductive strategies and the cellular processes that were responsible for the temporal variation found in the structure and dynamics of a population of *Euprymna tasmanica.* Many plants and animals do not reproduce all year round, but rather have seasonal peaks in reproductive activity (Brown and Shine, 2006). In some cases, reproductive seasonality is so precise it occurs within a few hours (e.g. coral spawning; Babcock*, et al.*, 1986). Strong seasonal synchronous spawning is a reproductive strategy common to many iteroparous fish, (i.e. *Hippoglossus stenolepis* (Loher, 2011); *Sparus aurata* (Chaves-Pozo, et al., 2005), octopus species (i.e. *Octopus cyanea* (van Heukelem, 1983); *O. vulgaris* (Mangold*, et al.*, 1993); and *O. mimus* (Cortez*, et al.*, 1995) and some terminal spawning squid (e.g. *Loligo opalescens* (Hixon, 1983); *Todarodes pacificus pacificus* (Ikeda*, et al.*, 1993). In these species, breeding is strongly seasonal and occurs over a short period with individuals producing their lifetime fecundity in one spawning episode lasting hours or days and results in virtually no overlap between adult populations (Boyle and Boletzky, 1996). In other cephalopod species reproduction occurs using a wide array of spawning strategies from simultaneous spawning events at the end of the animals life (e.g. *Loligo forbeso* (Pierce*, et al.*, 1994); *Sepioteuthis australis* (Moltschaniwskyj and Pecl, 2007), to continuous spawning during long periods of their life as seen in tropical species such as *Idiosepius pygmaeus* (Lewis and Choat, 1993).

During the initial observation of the size frequency distribution, the population of *E. tasmanica* displayed characteristics similar to that of a longer lived seasonal spawning species, with a peak in the number of mature individuals present over winter followed by a hatching period during summer (Chapter 2). However after focusing more on the life history characteristics of individuals within the population it became apparent that *E. tasmanica* spawn multiple times through an extended breeding season that lasts for several months, similar to that of *Loligo forbeso* (Pierce*, et al.*, 1994) and *Sepioteuthis australis* (Moltschaniwskyj and Pecl, 2007). The advantage of this spawning strategy is that the risk of recruitment failure is spread over several months, increasing the chance that at least some offspring will experience favourable conditions. The implication for this type of spawning strategy is that cohorts may be alternating between long and short generational times making it difficult to assigning one or two generic life history characteristics (Boyle and Boletzky, 1996). However, for the ease of explanation, a model with two alternate life history strategies has been proposed with the view that individuals will lie somewhere between the two, based on the environment they are laid.

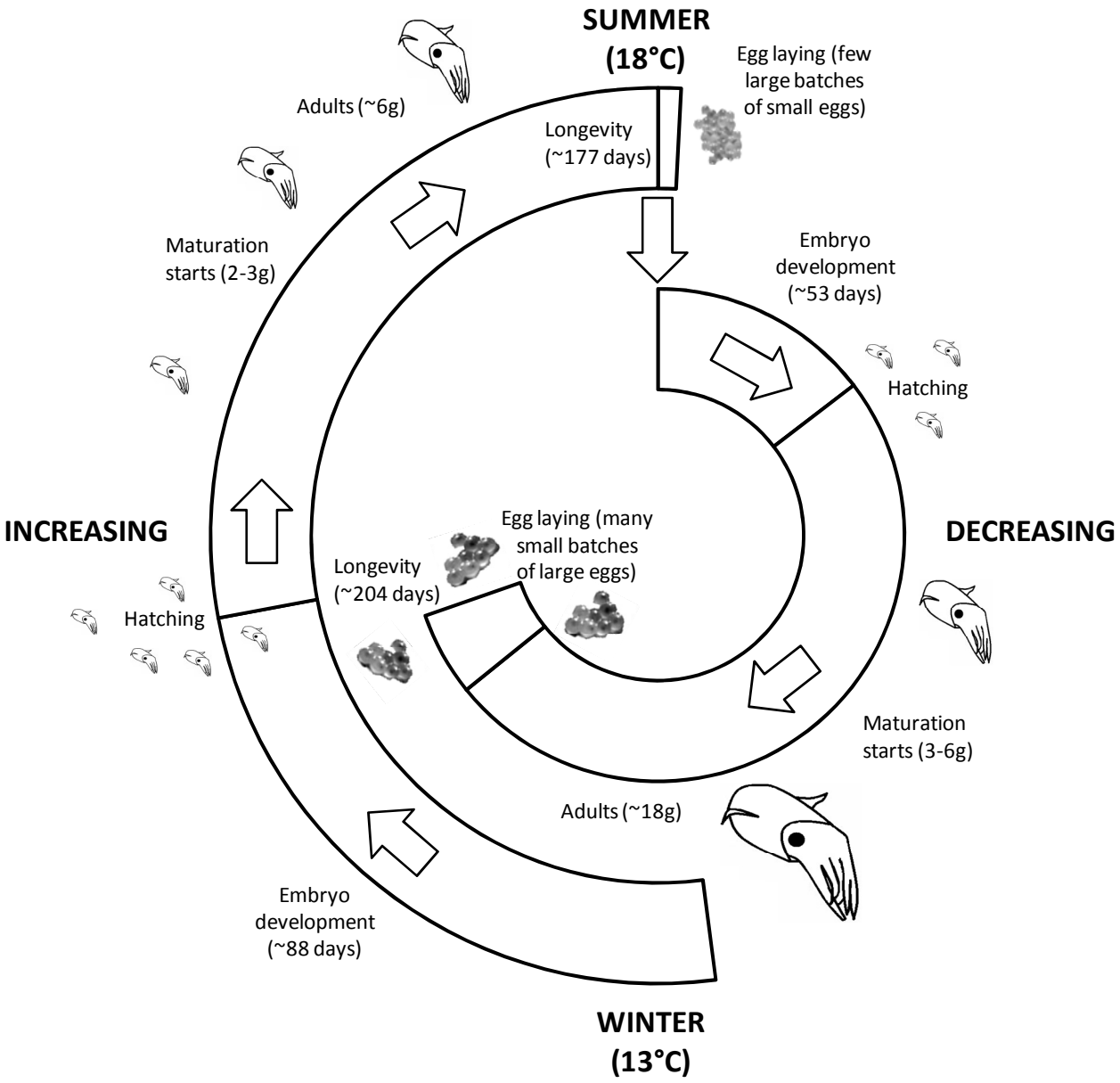

Figure 6.1: A conceptual model of the alternative life history strategies of *Euprymna tasmanica* based on the environmental conditions experienced.

6.1.1 Strategy one – Hatching during warming water temperatures

Assuming the length of embryonic development recorded in captivity is similar to that in the wild, eggs deposited in winter would hatch approximately 88 days later (Chapter 3), during spring (Figure 6.1). These hatchlings experience a period of warming water temperature and grow rapidly mainly via an increased rate of hypertrophy (Chapter 4) which appears to be fuelled by the faster rates of protein synthesis (Chapter 5). At approximately 3-4g, the initial period of fast growth (0.057g/day) of *E. tasmanica* ceased and individuals grew at a reduced rate (0.03g/day) for the remainder of their lives (Chapter 3). In fish (Wootton, 1990) and some cephalopod species (Pierce*, et al.*, 1999; Ho*, et al.*, 2004), change in the growth rate is often suggested to be associated with a reallocation of energy to reproduction. However, as individuals in this study had already started allocating energy to reproduction at around 2-3g, the observed change in growth rate is likely to be related to a change in the mechanisms behind growth rather than a reallocation of energy to reproduction and may be attributed to metabolic constraints that limits the size at which an individual can grow exponentially (Pauly, 1998). Although body mass continued to increase linearly with age (Chapter 3), the instantaneous growth rate (%BW/day) decreased as individuals got larger, this was shown by the decrease in protein synthesis with increasing body size (Chapter 5). Similar results were found in some species of octopus, where relative instantaneous growth rate in the late phase of growth decreased with age (Forsythe, 1993).

In *E. tasmanica* growth after this inflection became less dependent on temperature and more dependent on ration (Chapter 3). A similar pattern was shown in the patterns of growth at the lower level of organisation, with the initial 'temperature dependent' hypertrophic growth

being replaced by hyperplastic growth which was more dependent on body size (Chapter 4). The influence of ration and body size on growth are most likely related as larger animals have a better ability to gather food (Ceriola and Jackson, 2010). This is a common phenomenon in cephalopods, with growth in the early life stages often being more sensitive to the variability in environmental conditions (Mangold, 1987; Forsythe, 1993; Jackson, 1997; Robin and Denis, 1999; Hatfield, 2000; Martinez*, et al.*, 2000; Pecl, 2004; Pecl and Jackson, 2008).

After the initial fast growth, the weight at the onset of maturation was sex specific, with males allocating energy to reproduction at around 2g and females at 3g (Chapter 5). Once allocating energy to reproduction, the time to full sexual maturity was rapid (Chapter 5) and based on laboratory results (Chapter 3) females would start spawning during summer at the age of 3-4 months (Figure 6.1). Female *E. tasmanica* were rarely caught with full oviducts (Chapter 2), suggesting that eggs are not stored for long but laid soon after development. In captivity, females that experienced warm water were able to lay 1-2 batches of up to 130 eggs (depending on the ration), however on all occasions the second batch was generally smaller and of poorer quality (Chapter 3), which appears to be a consistent trait of *E. tasmanica* (Steer*, et al.*, 2004). As very few mature individuals were present during summer it is hypothesised that females once mature, lay eggs and die within a short period of time (Figure 6.1), which is a reproductive strategy similar to that of terminal spawning squid and octopus (i.e. *Loligo opalescens* (Hixon, 1983); *Todarodes pacificus pacificus* (Ikeda*, et al.*, 1993); *Octopus cyanea* (van Heukelem, 1983); *Octopus vulgaris* (Mangold*, et al.*, 1993); and *Octopus mimus* (Cortez*, et al.*, 1995)). These species invest large amounts of energy in reproduction during the final stages of life essentially trading future somatic growth for reproductive output, and as a consequence

compromise body condition once maturity is reached (Calow, 1987). In captivity the maximum lifespan of female *E. tasmanica* that used this reproductive strategy was around six months (Chapter 3) with individuals weighing no more than 6g (Chapter 5).

It is often thought that fast growth is a positive trait, not only as they become reproductively mature earlier but also because they reduce the chance of predation during their most vulnerable period of life (Roff, 1992; Stearns, 1992). As the lifespan of many insects is relatively short and the generational turnover is fast, many researchers have used insects to study the tradeoffs associated with fast growth. While many studies have found that fast juvenile growth rates is associated with shorter adult longevity (Sevenster and Van Alphen, 1993; Chippindale, et al., 1994), one study has documented that fast growing larvae were highly preyed upon due to their increased time spent foraging (Gotthard, 2000). These studies highlight that while one strategy may seem advantageous in a particular feature there will always be a trade-off that benefits other, possible unmeasured, characteristics. Therefore, a true advantage would be having the ability to switch to a strategy that best suits your immediate environment.

6.1.2 Strategy two – Hatching during cooling water temperatures

Offspring from the previous cohort would be laid during summer (Figure 6.1) and hatch around 55 days later (Chapter 3) as water temperature decreases. During cooling water temperatures, growth was slowed (presumably as a result to metabolic responses to temperature), and *E. tasmanica* appeared to trade a short generation time for increased longevity (Chapter 3). In doing so, although growing slower, individuals grew larger, with mature females recording an average body size twice that of their warm water parents (Chapter

2), which is an important life history characteristic, as larger animals are generally able to produce more offspring (Stearns, 1992). This increase in lifespan most likely occurred as a necessity due to delayed maturation, with the average body size at the onset of maturity being 3g for males and 5g for females (Chapter 5). For squid that adopt this strategy, sexual development occurs over a longer period and may result in multiple spawning (e.g. *Idiosepius pygmaeus* (Lewis and Choat, 1993); *Photololigo sp.* (Moltschaniwskyj, 1995); and *Sepioteuthis sp.* (Pecl, 2001)). In captivity, female *E. tasmanica* deposited their first batch at the age of approximately six months, with a weight of 6.6g (Chapter 3) and were able to lay up to nine batches over a period of approximately 20 days (Chapter 3). Although batches were smaller (50-70 eggs) than females held in warm water and fed a high ration, average egg volume was double (60mm^3). This strategy of depositing multiple batches over an extended period of time is likely to increase the chance that some offspring will hatch in suitable conditions (Rocha*, et al.*, 2001; Pecl, 2004; Boyle and Rodhouse, 2005).

6.1.3 Population structure after adopting the two strategies

The reason for the greater relative frequency of small immature individuals over the warmer months of the year (Chapter 2) can partly be explained by the increasing rate of embryonic development and reduced generational time (Chapter 3) of individual experiencing warming water. Faster embryonic development (Chapter 3), appears to be a common characteristic across species, as long as water temperature remains within the physiological range for development (Boletzky, 2003; Beukema*, et al.*, 2009). As water temperature increased embryo development, eggs that are laid in warming water will hatch at approximately the same period as earlier 'cool water' laid eggs. Similarly, in warm water, the interval between

generations decreases, thereby reducing the time between hatching and the deposition of the next batch of eggs. For example, if individuals hatch early in the summer their fast growth rate and short life span will enable them to reach maturity within a few months, allowing them to reproduce within the same season of hatching. This is a similar strategy to that found in tropical, continuous spawning species such as *Idiosepius pygmaeus* (Lewis and Choat, 1993). While fast growth may initially seem advantageous it appears that the maximum growth rate of individuals is not always possible and in some instances, i.e. cold water, fast growth is replaced by longevity. While a longer lifespan increases the chance of predation before becoming reproductively mature, longer living individuals also attain larger body sizes, which is likely to be a beneficial characteristic in escaping predation and acquiring food especially in an environment where resources may be limited. The combination of rapid growth and early maturity of warm-water individuals, combined with their extended life span and multiple spawning of cold-water individuals, makes it possible for the population of *E. tasmanica* to survive the year despite living in a variable environment and having such a short lifespan. Additionally, due to the extended lifespan of cold-water individuals and fast growth of warm-water individuals, it is likely that during late spring or early summer the population will be made up of a mixture of cohorts with very different life histories. This overlap between cohorts is essential for the stability of the population as it provides the genetic diversity and phenotypic plasticity required for population survival (O'Dor, 1998).

6.2 Summary and future direction

Since a review of the definition of cephalopod populations (see Boyle and Boletzky, 1996), an increased number of studies describing the relationship between short term

environmental variability and the reproductive strategies has increased our understanding into what influences the dynamics and structure of cephalopod populations. This has largely been achieved through increased number of laboratory based experiments, and through the application of ageing techniques to estimate the life history of individuals by back calculating hatch dates. In comparison with previous data, results from this study first confirm that environmental effects during the initial stages of growth are important stages in life that determine the growth and future reproductive capacity as adults (Hatfield, 2000; Moltschaniwskyj and Jackson, 2000; Pecl, 2004). Second, like many cephalopods growth of *E. tasmanica* occurs in two phases, an initial fast exponential growth phase, followed by a reduced more linear growth phase. It has been hypothesised that this change in growth occurs with the allocation of energy to reproduction (Forsythe and Van Heukelem, 1987; Mangold, 1987; Rodhouse, 1998). However results from this study suggest that, at least in *E. tasmanica*, this change in growth occurs with a change in the growth processes at the lower level of organisation and is possibly related to the metabolic constraints limiting the size at which an individual can grow exponentially (Pauly, 1998). Finally, individuals appear to fall somewhere between two distinct reproductive strategies that can be determined by direction of change in water temperature experienced (e.g. warming vs. cooling), especially during the early life history. The reproductive strategy of individuals that experienced warm water closely resembles a terminal spawning strategy found in almost all benthic octopus (van Heukelem, 1983; Mangold*, et al.*, 1993; Cortez*, et al.*, 1995; Rocha*, et al.*, 2001) and deep water squid (e.g. *Loligo opalescens* (Hixon, 1983); *Todarodes pacificus pacificus* (Ikeda, et al., 1993); *Moroteuthis ingens* (Jackson and Mladenov, 1994)), while cold water individuals that appear to be

intermittent terminal spawning with an extended spawning period, similar to that of many loliginid squid (Sauer and Lipinski, 1990; Rocha and Guerra, 1996; Rocha, et al., 2001). The ability of successive generations to switch between two or more reproductive strategies enables *E. tasmanica* to survive the variable environment despite their short lifespan. This ability to switch between multiple strategies is not unique to *E. tasmanica*, with other squid species displaying similar seasonally distinct reproductive strategies (e.g. *Idiosepius pygmaeus* (Jackson and Choat, 1992; Jackson, 1993); *Sepioteuthis lessoniana* (Jackson and Moltschaniwskyj, 2002) and *Loligo gahi* (Patterson, 1988). The fitness of an individual or population is classed by the generational time and reproductive output (Roff, 1992) and although one strategy may initially seem to be more advantageous than the other, one maximises fitness through short generation's time while the other does it through increased reproductive output. Although the strategies each have their own advantages and disadvantages it appears that *E. tasmanica* as a species is able to maximise its fitness by selecting a strategy that best suits the conditions they experience.

If the strategy described in this study for *E. tasmanica* is typical for an endangered or commercially important cephalopod species then management of fisheries must take into account how different environments influence the life history characteristics at all stages of life. To be able to untangle the complex web of variability that is apparent in populations of almost all cephalopod species, similar long term assessment studies that sample all stages of the population are needed, especially during the early life stages, where growth appears to be influenced most of all by water temperature (Mangold, 1987; Forsythe, 1993; Jackson, 1997; Robin and Denis, 1999; Hatfield, 2000; Martinez, et al., 2000; Pecl, 2004; Pecl and Jackson,

2008). For some species the assessment of the population dynamics is difficult, as individuals are either highly dispersed over large areas or the location of immature individuals is unknown. Additionally if food availability during the later stages of life is important for maintaining rapid growth in commercially important species, knowledge of the variability in prey species in and around spawning aggregation could be useful in determining the adult growth and reproductive potential of each spawning season. Although laboratory conditions cannot always replicate wild population habitats, this thesis highlights the importance of such studies in understanding the growth and reproductive strategies that may be adopted in the field. Similar laboratory studies can provide important information especially on the early life history in species where only the location of spawning aggregations is known, especially as the early life history is an important stage in determining growth and reproductive strategy (Hatfield, 2000; Moltschaniwskyj and Jackson, 2000; Pecl, 2004). Only until we know how the environment affects all stages of life in cephalopods can we begin to design successful management plans for the commercially important species of this unique group of short-lived animals.

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Appendix 1

Mantle RNA calculation - adapted from (Ashford and Pain, 1986)

A section of the frozen mantle tissue (0.05 g – 0.10 g) was weighed and placed in plastic polypropylene test tubes before being homogenized in 3ml of 0.2 M PCA using an IKA Homogenizer. Tissue was homogenized in short periods of less than 10 seconds to ensure that the tissue did not overheat. Between periods of homogenization, tissue stuck in the homogenizer blades were removed using fine tipped forceps and placed back in the PCA. The homogenized tissue was centrifuged at 5,500 rpm at 4 °C for 10 minutes. The supernatant was discarded and the pellet was washed with 2mL of 0.2M PCA, vortex mixed and centrifuged twice before the RNA concentration was determined. The pellet was re-suspended in 5mL of 0.3 M NaOH, vortex mixed and incubated in a 37ºC bath for 1 hour. Every 10 minutes the tissue was vortex mixed during incubation. After one hour the tubes were cooled for 5 minutes. Samples of 3.9 mL were removed into polypropylene test-tubes, the remaining sample was kept for protein analysis. 0.867 mL of 20%PCA was added to each tube and centrifuged at 5500 rpm at 4ºC for 10 minutes. The supernatant was immediately used for RNA analysis. A spectrophotometer was calibrated to zero using a RNA blank (39mL of 0.3M NaOH and 8.67 ml of 20% PCA). Each sample was then read at and absorbance of 260 nm and 232 nm. If any absorbance readings were more than 1.0 the sample was diluted by half using distilled water, and later multiplied by 2. The following calculations were used to find how much RNA was in each original sample:

- RNA (ug/ml) =(32.0 x A260 nm) – (6.11 x A232 nm)

- RNA in initial sample =RNA (ug/m;) x 4.767 x 5/3.9

  =RNA (ug/ml) x 6.112

- Divide by sample wt. to get ug RNA/mg sample.

Total weight (g) was used as the size measure to compare size at estimated age. Initial examination of the data showed exponential growth so weight was log10 transformed before using a linear regression to calculate growth rates for each season separately.