Understanding reproduction and energy storage, for broodstock conditioning, in *Mytilus galloprovincialis*

Jo-Anne Fearman

B. Sc. Marine Biology (Hons)

Submitted in fulfilment

of the requirements for the Degree of Doctor of Philosophy

University of Tasmania.

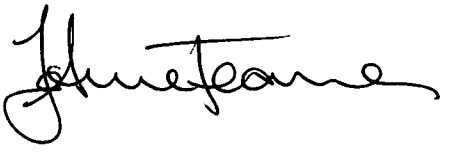
Launceston, Tasmania

June, 2010

This thesis is dedicated to my God

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person, except where due reference is made in the text of the thesis.



Jo-Anne Fearman

Authority of Access

This thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

Jo-Anne Fearman

Statement of Co-Authorship

The following people contributed to the publication of the work undertaken as part of this thesis:

Energy storage and reproduction in mussels, *Mytilus galloprovincialis*: the influence of diet quality (2009) *Journal of Shellfish Research* **28**:305 – 312

Fearman, J. (60%), Bolch, C.J.S. (10%), Moltschaniwskyj, N.A. (30%)

Warmer temperatures reduce rates of gametogenesis in temperate mussels, *Mytilus galloprovincialis. Aquaculture* (in press).

Fearman, J. (70%), Moltschaniwskyj, N.A. (30%)

- *N.A. Moltschaniwskyj, and C.J.S. Bolch contributed to the idea, its formalisation and development, and assisted with refinement and presentation.*

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: .....

Natalie Moltschaniwskyj
Supervisor
NCMCRS
University of Tasmania

.....

Christopher Bolch
Supervisor
NCMCRS
University of Tasmania

.....

Chad Hewitt
Head of School
NCMCRS
University of Tasmania

Date: .....

Abstract

Synchronous seasonal reproduction in *Mytilus galloprovincialis* has been well documented, however asynchronous reproduction is also observed in many populations. These reproductive events occur at different times of year, and therefore under different environmental conditions. Experimental evidence regarding the influence of environmental parameters, such as temperature and food, on reproduction and energy storage in this species is limited. Therefore, this research aimed to determine the important environmental conditions that influence the timing and rate of reproductive maturation in relation to energy storage and use in mussels, which can be used for broodstock conditioning in the mussel aquaculture industry.

Monthly samples of the population were collected over two years, and the timing and rate of maturation and energy storage was different between years and at different depths. Mussels used energy from food for reproduction in 2006, and used stored energy (glycogen) for reproduction in 2007. The use of these different reproductive strategies was potentially influenced by differences in temperature and food (phytoplankton concentration).

Diet composition was tested for its effect on reproduction and energy storage. Mussels were able to spawn more readily when fed a combination of *Chaetoceros calcitrans* and *Pavlova lutheri*. A monospecific diet of *Pavlova lutheri* provided PUFAs necessary for maturation, but may have provided less energy; and *Chaetoceros calcitrans* provided sufficient energy, which increased glycogen storage, but may have been limited in PUFAs.

The effects of temperature and ration were also tested, and the rate of maturation was fastest at 7°C and reduced in warmer water, up to 19°C, when held at

constant temperature. When held in temperatures that mimicked seasonal temperature changes in spring, summer and autumn, mussels produced oocytes and stored energy during summer, but during autumn production of oocytes was greater and stored glycogen was depleted. Mussels fed a higher ration in this experiment allocated greater amounts of energy to storage or energy demands, but oocytes were not increased, which suggests that temperature rather than ration regulates reproductive maturation.

The effects of food and temperature may be synergistic during seasonal cycles of reproduction. Reproduction appears to be prioritised over energy storage in mussels, which should result in year round reproduction in this species if energy (food or glycogen) is not a limiting factor. However warmer seasonal temperatures limit reproduction, therefore available energy is stored as glycogen. This information can be used to control the rate and timing of maturation of broodstock in a hatchery.

Acknowledgements

Firstly, my sincere thanks to my supervisor, Associate Professor Natalie Moltschaniwskyj, for letting me learn without letting me crash, for her knowledge of all things molluscan, and for her encouragement, advice and patience, thanks Nat!

My co-supervisor Dr. Chris Bolch provided valuable contributions to the experimental design and the preparation of manuscripts and my thesis, for which I am very grateful.

My industry supervisor Ian Duthie, the Hatchery Manager at Spring Bay Seafoods Pty. Ltd. (SBS), provided access to facilities and equipment in the hatchery, supplied algae for my experiments, provided advice on the construction and maintenance of my experimental system, and continual advice on mussel husbandry and hatchery operating procedures. Many thanks for allowing me to use the new hatchery Ian!

This research would not have been possible without the funding from Spring Bay Seafoods Pty. Ltd. Thanks to the Managing Director, Phil Lamb, for funding this research and allowing me to use SBS facilities. Funding was also provided by the Department of Agriculture Fisheries and Forestry through the Science and Innovation Awards, 2006.

The staff at SBS provided me with great support during my experiments. In particular I could not have run my experiments without the help of Zhongyuan "Anson" Ouyang who spent many hours feeding my mussels, as well as considerable over-the-phone troubleshooting – Thanks Anson!! Sereena Ashlin produced most of the algae I used for my experiments, and Hamish Morley and Jason Crisp also spent

many hours feeding mussels for me. I am extremely grateful to all of the hatchery staff for their willingness to help me out. The admin and factory staff were also very helpful and friendly during my stays at Triabunna, thanks to Andy, Gail, Roger, Amanda, Joan, Mel and many others for their support.

Grant Flemming and Andrew Fearman provided substantial time and effort to construct a temperature control system for my experiments. I am extremely grateful for their engineering expertise.

Finally, I would not have made it through this journey without the support and encouragement of my family and friends. I am especially grateful to my husband Andrew, it just would not have been possible without him!

Table of Contents

List of Figures

List of Tables

Chapter 1

General Introduction

1.1 Life-histories

Allocation of resources to growth and reproduction is one of the main characteristics differentiating life-histories (Stearns, 1992). Reproduction is energetically expensive, therefore many organisms exhibit trade-offs between reproduction and other energetically expensive processes such as survival and growth, because energy intake is limited by the environment and must be prioritized. Competition for resources within an animal occurs between the energetic costs of metabolism required for maintenance and survival, the cost of somatic growth, and the cost of reproduction (Stearns, 1992), and each of these processes is important in the life-history of an animal. For example growth is important because many traits, such as mortality and fecundity, are size dependant (Ramirez Llodra, 2002). Therefore in energy poor environments trade-offs occur between growth, reproduction and survival. Trade-offs between growth and reproduction usually involve differences in size at maturity, with some animals initially allocating energy to growth and later to reproduction, while other animals allocate energy to reproduction early in life and continue to grow after reaching maturity (Ramirez Llodra, 2002). Trade-offs between survival and reproduction result in different frequencies of reproduction; many small batches of eggs results in reduced fecundity with each reproductive event but increased survival between events, alternatively all available energy can be allocated to reproduction at the expense of survival (Charnov and Schaffer, 1973; Murphy, 1968). Within the energy allocated to reproduction trade-offs can also occur. For example an animal may increase the number of eggs produced and produce smaller eggs, or alternatively, the number of eggs may be

reduced to increase the size and energy allocated to each egg (Ramirez Llodra, 2002).

1.2 Energy storage

Some animals store energy during seasons of high food availability, to increase the energy available for use at a later date. Many bivalves store energy in somatic tissue, which is subsequently used as maturation of gametes occurs, for example, scallops (Barber and Blake, 1985; Brokordt and Guderley, 2004), clams (Darriba *et al.*, 2005; Drummond *et al.*, 2006), and oysters (Rodriguez-Astudillo *et al.*, 2005; Vite-Garcia and Saucedo, 2008) all exhibit seasonal changes in biochemical composition of the soma, associated with reproduction. This is known as capital reproduction, as energy for reproduction is derived from capital stores of energy; the alternative strategy is to use energy directly from food, which is known as income reproduction (Jonsson, 1997). Capital reproduction is favoured when the availability of food is seasonal but does not coincide with the timing of gamete maturation, while income reproduction is favoured when the availability of food is ample during periods of gamete maturation (Jonsson, 1997). The advantage of capital reproduction is that the energy for maturation of gametes is assured prior to the onset of gametogenesis, and in some animals reproduction will not start until a threshold energy store is reached (Bonnet *et al.*, 1998). There are also some disadvantages to capital reproduction, particularly in endothermic animals where mobility, and therefore foraging, may be reduced by stores of energy in the body (Bonnet *et al.*, 1998). The disadvantages of income reproduction are that unpredictable events may result in the inability to complete gamete maturation due to reduced availability of

resources (Bonnet *et al.*, 1998). Many animals exhibit a mix of income and capital reproductive strategies (Stearns, 1992), and bivalves can exhibit seasonal shifts in the reliance on capital reserves (Vite-Garcia and Saucedo, 2008). This has implications for the reproductive cycle of these animals, as the amount of stored reserves may strongly influence the extent or success of capital reproduction, as would fluctuations in food during income reproduction. Therefore, understanding changes in the energy source for reproduction may shed light on unexpected differences in reproductive cycles and aseasonal reproduction.

1.3 Reproduction

In bivalve molluscs, meiosis starts in oogonia and progresses to prophase I as oogonia become previtellogenic oocytes (Dohmen, 1983). Previtellogenic oocytes accumulate reserves within the cytoplasm becoming vitellogenic oocytes (Dohmen, 1983), which increase in size as vitellogenesis progresses (Pipe, 1987). The reserves accumulated within the oocyte are required to fuel the process of embryogenesis, and lipids are depleted within the oocyte as embryogenesis progresses (Gabbott, 1976), therefore vitellogenesis is the most energetically expensive step in the maturation of oocytes (Ramirez Llodra, 2002). During the final maturation stage prophase I vitellogenic oocytes reinitiate nuclear development and progress to metaphase I (mature) oocytes (Dohmen, 1983). After these gametes are released (spawning) connective tissue is rebuilt in the gonad, and/or maturation of new oocytes commences. Oocytes can become atretic if oocyte maturation is abandoned, and oocytes are broken down and reabsorbed (Le Pennec *et al.*, 1991). These processes

are regulated by endogenous triggers, which are influenced by changes in environmental conditions (Seed, 1976).

1.4 The effect of the environment

1.4.1 Effect of food

The influence of the environment on reproduction in marine invertebrates and bivalves has been reviewed extensively (Giese, 1959; Giese and Pearse, 1974; Mackie, 1984; Sastry, 1979; Seed, 1976). The availability of food, or food quantity, is variable throughout the year for temperate filter feeding organisms. The abundance of phytoplankton usually peaks during spring and has a second peak again in autumn (Waite and Suthers, 2007). Particulate organic matter is also important as a food source for bivalves (Hawkins *et al.*, 2002) and organic matter (non-phytoplankton) also increases during spring and again in autumn (Cheshuk, 2001). Filtration rates and/or absorption efficiency are influenced by the concentration of food in the water column (Cranford, 1995; Widdows, 1978), and the physiological status of the animal may also influence ingestion and assimilation efficiency (Cranford and Hill, 1999). Over stimulation of filtration results in the production of pseudo-faeces, which is energetically expensive, and is due to mussels reaching a maximum ingestion rate (Bayne *et al.*, 1976).

The food quality available to an organism may also vary seasonally, as mentioned above, with the organic content of seston changing seasonally. Also, differences in food quality have been found to effect growth and reproduction, for example diets supplemented with essential lipids and/or carbohydrates have

influenced reproduction in bivalve adults and subsequent growth and survival in larvae (Martinez *et al.*, 2000; Soudant *et al.*, 1996a; Soudant *et al.*, 1996b). Many animals exhibit behavioural responses to variability in food quality, and in bivalve molluscs filtration rate decreases with increased food quality (Cranford and Hill, 1999; Utting and Millican, 1998).

1.4.2 Effect of temperature

Temperature is one of the major environmental factors influencing reproduction (Giese, 1959), and as temperature fluctuates seasonally in almost every marine environment, animals are exposed to a range of temperatures throughout an annual cycle. At a molecular level temperature influences the rates of reactions, and the stability and activity of molecules (Somero, 2002). Consequently, cellular function and organ function are also affected, and as a result, all biological processes and functions are influenced by temperature (Somero, 2002). Ectotherms, and in particular sessile marine invertebrates, are heavily influenced by temperature. As these animals have no physiological or behavioural mechanisms for regulating body temperature, changes in the environmental temperature affect their own body temperature (Bayne *et al.*, 1976). Many ectothermic animals are able to compensate for changes in temperature by adjusting feeding and respiration rates so that they are independent of environmental temperature, allowing them to maintain energy intake at various temperatures (Bayne *et al.*, 1976; Griffiths and Griffiths, 1987). However, this acclimation can only occur within the optimal temperature range, and beyond this range a breakdown in compensating mechanisms occurs (Widdows, 1973). Gamete maturation also occurs within a temperature range, and threshold

temperatures for gamete maturation are observed in some species, for example in scallops, *Aequipecten irradians*, maturation of oocytes does not occur below 15°C (Sastry, 1968). Orton (1920) proposed an optimum window for reproduction in seasonal environments, with a temperature threshold at which maturation of gametes commences; however, this theory does not apply in all situations, mainly because other environmental factors, such as food, also have a substantial influence on reproduction (Olive, 1995).

Responses to temperature are also seen over the geographical range of a species. Populations at lower latitudes often exhibit extended reproductive periods, or earlier in the year, compared to populations of the same species at higher latitudes (Seed, 1976), and it is implied that these differences are due to differences in temperature ranges across these latitudes. However, compensation, by faster rates of maturation of gametes and growth, is seen in animals adapted to cooler regions compared to warmer adapted populations, due to the shorter growing and/or reproductive seasons at higher latitudes; so that total growth and reproductive effort are equivalent amongst thermal regimes (Kokita, 2003; Yamahira and Conover, 2002). Latitudinal variation in the timing and rate of maturation is not always attributed to temperature however, and in mussels on the east coast of the US these differences were attributed to differences in the availability of food (Newell *et al.*, 1982).

1.5 Study species

The Australian mussel has been referred to as *Mytilus edulis planulatus* in the past, however it has the same allele frequency and morphology as *Mytilus galloprovincialis* (McDonald *et al.*, 1991). Long term comparisons of *Mytilus* in New Zealand, over a large geographical range (1800km), suggest that all populations there are *M. galloprovincialis* (Gardner, 2004). Hybridisation occurs amongst *Mytilus* species such as *M. galloprovincialis*, *M. edulis*, and *M. trossulus* (Shields *et al.*, 2008), and morphological differentiation between species is difficult in some sympatric populations; therefore *Mytilus galloprovincialis* is thought to be a subspecies by some authors (Gardner, 1992), while other authors consider it a separate species (McDonald *et al.*, 1991), depending on the species definition used. It is assumed in this body of work that previous studies on populations of *Mytilus edulis planulatus* in Australia are all the same species, which will be referred to here as *Mytilus galloprovincialis*. *Mytilus galloprovincialis* is a Mollusc of the order Bivalvia, common name Mediterranean mussel. Bivalve molluscs are ectothermic filter feeders, that extract phytoplankton and detritus from surrounding water using their gills (Bayne *et al.*, 1976). Mussels are gregarious, using a byssal thread to attach to the substrate, and are sessile for the remaining life-history (Yonge, 1976). They are global in distribution, from frozen Arctic conditions to Mediterranean climates, and they broadcast spawn to produce planktotrophic larvae (Seed, 1976). Reproductive maturity is reached at a small size and early age (<10mm), and growth continues once reproductive maturity is attained (Salkeld, 1995).

Reproduction in mussels occurs seasonally, with maturation usually occurring during autumn and winter, and spawning in early spring for most populations (Table 1.1). After spawning, mussels accumulate glycogen in the mantle tissue, in adipogranular cells and vesicular connective tissue (glycogen storage tissue), which occurs during spring and summer (Gabbott and Peek, 1991), when algal concentrations in the water column are greatest. Aseasonal reproduction is also observed in some mussel populations; some populations mature oocytes again through spring and summer, other populations do not mature oocytes again until the next autumn, and some populations mature oocytes and spawn throughout the year (Table 1.1). These differences are often attributed to differences in the environment that each population inhabits (Seed, 1976).

Table 1.1. Seasonal timing and frequency of gamete maturation and spawning in *Mytilus* species.

Species	Location	Reproductive maturation	Spawning	Reference
Mytilus galloprovincialis	Vigo, Spain	Summer - autumn	Winter-spring, late autumn	1.
	Vigo, Spain	Late autumn-early winter	Late winter / early spring through to summer	2.
	Vigo, Spain & Arousa & Muros Bays	Autumn - early winter, again in spring.	Two spawnings in spring	3.
	Ares-Betanzos Bay	Maturation took until spring	One spawning in summer	
	NW Mexico	Autumn - early spring	All year around, major in winter	4.
	South Africa		Summer and winter	5.
	South Africa	Autumn-winter	Spring-summer and late summer-autumn	6.

	Japan	Autumn-winter (info on males only)		
Mytilus edulis aoteanus	New Zealand	Autumn - winter	Spring and throughout the year	7.
Mytilus edulis planulatus	Australia (various latitudes)		Late autumn through to early summer (except Tasmania – winter and spring)	8.
	Tasmania, Australia	Autumn - winter	Spring and summer	9.
	Tasmania, Australia	Autumn - winter	Spring and summer, and minor throughout the year	10.
	Western Australia	Autumn and winter	Spring	11.
	Sydney, Australia		Winter	12.
Mytilus edulis	England	Autumn to spring	Spring	13.
	England	Autumn and winter, again in summer	Spring and summer/autumn	14.
	N. America		Summer	15.
	Baltic (Sweden)	Autumn to spring	Late spring / summer	16.

Maine, USA	Spring	Summer	17.
West coast Ireland	Autumn through winter	Spring and summer	18.
Iceland	Winter	Summer to autumn	19.
California, USA		Spring and autumn	20.
Netherlands		Spring	21.
Boston / Cape Cod, USA		Spring and summer	22.

* For more population comparisons see (Seed, 1976)

1. (Suarez *et al.*, 2005) 2. (Caceres-Martinez and Figueras, 1998) 3. (Villalba, 1995) 4. (Curiel-Ramirez and Caceres-Martinez, 2004) 5. (van Erkom Schurink and Griffiths, 1991) 6. (Zardi *et al.*, 2007) 7. (Kennedy, 1977) 8. (MacIntyre *et al.*, 1977) 9. (Dix and Ferguson, 1984) 10. (Cheshuk, 2001) 11. (Wilson and Hodgkin, 1967) 12. (Wisely, 1964) 13. (Chipperfield, 1953) 14. (Lowe *et al.*, 1982) 15. (Myrand *et al.*, 2000) 16. (Kautsky, 1982) 17. (Maloy *et al.*, 2003) 18. (King *et al.*, 1989) 19. (Thorarinsdottir and Gunnarsson, 2003) 20. (Page and Ricard, 1990) 21. (Pieters *et al.*, 1980) 22. (Kimball and McElroy, 1993)

1.6 Broodstock conditioning and aquaculture

The culture of mussels has been commonplace for hundreds of years, originating in France in the 13th century (Maclean, 1972), they have become an important food source (1.6 million tonnes in 2007 globally), and are increasing in value in many countries (F.A.O, 2009). To support the culture of mussels, juvenile mussels (spat) must be collected and grown-out for harvest. However, the collection of juvenile mussels is seasonal, and often unreliable, particularly on the east coast of Tasmania. The supply of juvenile spat from a hatchery year-round would overcome this problem, and allow for expansion of the mussel aquaculture industry. Hatchery production of mussel larvae would also substantially reduce the 'fishing' pressure on juveniles in the natural population and provide a sustainable solution.

Hatchery techniques and protocols for the production of mussel larvae are currently experimental and require development. Understanding and controlling reproduction in adult mussels is the first step in controlling the production of larvae year-round. Conditioning adults for the production of mussel larvae in hatcheries is a newly emerging practice, and protocols are usually developed by individual companies and not widely available. Conditioning bivalve broodstock in a hatchery is achieved by holding adults at optimal environmental conditions that facilitate the maturation of gametes, and these optimal conditions are different for each species. Temperature (Martinez and Perez, 2003; Martinez *et al.*, 2000), diet (Pronker *et al.*, 2007; Utting and Millican, 1998), and photoperiod (Chavez-Villalba *et al.*, 2002; Fabioux *et al.*, 2005) have all been used to control the rate or success of maturation of gametes and subsequent development of larvae in hatcheries.

In this study, trials were conducted at the mussel hatchery in Triabunna, Tasmania, Australia (Figure 1.1), and mussels were obtained from the farm lease near Maria Island (Figure 1.1), a well mixed body of water (Jordan *et al.*, 1995), where the water temperature ranges from ~15 °C to 10 °C during autumn and winter, ~11 °C in early spring, and ~12 °C to 18 °C during spring and summer.

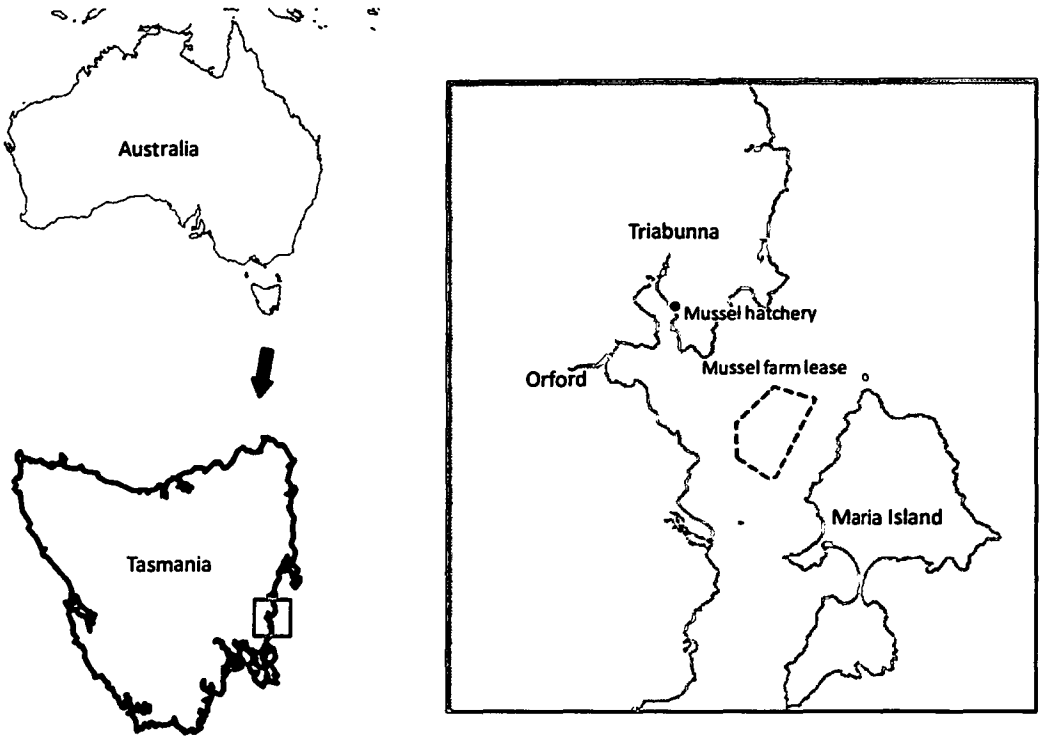


Figure 1.1 Map showing the location of the mussel hatchery and farm lease, in Tasmania, Australia.

1.7 Aims and thesis structure

The influence of the environment on the allocation of energy to reproduction and/or energy storage is not well understood. It is not clear to what extent the three

factors; temperature, food quality, and food quantity drive the amount of energy stored as glycogen (y-axis in Figure 1.1A), the rate of maturation of oocytes and the proportion of oocytes in the mantle (x-axis in Figure 1.1B), or the decisions or constraints in allocating energy to reproduction or storage (Figure 1.2C).

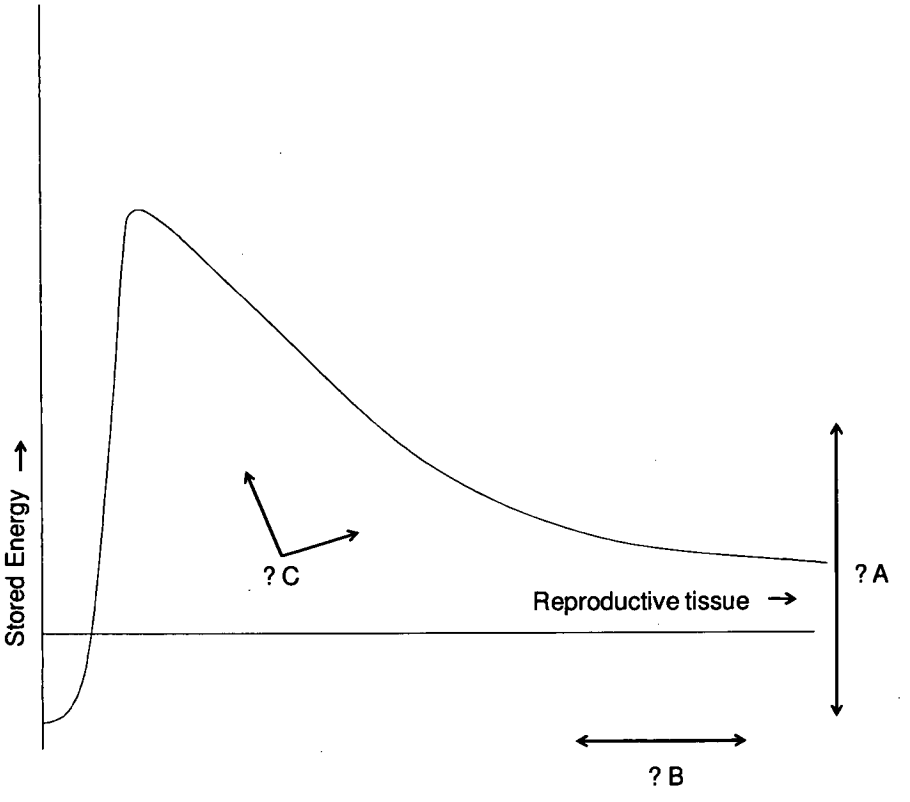


Figure 1.2 Potential relationship between energy storage and reproduction

This research focused on determining the environmental factors that drive mussels to allocate energy to reproduction or energy storage or both, and to determine whether these factors act independently or synergistically; to elucidate the factors driving seasonal and aseasonal reproduction.

Specifically, the aim of this work is to examine the influences of temperature and food on the allocation of energy to reproduction or energy storage. This aim was addressed in each of the data chapters as follows:

Chapter 2 – Oocyte maturation and energy storage in *Mytilus galloprovincialis*, in relation to seasonal environmental conditions

Seasonal changes in reproduction and energy storage of mussels in their natural environment are examined temporally and spatially in this chapter. Oocyte maturation was examined at different depths experiencing different temperatures, and over two years experiencing different temperatures and food availability. The changes in the timing and rate of oocyte maturation and energy storage were related to differences in temperature and food, developing hypotheses about the specific influence of temperature and food on the allocation of energy to reproduction and storage.

Chapter 3 – Energy storage and reproduction in mussels, *Mytilus galloprovincialis*: the influence of diet quality

The nutritional requirements for oocyte maturation are examined through the manipulation of diet; mussels were fed two monospecific diets, lacking in essential components or energy, and a diet combining the two. The extent of oocyte maturation and energy storage in relation to the mussels that spawned at the end of the experiment is used to interpret the allocation of energy to storage or reproduction in mussels lacking essential nutrients or energy.

Chapter 4 – Warmer temperatures reduce rates of gametogenesis in temperate mussels, *Mytilus galloprovincialis*.

The influence of temperature is examined through the manipulation of temperature, with the rate of oocyte maturation and energy storage compared amongst females held at constant temperatures. The role of temperature in regulating the rate of maturation is discussed in relation to other species and observations in natural populations.

Chapter 5 – Does changing temperature or ration drive energy storage and reproduction in mussels, *Mytilus galloprovincialis*?

This chapter presents findings from the third manipulative experiment that sought to examine potential interactions between changing temperatures, representing different seasons, and food availability. Oocyte maturation, energy storage and mussels that spawned are compared as a function of season and ration, and responses of mussels to these conditions are used to interpret factors driving the extent of capital and income reproduction during different seasons.

Chapter 2

Oocyte maturation and energy storage in *Mytilus galloprovincialis*, in relation to seasonal environmental conditions

2.1 Introduction

The environment has long been suggested as a major influence on the timing and rate of gamete maturation and the timing of spawning in marine invertebrates (Giese, 1959), and reproduction in marine mussels has been studied for many years (Chipperfield, 1953; Seed, 1976). Latitudinal differences in timing and rate of maturation exist among populations of marine invertebrates, and are often attributed to differences in local environmental conditions, such as food and temperature (Mackie, 1984; Seed, 1976). In *Mytilus edulis* populations in Europe, differences in the timing of maturation are attributed to the thermal regimes experienced by mussels at different latitudes, with more southern (warmer) populations reproducing earlier and over longer periods, and more northern (cooler) populations reproducing later and over shorter periods (Seed, 1976). In temperate environments, temperature is an external cue that synchronises reproduction (Giese and Pearse, 1974). However, populations of *Mytilus edulis* at different latitudes on the east coast of the US experience different temperature regimes, but the rate and timing of maturation is attributed to differences in food availability, rather than temperature (Newell *et al.*, 1982). Therefore environmental conditions such as temperature and food are very important in regulating the timing and rate of maturation in mussels.

Seasonal changes in reproduction in bivalves are strongly linked to changes in stored energy reserves. Energy required for maturation can be derived either directly from food, which is known as income (or opportunistic) reproduction, or from energy stores within the animal, known as capital (or conservative) reproduction (Jonsson, 1997). Mussels store energy as glycogen, which accumulates

during resting periods and is subsequently used during gametogenesis (Bayne *et al.*, 1982). These cycles of glycogen use and storage, and gametogenesis and spawning, are well documented in many bivalve species, e.g. oysters (Honkoop, 2003; Ren *et al.*, 2003), scallops (Farias *et al.*, 1997), clams (Darriba *et al.*, 2005), and mussels (Zwaan and Zandee, 1972).

The strategies of capital versus income reproduction have advantages and disadvantages for mussels. The quantity and quality of food is often seasonal in temperate environments, and the temporal availability of food may not coincide with the timing of maturation, making energy storage (capital) an advantage for subsequent maturation of oocytes. Mussels also have lower energetic requirements for basal metabolism and limited behavioural or energetic disadvantages associated with storing energy compared to mobile endothermic animals (Bonnet *et al.*, 1998). However, the amount of energy mussels can store is physically limited, as the soft tissue body components cannot increase beyond the internal volume of the shell. Income reproduction allows for the direct allocation of energy to maturation of oocytes, and may reduce the 'double handling' energy costs of storing energy and converting it to another form at a later date (Jonsson, 1997). However, changes in the availability of food during oocyte maturation may result in bivalves abandoning maturation during income reproduction (Galap *et al.*, 1997). Bivalves use both capital and income reproductive strategies to various extents during different seasons, for example pearl oysters use income reproduction during summer and capital reproduction during winter (Vite-Garcia and Saucedo, 2008).

Condition index and reproductive stages are often used to describe seasonal maturation in marine invertebrates (Giese and Pearse, 1974). However somatic and reproductive tissue are included in the condition index in mussels, which both vary seasonally, and the use of condition indices can be misleading if somatic condition is also seasonal (West, 1990). Energy storage and maturation of gametes occur in the mantle tissue of mussels, where large changes in the proportion of different cell types occur during the reproductive cycle. During the resting phase before gametogenesis begins, adipogranular cells and vesicular connective tissue (glycogen storage tissue) are dominant features of the mantle (Seed, 1976). As gametes develop within follicles in the mantle tissue, glycogen storage tissue diminishes as the number and size of gametes increase. Meiosis starts in oogonia and progresses to prophase I as oogonia become previtellogenic oocytes (Dohmen, 1983). Previtellogenic oocytes accumulate reserves within the cytoplasm becoming vitellogenic oocytes (Dohmen, 1983), which increase in size as vitellogenesis progresses (Pipe, 1987). During final maturation prophase I vitellogenic oocytes reinitiate nuclear development and progress to metaphase I (mature) oocytes (Dohmen, 1983). After spawning glycogen storage tissue is rebuilt in the mantle, and/or maturation of new oocytes commences. Oocytes can become atretic if oocyte maturation is abandoned, and oocytes are broken down and reabsorbed (Le Pennec *et al.*, 1991). Exogenous triggers for the timing and rate of maturation and strategies of energy allocation are not well understood. In scallops a threshold temperature exists for vitellogenesis to begin (Sastry, 1968), however in mussels maturation of oocytes occurs at 0°C in Arctic areas with sufficient food (Kautsky, 1982).

The aim of this study was to determine the influence of environmental factors, temperature and food, on the timing and rate of oocyte maturation and the use of capital and income energy sources. Spatial sampling was designed to determine the influence of the cooler (deep) site and the warmer (surface) site, during the same seasonal period of autumn oocyte maturation and summer glycogen accumulation, on the timing and rate of oocyte maturation and the use of capital and income energy sources. Temporal sampling was designed to determine the influence of predictable seasonal cycles of temperature and food, and annual variation in these cycles, on the timing and rate of oocyte maturation and the use of capital and income energy sources.

2.2 Materials and Methods

2.2.1 Population sampling

A sample of 20 mussels was collected monthly from January 2006 to May 2008 from surface and 15m deep mussel grow-out lines, in Mercury Passage (42.54°S, 148°E) on the east coast of Tasmania. Morphological measurements were taken from each mussel, including shell length, total weight, submerged total weight, wet meat weight, submerged wet meat weight, shell weight, and submerged shell weight; and the sex of the mussel recorded. Shell volume (mL) was estimated to be as half of the shell length * shell width * shell depth. Condition index was calculated as wet meat weight divided by shell volume, and the density of the meat was calculated as the wet meat weight divided by the difference between submerged meat weight and wet meat weight. From each monthly sample, 3-10 female mussels were

fixed in FAACC (formalin, acetic acid, calcium chloride) for histological processing and 2-10 females were stored at -80 °C for estimation of glycogen concentration.

Estimates of relative phytoplankton concentrations (cells.ml^{-1}) (as a proxy for food availability) were derived from monthly phytoplankton count data collected by the Tasmanian Shellfish Quality Assurance Program – Department of Health and Human Services (TASQAP – DHHS). Integrated water samples were collected from a 0-15m depth range from a site in Spring Bay adjoining the Mercury passage farm site. Estimates of total and species abundance of centric diatoms, pennate diatoms, and dinoflagellates was carried out using standard Utermohl settling techniques (Hasle, 1978). Water temperature was recorded daily on the surface throughout 2006 to 2007, and only during 2007 at 15 m below the surface due to loss of the data-logger. Satellite SST data was sourced from the Integrated Marine Observing System (IMOS).

2.2.2 Determination of glycogen concentration

Frozen mussel samples were freeze-dried and weighed to determine the water content, ground using a mortar and pestle and homogenised with 8 mL deionised water and 10 mL 0.6 M perchloric acid. Glycogen was enzymatically broken down into glucose units according to the method of Keppler and Decker (1983). Glucose concentration (mM) was determined using an ANALOX GM7 Micro-Stat Analyser according to the manufacturers' instructions and protocols. Stored glycogen concentration was determined by subtracting the initial glucose concentration from the concentration produced by the breakdown of glycogen.

Oyster glycogen (Sigma) was used as a control standard for the Keppler and Decker (1983) method. The standard curve was linear (R^2=0.99) to 150 mg of oyster glycogen, and the glycogen (mg) in the sample was equal to 3.956*mM of glucose. The standard curve was used to convert the glucose concentration (mM) to the concentration of glycogen (mg), within the mussel tissue sample (g).

2.2.3 Determination of cellular mantle composition and oocyte size

A piece of fixed tissue from the midsection of each mussel was embedded in paraffin, sectioned to 5 μm, and sections stained with Haemotoxylin and Eosin and mounted with DPX. Eight histology images from each female were haphazardly selected by moving the field of view from left to right. Images were captured at 10 x magnification using a Leica DC300F camera mounted on an Olympus BH2 microscope. To determine the fraction of each cell type in the mantle tissue, a grid was overlaid on the histology image and used for point counts (Weibel, 1979). A 3cm grid was used (~60 μm at 10 x magnification) for vitellogenic (including pedunculated vitellogenic, and 'free' mature oocytes as their attachment to the follicle wall was difficult to determine using two-dimensional images), atretic and adipogranular cells (glycogen storage tissue); this grid size was determined to be appropriate based on the average size of these cells. For the smaller oogonia and previtellogenic oocytes a 1 cm (~20 μm at 10 x magnification) grid was used. Based on stereological principles that point counts represent volume density (Weibel, 1979), the proportion of point counts for each cell type was used to represent the volume fraction of each cell type in the mantle tissue, an approach verified for

mussels (Lowe *et al.*, 1982), with points that overlayed empty space in the mantle tissue on the histology image used as an indication of spawning.

Additional 3μm sections were obtained from mussels where large oocytes, suspected to be in germinal vesicle breakdown, were present. To confirm the stage of nuclear development, tissue sections were blocked with horse serum to minimise non-specific binding, and stained with a 0.5 μg/mL Hoechst (Sigma) fluorescent solution specifically targeting DNA (Cuomo *et al.*, 2005). Images were captured using UV illumination. Both targeted DNA fluorescent staining, and Haemotoxylin and Eosin staining, were used to confirm that large oocytes with no nucleus were oocytes transitioning from prophase I to metaphase I through the process of germinal vesicle breakdown (Figure 2.1 A), and showed the condensed chromosomes and meiotic asters associated with the final stages of oocyte maturation (Figure 2.1 B and C).

To assess the changes in the oocyte size frequency distribution in females during each year and at each depth, four females from each month were randomly selected and the diameter of 100 randomly selected oocytes, from all stages of development, in each female were measured. Measurements were made across the shortest diameter of the oocyte, to avoid measuring the stalk of pedunculated oocytes, and only oocytes containing a nucleus were measured.

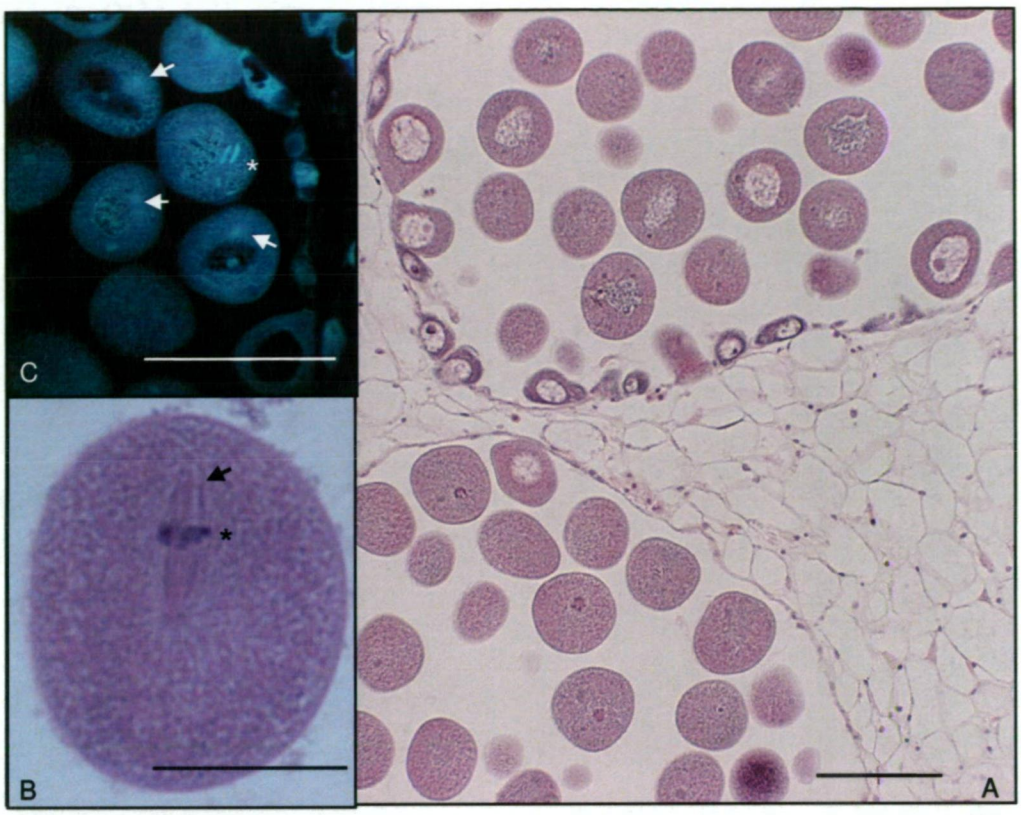


Figure 2.1. Histological section of the mantle tissue A) stained with Haemotoxylin and Eosin showing various oocytes in germinal vesicle breakdown - scale bar 100 µm, and B) an oocyte in germinal vesicle breakdown with chromosomes aligned between the meiotic asters, scale bar 25 µm. C) Hoescht DNA targeted fluorescent staining confirms the presence on condensed chromosomes, scale bar 100 µm. Arrows indicate asters associated with meiosis, and asterisk indicates condensed chromosomes.

2.2.4 Data analysis

A range of biological characteristics of female mussels were examined as predictors of reproductive maturation and glycogen concentration using multiple regression (all subsets) analyses. A χ^2 test of independence was used to compare the size frequency distribution of oocyte diameters among months during 2006 and 2007, and between the surface and deep sites. To determine if variation in the

concentration of glycogen among females and water temperature was a function of year, month, and depth, factorial ANOVAs were used (year and month as orthogonal factors of interest, or depth and month as orthogonal factors of interest), with Tukeys post-hoc tests. As only one assessment of phytoplankton concentration was made per month, the concentration of phytoplankton was compared among seasons and years, with months used a replicates for each season. To determine if variation in the concentration of phytoplankton was different for the same season between the two years planned contrasts were used. Differences in the composition of the cell types in the mantle between years and depths, among months, was examined using average fractional volume of the different cell types in a MANOVA (year and month as orthogonal factors of interest, or depth and month as orthogonal factors of interest), followed by a canonical discriminant analysis to determine which levels in the significant factors were differing, and the cell types driving the differences. For the cell types that were driving differences among the groups a Tukeys post-hoc test was used to determine where the differences were in average cell fractional volume for the significant factors. For all data used in analyses of variance, residual plots were used to check for equal variances, and data was square root transformed when the assumption of equal variances was violated.

2.3 Results

2.3.1 Condition index and meat density

This population of mussels was predominantly dioeceous, with a 0.3% occurrence of hermaphrodites containing mature oocytes and spermatozoids, and a

population sex ratio of 1:1. Increases in the proportion of vitellogenic oocytes ($F=30.291$, df 2,151, $p<0.001$) and glycogen storage tissue ($F=10.158$, df 2, 151, $p<0.001$) resulted in significant increases in condition index, and spawning (measured as increased empty space in the mantle tissue) resulted in significant decreases in condition index ($F=7.909$, df 2, 151, $p=0.001$). However, whole body condition index was not a good predictor of mantle tissue characteristics, as it only explained 28% of the variability in volume fraction of vitellogenic oocytes, 11% of the variability in volume fraction of glycogen storage tissue, and 30% of the variability in volume fraction of space in the mantle tissue. Meat (soft tissue) density did not explain any of the variability in the cellular characteristics of the mantle tissue. Glycogen concentration increased linearly as meat density increased ($F=4.227$, df 2,172, $p=0.016$), however as meat density only explained 22% of the variability in glycogen concentration, it could not be used to predict glycogen concentration.

2.3.2 Temporal variability at 15m

Average glycogen concentration changed among months, however the monthly pattern differed between the two years ($F_{Month*Year}=14.28$, df 11,167, $p<0.001$). Glycogen concentration was relatively constant during most of 2006, averaging approximately 64 mg/g up to September (Figure 2.2), followed by a three-fold increase in glycogen concentration during the 2006 austral spring and summer (October 2006 to February 2007). In spring 2007 glycogen concentration also increased approximately three-fold, but was delayed by a month compared with 2006 (Figure 2.2). The glycogen concentration of females sampled in January 2007 was

80% greater than for females sampled in January 2006 (Figure 2.2), and the glycogen concentrations remained at greater levels for longer during the 2007 summer and early autumn compared with 2006 (Figure 2.2).

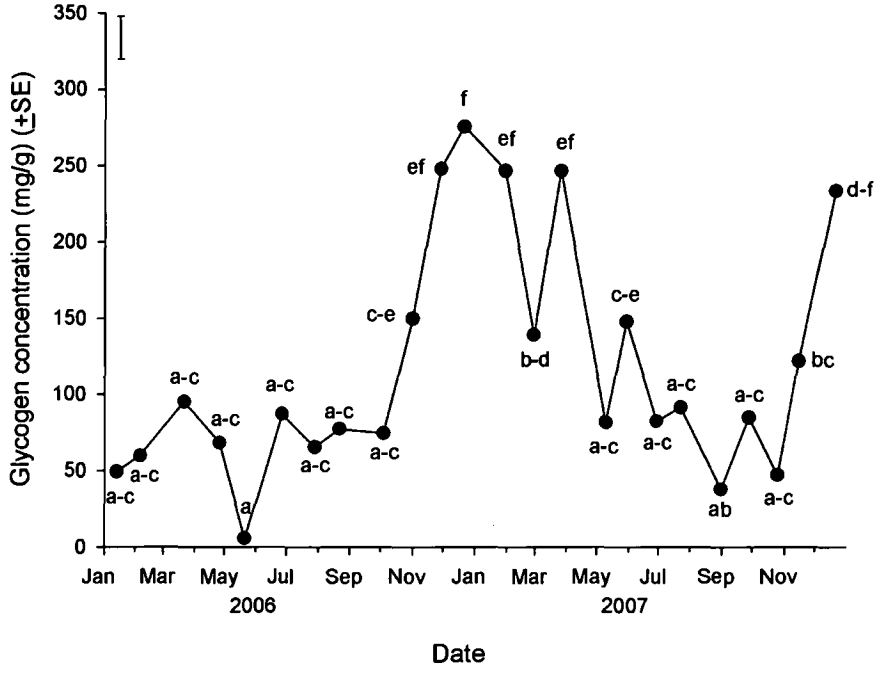


Figure 2.2. Mean monthly glycogen concentration in mussels collected from the deep site during 2006 and 2007. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different. An average standard error across all means is provided in the top left corner.

The composition of the different cell types in the mantle tissue varied monthly, but the monthly pattern of change differed among the two years ($F_{Month*Year}$=2.900, df 55, 925, $p<0.001$). About 50% of the variation in the composition of cell types in the mantle was due to differences in the volume fraction of glycogen storage tissue and vitellogenic oocytes present (Figure 2.3). During 2006, glycogen storage tissue reached a maximum of 44% of the mantle tissue in November, and was variable throughout the rest of the year (Figure 2.4A).

Vitellogenic oocyte fractional volume reached a maximum of 45% of the mantle in March 2006, and became more variable among individuals before dropping to an average minimum of 13% of the mantle in August and October, which coincided with maximum space (57%) in the mantle tissue during August (Figure 2.4B & C). A further 23% of the variability was correlated with space in the mantle tissue (Figure 2.3). During January, April, May and November 2006, space in the mantle tissue averaged a minimum of 26% of the mantle, with the minimum space during November coinciding with maximum glycogen storage tissue (Figure 2.4C). During 2007, glycogen storage tissue increased by 40% from October to November (Figure 2.4A), while vitellogenic oocytes reached a maximum of 43% in August, and decreased by 80% from August to December (Figure 2.4B). Space in the mantle tissue averaged a minimum of 23% from January to May and July in 2007, and did not change significantly throughout the year (Figure 2.4C).

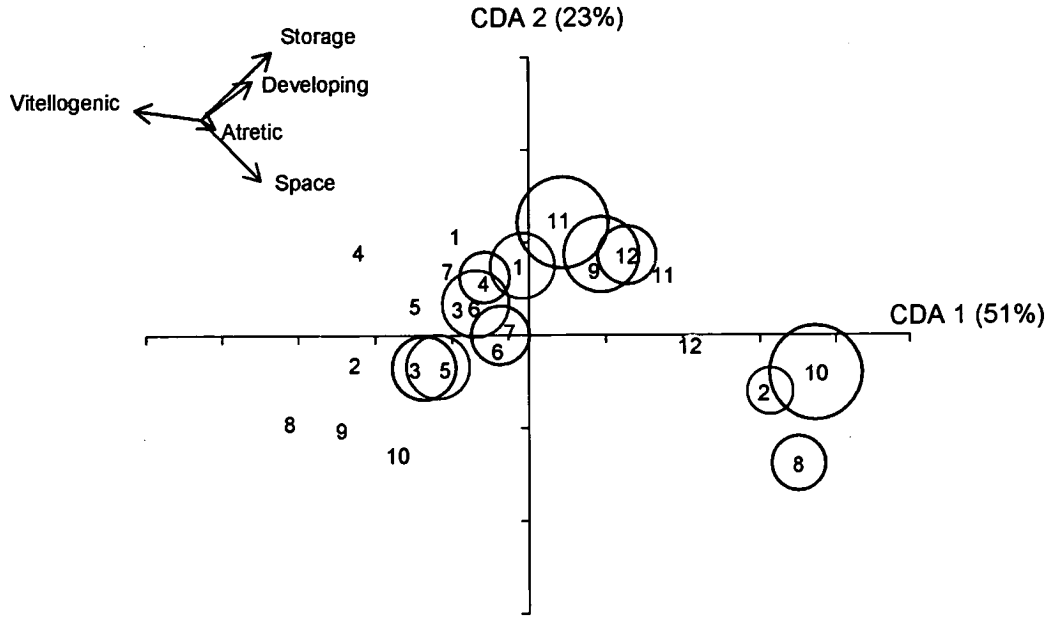


Figure 2.3. Plot of differences between 2006 and 2007 compared amongst all cell types in the mantle tissue, based on Canonical Discriminant Analysis, in multi-dimensional space. Grey circles represent months during 2006, bold circles represent months during 2007, and numbers represent month in chronological order.

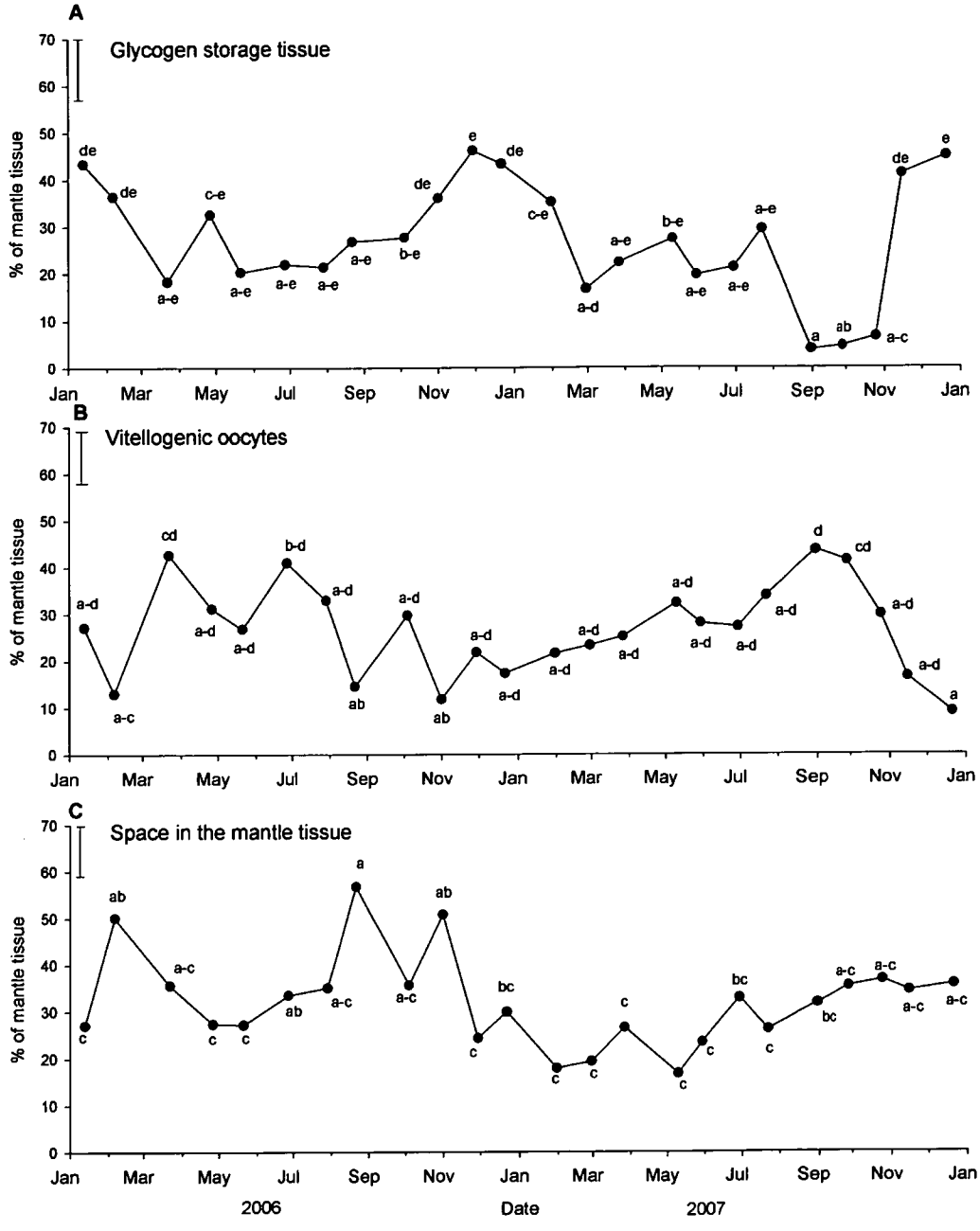


Figure 2.4. Mean monthly volume fraction of A) glycogen storage tissue, B) vitellogenic oocytes and C) space in the mantle tissue from mussels collected from the deep site during 2006 and 2007. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different. An average standard error across all means is provided in the top left corner.

Previtellogenic oocytes ranged from 3 - 29 μm, vitellogenic oocytes from 18 - 49 μm, and metaphase I oocytes (finished germinal vesicle breakdown) ranged from 38 - 63 μm. The size frequency distribution of oocytes varied monthly, but the monthly pattern of change differed during 2006 and 2007 (χ^2=1592, df 207, $p<0.001$). The frequency of large metaphase I oocytes (50-55 μm), peaked in February 2006 along with a pulse of previtellogenic oocytes (10 μm) (Figure 2.5B). Vitellogenic (30-40 μm) oocytes were most abundant during March, and these oocytes matured to metaphase I oocytes (50-55 μm) by May (Figure 2.5B). Proliferation of new oocytes was evident in the mantle of females sampled in July in 2006, with almost 100% more small oocytes (10 μm) present than expected (Figure 2.5B). Continued production and maturation of these oocytes was evident with small (15-25 μm) oocytes being abundant from August to December (Figure 2.5B). During 2007 maturation of oocytes was evident from July to October, with fewer previtellogenic (10-25 μm) oocytes, and more abundant vitellogenic oocytes (30-45 μm) than expected (Figure 2.5C). Proliferation of new oocytes occurred during November and December 2007, with previtellogenic oocytes (10-20 μm) 75% more abundant than expected (Figure 2.5 C). Metaphase I (50 μm) oocytes were more abundant than expected during December 2007 (Figure 2.5 C).

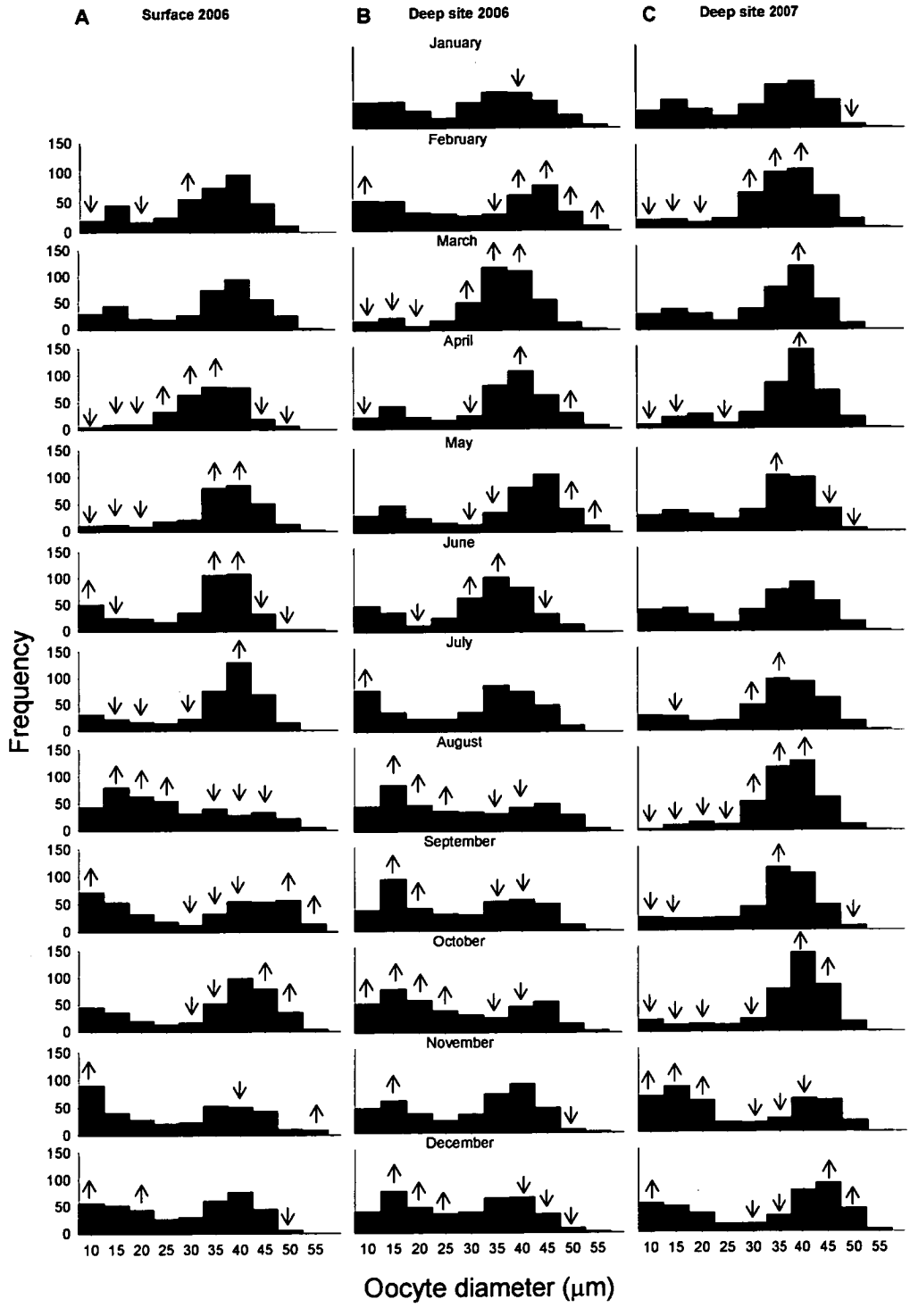


Figure 2.5. Oocyte size frequency distributions from A) surface site 2006, B) deep site 2006 and C) deep site 2007, from January through to December. Arrows indicate departure from expected frequencies based on the assumption that oocyte diameter is independent of month and site (The arrows indicate observed values greater than expected ↑, and less than expected ↓).

Surface water temperature varied among months, and the monthly pattern differed between the two years ($F_{Month*Year}$=15.502, df 11, 266, p<0.001). In 2007 water temperatures from April to June were on average 2 °C warmer than in 2006; from July to October water temperatures were on average 1 °C warmer than in 2006, and in November and December, water temperatures were an average of 2 °C warmer than in 2006 (Figure 2.6). There were no significant differences in water temperatures from January to March of 2006 and 2007 (Figure 2.6). Satellite images show a greater presence of the East Australian Current in Tasmanian waters during 2007, particularly from April to July, and September to November (Figure 2.7), compared to 2006 (IMOS, 2009).

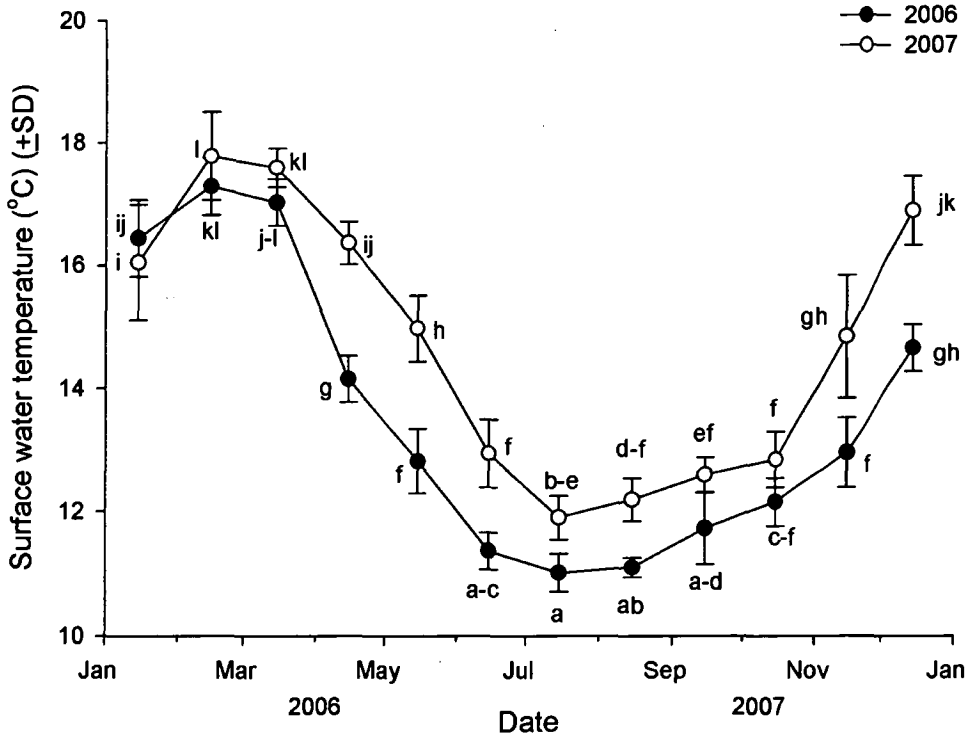


Figure 2.6. Mean monthly surface water temperature in 2006 and 2007. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different. Monthly variability is indicated by the standard deviation (SD)

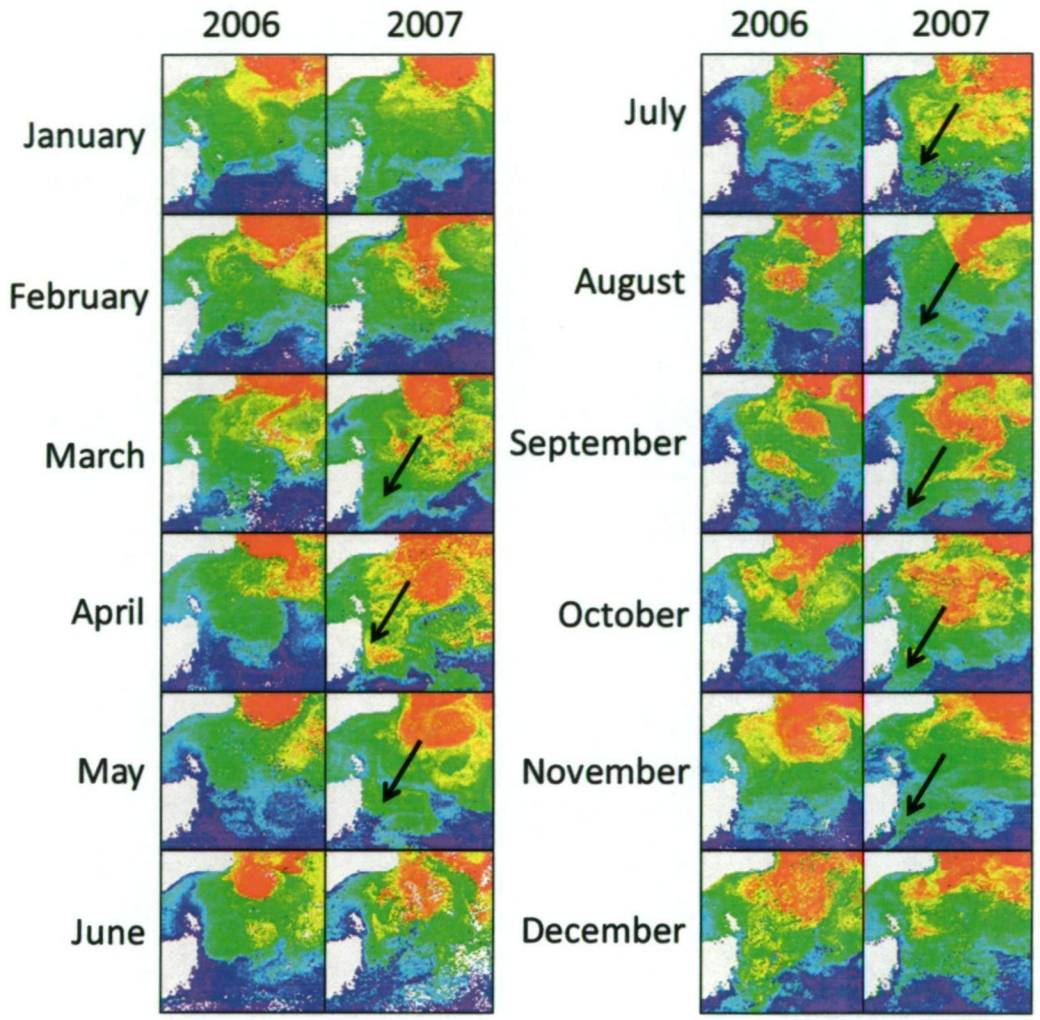


Figure 2.7. Satellite images from the National Oceanic and Atmospheric Administration AVHRR instrument series polar operational environmental satellite, spatially constructed from a 14 day sea surface temperature mosaic, in eastern Tasmanian waters during A) 2006 and B) 2007. The colour range from blue, green, yellow to red indicates increasing sea surface temperatures. Warmer sea surface temperature, associated with the East Australian Current (EAC), can be seen extending further south along the Tasmanian coast line (green, yellow, red) from March to May, and from July to November in 2007. Arrows indicate the extended warm water associated with the EAC.

Total phytoplankton concentration ($R=0.58$, $n=12$, $p=0.049$) and glycogen concentration ($R=0.62$, $n=12$ $p=0.031$) increased as temperature increased during

2007, but not 2006. Vitellogenic oocytes (R=-0.71, n=12, p=0.010) decreased as temperature increased during 2007 but not 2006.

The concentration (cells.mL^{-1}) of phytoplankton, grouped into centric diatoms, pennate diatoms, and dinoflagellates, varied throughout the year (Figure 2.8). Phytoplankton concentration was highly variable within seasons, however planned contrasts showed that the concentration of pennate diatoms was 96% greater in winter 2006 compared to 2007 (F_{Year}=2.397, df 1,15, p=0.03) (Figure 2.8C). Also, the concentration of pennate diatoms increased as total phytoplankton increased during 2006 (R=0.75, n=12, p=0.005), but not during 2007 (R=0.53, n=12, p=0.073). A peak in phytoplankton concentration occurred in August 2006, but not other winter months in 2006, and a similar peak did not occur in any winter months of 2007. No differences between seasons or years could be detected in total phytoplankton concentration (F_{Year}=1.284, df 1,15, p=0.275, F_{Season}=0.823, df 3,15, p=0.501) due to the high variability (CV>1) in winter and spring months. Centric diatoms (F_{Year}=0.714, df 1,15, p=0.411, F_{Season}=1.065, df 3,15, p=0.393), and dinoflagellates (F_{Year}=0.276, df 1,15, p=0.607, F_{Season}=1.708, df 3,15, p=0.208) also showed no differences between years and months.

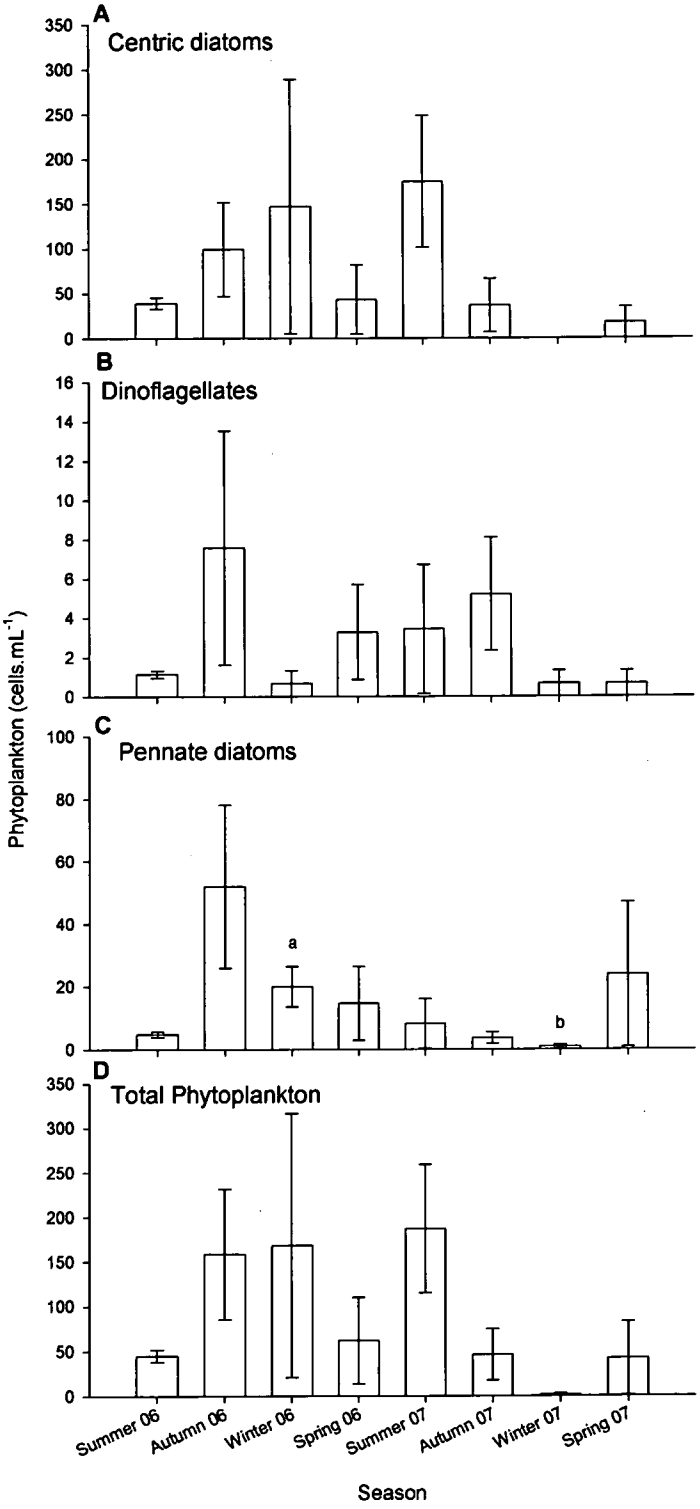


Figure 2.8. Changes in phytoplankton concentration from classes A) Centric diatoms B) Dinoflagellates C) Pennate diatoms and D) total phytoplankton in Spring Bay, sampled from January 2006 to December 2007.

2.3.3 Surface vs deep sites

Average glycogen concentration changed among the months, and the monthly pattern differed between females collected from surface and 15 m ($F_{Month*Depth}$=6.421, df 10, 145, $p<0.001$). Females deeper in the water column increased their glycogen concentrations by 70% during the austral spring (October to December), while at the surface the concentration of glycogen in females increased more gradually during spring followed by a 32% increase from November to December (Figure 2.9).

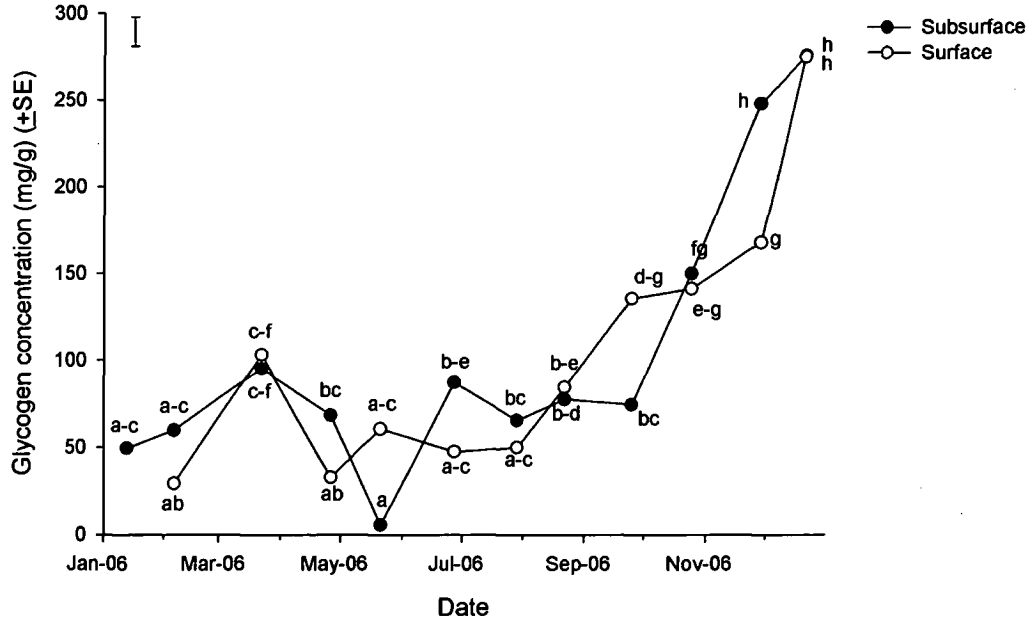


Figure 2.9. Mean monthly glycogen concentration in mussels collected from surface and deep sites during 2006. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different. An average standard error across all means is provided in the top left corner.

The composition of cell types in the mantle tissue changed among months and the pattern of change varied with depth ($F_{Month*Depth}$=2.721, df 50, 610, $p<0.001$). Approximately 51% of the variation in cell types was due to variation in the volume fraction of developing oocytes and vitellogenic oocytes (Figure 2.10). On the surface, vitellogenic oocytes decreased rapidly by 75% from July to August (Figure 2.11A), which coincided with an 80% increase in the volume fraction of developing oocytes (Figure 2.11B). In mussels from the deep site, vitellogenic oocytes were variable, and fell to a minimum (12%) in October (Figure 2.11A). Developing oocytes were also variable throughout the year, and reached a peak in September (Figure 2.11B). A further 23% of the variability was correlated with space in the mantle tissue (Figure 2.10). Space in the mantle tissue did not change significantly on the surface, however at the deep site space in the mantle tissue peaked at 57% during August (Figure 2.11C).

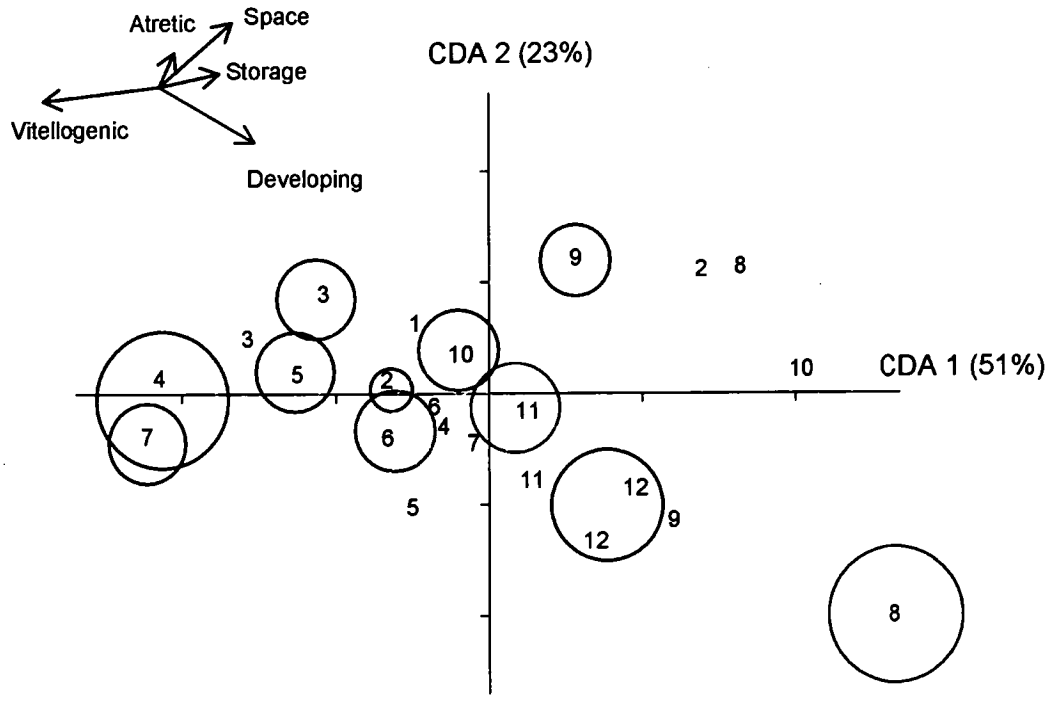


Figure 2.10. Plot of differences between surface and deep sites compared amongst all cell types in the mantle tissue, based on Canonical Discriminant Analysis, in multi-dimensional space. Bold circles represent months on the surface, grey circles represent months at the deep site, and numbers represent month in chronological order.

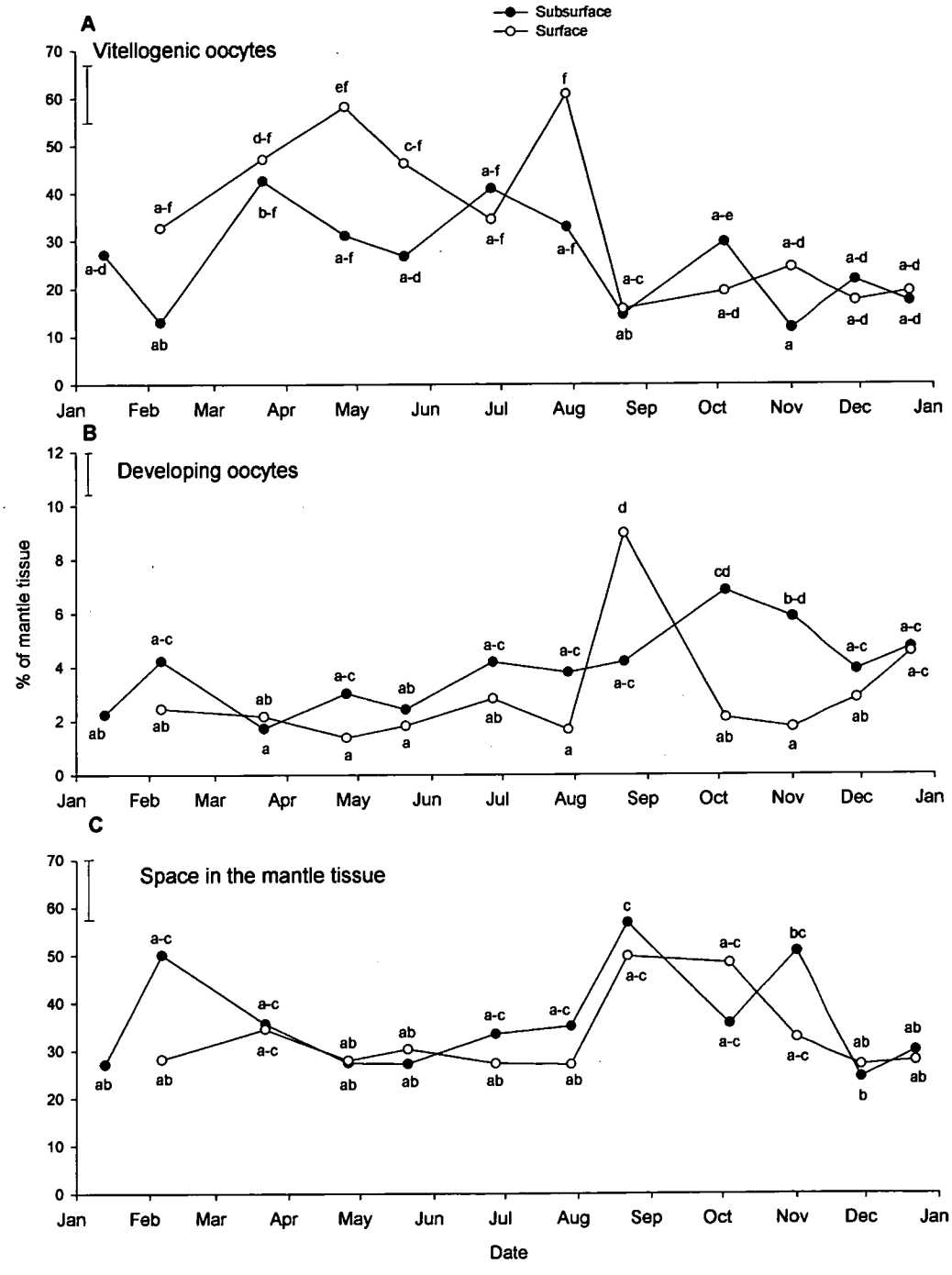


Figure 2.11. Mean monthly volume fraction of A) vitellogenic oocytes, B) developing oocytes, and C) space in the mantle tissue from mussels collected from surface and deep sites during 2006. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different. An average standard error across all means is provided in the top left corner.

The frequency of different sized oocytes changed among months, and the pattern of change varied between surface and deep sites (χ^2=1657, df 198, p<0.001). On the surface, maturation of vitellogenic oocytes (25-35 μm) occurred from April to July (Figure 2.5A). Proliferation of new oocytes (10 μm) was evident in June on the surface, and these oocytes matured to 15-25 μm oocytes by August, accompanied by a reduction in vitellogenic (35-45 μm) oocytes (Figure 2.5A). Metaphase I oocytes (45-55 μm) were most abundant from September to November on the surface along with another pulse of previtellogenic oocytes in September and November (10 μm) (Figure 2.5A). At the deep site metaphase I oocytes (50-55 μm) peaked in February along with a pulse of new oocytes (10 μm) (Figure 2.5B). During March vitellogenic oocytes (30-40 μm) were most abundant, and these oocytes matured to metaphase I oocytes (50-55 μm) by May (Figure 2.5B). Proliferation of new oocytes was evident in the mantle of females sampled in July at the deep site, with almost 100% more previtellogenic oocytes (10 μm) present than expected (Figure 2.5B). Continued production and maturation of these oocytes was evident with previtellogenic (15-25 μm) oocytes being abundant from August to December (Figure 2.5B).

Patterns in the monthly changes in water temperature were similar at the surface and deep sites ($F_{Depth*month}$=1.82, df 8, 176, p=0.077), and on average water temperature was 0.4°C cooler at the deep site than on the surface (F_{Depth}=37.75, df 1,176, p<0.001).

2.4 Discussion

Spatial and temporal differences in the timing and rate of oocyte maturation and the reliance on capital or income energy sources were strongly driven by temperature and food in this study. Warmer temperatures resulted in delayed and slower maturation of oocytes, and/or storage of glycogen. Limited food availability increased the dependence on capital energy stores and may have delayed oocyte maturation when energy stores were limiting. Condition index and meat density were inappropriate for estimating the number of oocytes and the concentration of glycogen in female mussels.

2.4.1 Condition index and meat density

This study was unable to validate condition index as a good predictor of reproductive stage or glycogen concentration in mussels, despite condition index commonly and extensively being used as an indication of reproductive stage in mussels (Cheshuk *et al.*, 2003; Dix and Ferguson, 1984; Kimball and McElroy, 1993; Suarez *et al.*, 2005). As seasonal changes in glycogen storage tissue also result in changes to the condition index in mussels, it appears that condition index is a measure of both somatic and reproductive condition and cannot be used to predict the onset or completion of spawning activity. In other studies the variability between months in glycogen storage tissue and follicle tissue, evident using stereological measurements, was not captured by the condition index (Kimball and McElroy, 1993); and condition index did not differentiate between changes in stored glycogen and reproductive changes in one and two year old mussels (Duinker *et al.*, 2008).

Predictions of gonad maturity are not always accurate using relationships between gonad size and body size, particularly when somatic condition also changes seasonally (West, 1990). Therefore, condition index appears to reflect changes in both somatic and reproductive condition, and may be inappropriate for measuring reproductive state.

Meat density was investigated as a potential indicator of the state of oocyte maturation, as increased lipid content is known to decrease density (increase buoyancy) in fishes (Phleger, 1998), and bivalve oocytes have a high lipid content (Pipe, 1987). However, no relationship between meat density and the proportion of vitellogenic oocytes in the mantle was observed. The yolk of bivalve oocytes also includes glycogen-rich vesicles and may include proteinaceous yolk (Pipe, 1987), which may also influence the density of oocytes. Glycogen concentration was positively related to meat density, however, variability in the relationship was quite large and as a result the relationship was not considered useful for predicting glycogen concentration.

2.4.2 Temporal variability in maturation of oocytes and energy storage

Energy dynamics and the timing and rate of oocyte maturation in female mussels differed between the years, which may be attributed to temporal differences in water temperature. A delay in the maturation of oocytes and greater concentrations of glycogen in 2007 coincided with warmer autumn water temperatures due to the increased influence of EAC water along the eastern Tasmanian coast during 2007. Warmer water temperatures may slow maturation of oocytes and/or increase energy

storage and influence the extent of capital and income reproductive strategies in mussels. Warmer summer water temperature in Western Australia inhibits maturation of oocytes during summer until autumn when water temperatures fall below 21 °C (Wilson and Hodgkin, 1967), while in Tasmania summer water temperatures are cooler, and maturation of oocytes during summer occurs when SST temperatures are 18-20 °C (Cheshuk, 2001; Dix and Ferguson, 1984). The delayed maturation of oocytes was unlikely to be due to limited availability of energy, as glycogen was available in the mantle tissue to be used as energy for maturation of oocytes.

Mussels showed inter-annual differences in the use of different sources of energy - capital and income - for reproduction. Females had little glycogen reserves during 2006 relative to 2007, suggesting that energy for reproduction must have been derived mainly from food (income). In contrast, substantial glycogen reserves present during January 2007 were depleted over autumn, suggesting that energy for reproduction during autumn and winter in 2007, when little food was available, was probably derived from glycogen stores (capital) rather than from food. Scallops also adopt either income or capital reproductive strategies depending on food availability (Luna-Gonzalez *et al.*, 2000). The use of capital energy sources for reproduction is considered to be a response to the temporal variability of food, while maturation of oocytes using energy from food (income) is an opportunistic response to ample food (Bonnet *et al.*, 1998). It is possible that mussel populations on Tasmania's east coast could experience years when individuals have been unable to accumulate glycogen stores combined with the influence of the nutrient-poor EAC water, which would

reduce available energy and potentially limit reproduction, resulting in poor recruitment of juveniles into the adult population or survival of adults over summer. Similar results have been observed in populations of the clam *Macoma balthica*, where warmer winters result in reduced reproduction and warmer summers result in reduced survival (Beukema *et al.*, 2009). In this case the quality of mussels on the grow-out lines may also be affected, as the mantle may contain little glycogen or oocytes, which would result in reduced meat yield from these mussels.

2.4.3 Surface vs deep sites

The timing of energy accumulation and the timing and rate of maturation of oocytes was different between surface and deep sites as seen in the generally higher proportion of vitellogenic oocytes and delayed accumulation of glycogen at the surface. This is unlikely to be related to the differences in temperature between the surface and deep sites, as the deep site was consistently cooler than the surface, yet vitellogenic oocytes were fewer. If warmer temperatures slow maturation of oocytes (see discussion of temporal differences), the deep site would be expected to produce more vitellogenic oocytes than the surface site rather than the differences observed here. The loss of vitellogenic oocytes, usually through spawning, and the increase in developing oocytes was more pronounced on the surface in August compared to the deep site and may have been influenced by temperature. Many stimuli have been recorded for spawning in mussels, including temperature change, algae, wave action and lunar periodicity (Bayne, 1965; Chipperfield, 1953; Seed, 1976), all of which are likely to be greater at the surface compared to the deep site.

Glycogen concentration increased later and more slowly in mussels on the surface than in mussels at the deep site. This may be due to the differences in temperature. Warmer temperature increases the energetic cost of metabolism in ectothermic animals (Somero, 2002), and even after acclimation of filtration and oxygen consumption rates, mussels held at warmer temperatures were in negative energy balance, due to decreased assimilation efficiency, compared to cooler temperatures (Widdows and Bayne, 1971). Therefore energetic requirements are likely to be greater, and energy availability lower, for mussels situated in the warmer surface waters, explaining slower and later energy (glycogen) storage on the surface observed in this study.

Glycogen concentration was low at both surface and deep sites for the early part of 2006, therefore energy for reproduction must have been derived mainly from food at both depths. Comparisons of phytoplankton at the surface and deep sites were not made, however, the waters on the east coast of Tasmania are well mixed to 30 m (Jordan *et al.*, 1995) therefore phytoplankton (food) concentrations would be unlikely to differ significantly from the surface and 15 m. In scallops, somatic and gonad production were greater in shallow water than deep water, where food and temperature were lower at deeper sites (MacDonald and Bayne, 1993). Also in Californian mussels, the concentration of chlorophyll-a at 9 m and 18 m was greater than at 2 m but growth was more strongly correlated to particulate organic matter than chlorophyll-a (Page and Hubbard, 1987). In some locations up to 80% of the ingested diet in scallops is particulate organic matter (non-phytoplankton) (Hawkins *et al.*, 2002; Slater, 2005). Therefore greater information on the differences in

phytoplankton and particulate organic matter, spatially and temporally, is required to determine the influence of food on reproduction and the energy allocation strategy of mussels in this population.

2.4.4 Conclusion

The differences in the rate and timing of oocyte maturation and energy storage between years and depths demonstrate substantial plasticity in these processes in mussels. The causal relationships between reproduction, energy storage and environmental variables such as temperature and food are not completely understood, however it is clear that environmental conditions influence reproduction in many bivalve species (Sastry, 1979), and that changes in reproduction and energy storage co-vary with changes in environmental conditions such as temperature and food. Further research into the specific effects of temperature and food on reproduction and energy storage in mussels would be of considerable value for predicting changes in natural populations and controlling reproduction in mussel hatcheries.

Acknowledgements

Integrated Marine Observing System (IMOS) - IMOS is supported by the Australian Government through the National Collaborative Research Infrastructure Strategy and the Super Science Initiative.

Chapter 3

Energy storage and reproduction in mussels, *Mytilus galloprovincialis*: the influence of diet quality

---

Part of this chapter has been published:

Fearman, J., Bolch, C.J.S., Moltschaniwskyj, N.A. (2009) *Journal of Shellfish Research* **28**:305 - 312

3.1 Introduction

Reproduction in bivalve molluscs usually follows an annual seasonal cycle. Gametogenesis and energy storage occur in the mantle tissue, where a large shift in cell types is evident throughout the annual cycle. During the resting phase, before gametogenesis begins adipogranular cells and vesicular connective tissue (glycogen storage tissue) are dominant features of the mantle tissue. As gametes develop within follicles in the mantle tissue, the adipogranular and vesicular tissue diminishes, as the number and size of gametes increase. *Mytilus galloprovincialis* in Tasmania undergoes gametogenesis in autumn and winter and spawning occurs in early spring, which may be repeated in late summer and early autumn (Dix and Ferguson, 1984). Associated with the seasonal change in the abundance of glycogen storage cells, are changes in stored energy reserves. Mussels store energy as glycogen, which accumulates in the mantle tissue during resting periods and is subsequently used during gametogenesis (Bayne *et al.*, 1982). These cycles of glycogen storage and gametogenesis are well documented in many bivalve species, e.g. oysters (Honkoop, 2003; Ren *et al.*, 2003), scallops (Farias *et al.*, 1997), clams (Darriba *et al.*, 2005), and mussels (Zwaan and Zandee, 1972). As stored glycogen is used as an energy source during gametogenesis, the quantity of glycogen stored during the resting period may substantially influence the reproductive capacity of an individual with respect to fecundity, oocyte quality and larval development.

Both food and temperature are important factors in regulating the timing and rate of energy storage and reproduction in mussels and other bivalve species (Alfaro *et al.*, 2001; Bayne *et al.*, 1975; Bayne *et al.*, 1982; Chavez-Villalba *et al.*, 2002;

Cranford and Hill, 1999; Darriba *et al.*, 2005; Dix and Ferguson, 1984). The direct effect of food quantity is straightforward; when food is plentiful, the energetic costs of metabolism are met, and excess energy is available for somatic growth, energy storage and reproductive maturation (Widdows and Johnson, 1988).

The effect of food quality however, is not well understood. The gross biochemical composition of the diet has an influence on the physiology of bivalves, particularly if a major component is lacking; however, specific forms of lipids, proteins or carbohydrates, such as polyunsaturated fatty acids and sterols, also influence bivalve physiology. Bivalve molluscs have a limited ability to elongate and desaturate fatty acid precursors; therefore polyunsaturated fatty acids (PUFAs), particularly DHA and EPA, are essential dietary requirements in adults and larvae (Knauer and Southgate, 1999; Utting and Millican, 1998). Lipids are accumulated in maturing oocytes during vitellogenesis and, among other things, are an endogenous energy reserve for fertilised oocytes during development and metamorphosis into larvae, prior to dietary energy sources becoming attainable via feeding (Gallager and Mann, 1986). The availability of biochemical components of food, and particularly essential nutritional requirements such as polyunsaturated fatty acids, has a demonstrable effect on reproduction in other bivalve species. In particular, lipids and fatty acids are essential for the development of mature gonads and subsequent larval growth and metamorphosis in the scallop *Pecten maximus* (Soudant *et al.*, 1996a; Soudant *et al.*, 1996b). Similarly in the small clam, *Macoma balthica*, broodstock conditioned with algae and a PUFA lipid emulsion produced more and larger oocytes at spawning (Hendriks *et al.*, 2003). Broodstock diets high in carbohydrate

supplements (Martinez *et al.*, 2000) also have beneficial effects on subsequent larval growth and development. The influence of specific biochemical components on the timing and rate of reproductive maturation and energy storage, however, is not clear.

In hatcheries reproductively mature individuals are provided with cultured microalgae as food until ready for controlled spawning. However, the quality of algae, measured through biochemical composition, varies among species and growing conditions; particularly in the proportion of fatty acids (Brown *et al.*, 1997; Ponis *et al.*, 2006). The reported biochemical compositions of the two algal species used in this study, *Chaetoceros calcitrans* and *Pavlova lutheri* differ considerably (Table 3.1). Generally, *C. calcitrans* contains very little DHA, while *P. lutheri* has greater relative concentrations of DHA (Table 3.1). The quantity of sterols reported in *C. calcitrans* and *P. lutheri* varies, however, the sterols in *P. lutheri* consist of ~40-50% pavlovals which are not readily assimilated by bivalve larvae (Soudant *et al.*, 1998). The amino acids and proteins of the two algal species do not differ substantially in quality or quantity (Brown, 1991). There are varying reports on the quantity of carbohydrates in *C. calcitrans* and *P. lutheri*; in general they have similar quantities, but the quality differs (Table 3.1). The carbohydrate component of *C. calcitrans* includes a high percentage of glucose and galactose – simple sugars that are efficiently digested by bivalves, while *P. lutheri* contains less glucose and galactose, and relatively greater quantities of other sugars such as mannose, xylose, and arabinose (Table 3.1) – thought to be less easily digested by bivalves (Epifanio, 1979).

Table 3.1. Table summarizing reported biochemical compositions of *Pavlova lutheri* and *Chaetoceros calcitrans*.

		Pavlova lutheri	*Chaetoceros calcitrans*
Proximal composition (% dry weight)			
	Protein	29 - 31.3 [a,b.]	34 - 49.3 [a,b,c.]
	Carbohydrate	9 - 13.5 [a,b.]	6 - 27.4 [a,b,c.]
	Lipid	12 - 26.6 [a,b.]	15.5 - 25.1 [1 a,b,c.]
Sterols (% total lipid)			
	Cholesterol	0.2 [b,d.]	28.2 - 46.2 [d,e,f.]
	Desmosterol	2.2-2.7 [b,d.]	3 [d, e,f.]
	Campesterol	5 - 6.4 [b,d.]	1.4 [d, e,f.]
	Stigmasterol	8.8 - 16 [b,d.]	0.8 [d, e,f.]
	4α-methylporiferosterol	16.1 - 20.5 [b,d.]	-
	β-sitosterol	29.9 - 31.6 [b,d.]	-
	24-MethyleneCholesterol	-	5.8 - 56 [d, e,f.]
	Isofucosterol	-	17.3 - 45 [d, e,f.]
	Methylpavlovol	20.7 - 21.2 [b,d.]	-
	Ethylpavlovol	3.5 - 7.2 [b,d.]	-
Fatty acids (% total lipid)			
	EPA	19.7-28.3 [d,g,h,i.]	15.4 - 26.9 [c,e,j.]
	DHA	9.4-11.5 [d, g,h,i.]	1.7 - 4.5 [c,e,j.]
Lipid Classes (% total lipid)			
	Hydrocarbons	0.2 [g,i.]	0.4 [i.]
	Triacylglycerol	4 [g,i..]	8.4 [i.]
	Free fatty acids	-	11.4 [i.]
	Sterols	5.5 - 6.3 [g,i.]	6.1 [i.]

	Polar lipids	52 - 78.3 [g,i.]	72.8 [i.]
	Other	11 [g,i.]	0.9 [i.]
Polysaccharides (% total carbohydrate)			
	Arabinose	11.7 [a.]	0.2 [a,k.]
	Fucose	3.6 [a.]	6.4 - 14.3 [a,k.]
	Galactose	12.9 [a.]	16.4- 20.5 [a,k]
	Glucose	42.6 [a.]	54.7 - 59.9 [a,k.]
	Mannose	13.2 [a.]	1.6 - 2 [a,k.]
	Rhamnose	2.1 [a.]	1.4 - 3.3 [a,k.]
	Ribose	3.5 [a.]	3.3 - 11 [a,k.]
	Xylose	10.3 [a.]	0.7 - 1.7 [a,k.]
	Inositol	-	2.7 [a,k.]

a. (Brown, 1991), b. (Wikfors *et al.*, 1992) calculated from presented data,

c. (Natrah *et al.*, 2007), d. (Ponis *et al.*, 2006), e. (Rico-Villa *et al.*, 2006),

f. (Tsitsa-Tzardis *et al.*, 1993), g. (Milke *et al.*, 2004), h. (Milke *et al.*, 2008),

i. (Volkman *et al.*, 1989), j. (Ackman, 1981), k. (Whyte, 1987).

Understanding the influence of diet quality on the timing and rate of reproduction and energy storage has major implications for understanding and managing natural mussel population dynamics, where asynchronous reproduction, spawning failure, and partial population spawning feature regularly (Seed, 1976). Hatchery techniques to control the timing and quality of female mussel broodstock are currently under-developed. Until recently the supply of juvenile mussels from natural populations has been sufficient to supply the mussel aquaculture industry. However, increasing worldwide demand for mussels combined with the limited natural spatfall in some areas has increased the need for hatchery supply of spat. To

consistently produce spat, broodstock conditioning techniques must be developed. This requires an understanding of the influence of environmental conditions on physiological processes associated with reproduction.

Few studies have examined the effect of food quality on reproduction in adult mussels and its influence on glycogen accumulation and gametogenesis is not well understood. This study examines the effects of diet composition on energy storage and reproductive indicators such as maturation stage, spawning ability, fecundity, oocyte quality and larval production in the Mediterranean mussel, *Mytilus galloprovincialis*.

3.2 Materials and Methods

3.2.1 Experimental Set-up

A total of 390 mussels were collected from subsurface mussel lines on a mussel farm in Mercury Passage, Tasmania, in October 2006. To determine the initial condition and reproductive state, 30 mussels were randomly selected and shell length, width, and depth recorded, along with total wet weight, wet shell weight, and wet meat weight. The meat of each mussel was sectioned along the dorso-ventral margin; one half was fixed for histological analysis in FAACC (formalin, glacial acetic acid, and calcium chloride) and the other half frozen and stored at -80°C for estimation of glycogen concentration.

The remaining mussels were measured (shell length and total weight) before being randomly allocated to one of the 12 50 L experimental tanks and allowed to acclimate overnight. The experimental tanks each held 30 mussels and were attached

to a raw seawater flow-through system. Ambient water temperature (increasing from 12–15 °C) was used throughout the experiment. Each tank of mussels was randomly allocated to one of four treatments; raw seawater (control), and three supplemental feeding diets, with three replicate tanks in each treatment. The control treatment was not supplied with additional microalgae and had access only to the algae in the raw seawater. The three supplemental feeding diets were equivalent dry weights of: 100% *Chaetoceros calcitrans*; 100% *Pavlova lutheri*; or a 1:1 dry weight combination of *C. calcitrans* and *P. lutheri*. Algae was grown in batch culture with Walne nutritional medium, under continuous light, at 24 + 1.5 °C, and the pH was maintained between 7.73 and 8.85 with addition of CO_2 at 1% (volume) in the supplied air. *Chaetoceros calcitrans* was grown in 17 L carboys with the addition of silicate and was harvested during the late logarithmic phase, while *Pavlova lutheri* was grown in 500 L polypropylene bags and harvested during the stationary phase. Algal densities were determined daily by standard algal cell counts and the volume of algae fed daily was based on providing ~2% dry weight (0.05 g) of algae to dry weight of mussel per day, calculated from average dry weights of mussels from field data, and published algal dry weights (Brown, 1991). Mussels were batch-fed the microalgae once daily from a 10 L container through a 4 mm valve.

Mussels were held in experimental conditions for six weeks. Mortalities were removed daily and the daily ration adjusted to account for their removal. Tanks were also monitored daily for evidence of spawning, which was the presence of remnant sperm or eggs in the water or on the bottom of the tank. At the end of the six weeks

10 mussels were randomly selected from each tank and processed in the same manner as the initial sample.

3.2.2 Spawning and larval rearing

The mussels remaining in each tank at the end of the experiment were induced to spawn to assess the number of mussels able to spawn. From the mussels that spawned, fecundity, average oocyte diameter and the hatching rate from fertilized oocytes was determined. Spawning was induced by cycling water temperatures between ~24 °C and ~18 °C hourly with 5 minutes of air exposure between cycles for 8 hours daily, and the entire spawning and larval rearing process was carried out over three consecutive days. As individuals began spawning they were removed and placed in individual containers. After spawning for one hour, mussels were relocated into new individual containers and allowed to continue spawning. Oocytes released during the first hour were fertilised using sperm from males held in the same diet treatment where possible, and from the control for the females in the *C. calcitrans* treatment as no equivalent sperm were available. Fertilised oocytes from each female were pooled into their treatment replicates and placed in 1 L larval rearing tanks at a density of 40 oocytes/mL. At 48 h post-fertilisation, duplicate 1 mL samples of D-veliger larvae were examined and counted using a Sedgewick-Rafter counting cell and a compound microscope, and the total number of D-larvae in each larval rearing tank was calculated.

A 20 mL sample of oocytes released within the first hour and a 20 mL sample of oocytes released after the first hour were taken from each individual and fixed in formalin for later analysis. Four 25 µL subsamples from each 20 mL sample of

oocytes were counted as described above and total individual fecundity determined by combining the total from the first hour sample with the total from the sample taken after the first hour.

3.2.3 Determination of glycogen concentration

Frozen mussel samples were freeze-dried and weighed to determine the water content, ground using a mortar and pestle and homogenised with 8 mL deionised water and 10 mL 0.6 M perchloric acid. Glycogen was enzymatically broken down into glucose units according to the method of Keppler and Decker (1983). Glucose concentration (mM) was determined using an ANALOX GM7 Micro-Stat Analyser according to the manufacturers' instructions and protocols. The stored glycogen concentration was determined by subtracting the initial glucose concentration from the concentration produced by the breakdown of glycogen.

3.2.4 Determination of reproductive stage

The fixed tissue was cut through the midsection of each mussel, embedded in paraffin, sectioned to 5 μm, and sections stained with Haemotoxylin and Eosin and mounted with DPX. Eight histology images from each female were haphazardly selected by moving the field of view from left to right. Images were captured at 10 x magnification using a Leica DC300F camera mounted on an Olympus BH2 microscope. To determine the stage frequency distribution of cells in the tissue, a 3 cm grid was overlaid on the histology image and used for point counts of vitellogenic, atretic and adipogranular cells, and similarly a 1 cm grid was used for previtellogenic oocytes and oogonia, according to standard stereological techniques

(Weibel, 1979). Pedunculated vitellogenic oocytes and free mature oocytes (Suarez *et al.*, 2005) were difficult to visually differentiate on histological slides as their attachment to the follicle wall was difficult to determine, therefore pedunculated vitellogenic oocytes and free mature oocytes were combined and classified as vitellogenic oocytes.

The reproductive stage of each female mussel was determined, using the relative frequency of oocyte stages and storage cell types, as an alternative to using the most frequent cell type or the volume fraction of each cell type. The most frequent cell type was storage tissue in all but two of these mussels, which provides no description of reproductive changes. Comparing the relative frequency of cell types provides a reproductive description of each female focused on the cell types whose frequencies are greater or fewer relative to other female mussels. Chi-square analysis of independence was used to determine which oocyte stage or cell type was more prevalent than expected for each female and this measure then used to assign the female into the following reproductive stages: resting, developing, vitellogenic, atretic and spent (Table 3.2).

Table 3.2. Reproductive stages according to the most frequent cell type

Reproductive Stage	Most frequent cell type
Developing	Oogonia and Previtellogenic oocytes
Vitellogenic	Vitellogenic oocytes
Atretic	Atretic oocytes
Spent	Empty follicles in mantle
Resting	Adipogranular cells

3.2.5 Data analysis

A one-way ANOVA, and planned contrasts, were used to compare the final glycogen concentration of mussels in each diet with the initial glycogen concentration, allowing the change in glycogen concentration over time to be determined. A nested ANOVA (diet as the factor of interest and tanks nested within diet), with Tukeys post-hoc test, was used to analyse the effect of diet on egg diameter and fecundity. In all ANOVAs the assumption of equal variances was examined using residual plots, and the data met the assumptions and did not require transformation. The frequency of cell types in the mantle tissue, the frequency of spawning, and the production of D-veliger larvae in each diet were analysed using χ^2 test of independence. Similarly the frequency of reproductive stages was analysed using six χ^2 tests, comparing the stage frequency distributions between the start and the end of the experiment in each diet. As the combination and *C. calcitrans* diet produced similar differences from the start of the experiment, as did the *P. lutheri* and control, the stage frequency distributions between the *C. calcitrans* and the combination diet, and between the *P. lutheri* and control diet at the end of the experiment were tested. In order to minimize the risk of Type 1 error, α values were adjusted to 0.008 using a Bonferroni adjustment to maintain a total error of 0.05 across the six tests (Quinn and Keough, 2002).

3.3 Results

Mussels showed varied responses to the four diets, both in energy storage and reproduction. The concentration of glycogen in the treatments was significantly different among diets ($F=11.34$, df 4,133, $P=0.008$). Mussels fed the *Chaetoceros*

calcitrans diet had 26% increase in mean glycogen concentration between the start of the experiment and six weeks later at the end of experiment (Figure 3.1). Mussels fed the monospecific diet of *Pavlova lutheri* and those in the control treatment, decreased in glycogen concentration from the start of the experiment by 23% and 21% respectively. The combination diet showed no significant change in glycogen from the initial concentration (Figure 3.1).

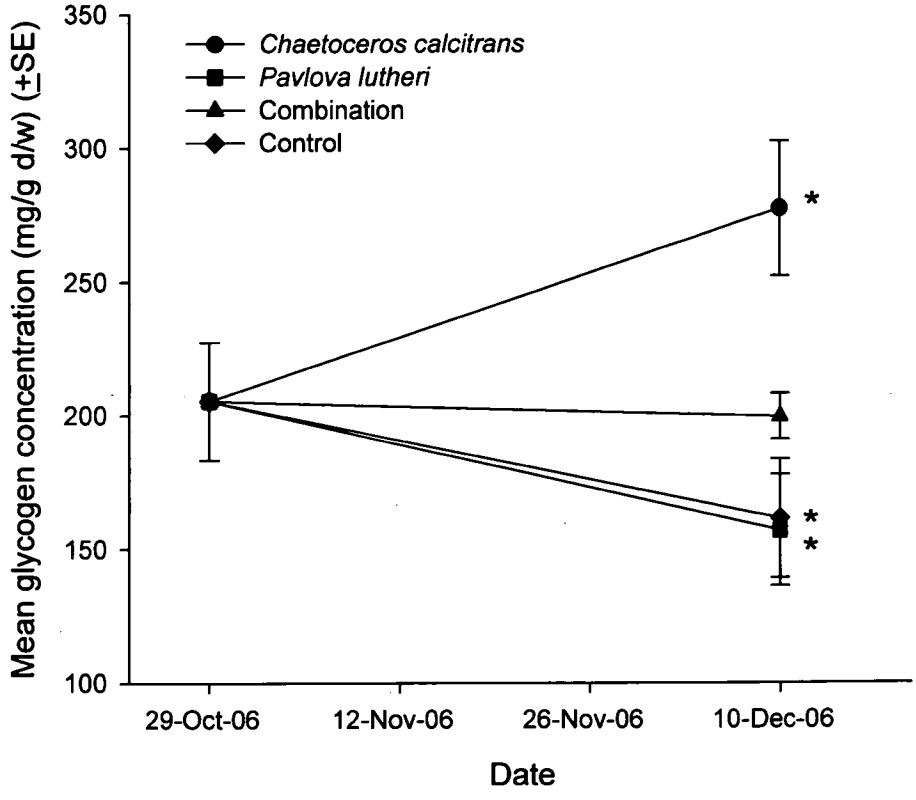


Figure 3.1. Change in mean glycogen concentration in mussels fed three different algal diets over six weeks, compared to a raw seawater control. Means indicated by an asterisk are significantly different from the initial mean glycogen concentration.

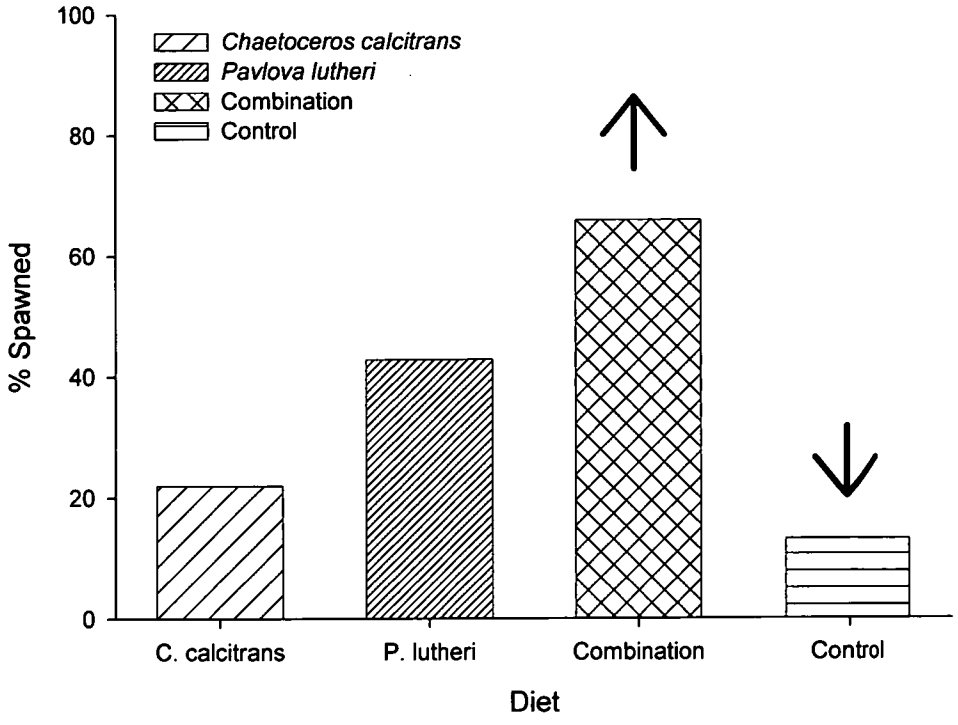


Figure 3.2. Percentage of male and female mussels that spawned when fed three different algal diets over six weeks, compared to a raw seawater control. Arrows indicate departure from expected frequencies based on the assumption that spawning is independent of diet (The arrows indicate observed values greater than expected ↑, and less than expected ↓).

The number of individuals, both male and female, that spawned was dependent on diet (χ^2=32.54, df 3, p<0.001), and no spawning was observed in the replicate tanks during the experiment. In the combination diet treatment, 13 more mussels spawned than expected; in the control diet, 10 less mussels spawned than expected. The number of mussels that spawned from the monospecific diets showed no departure from the expected χ^2 values (Figure 3.2).

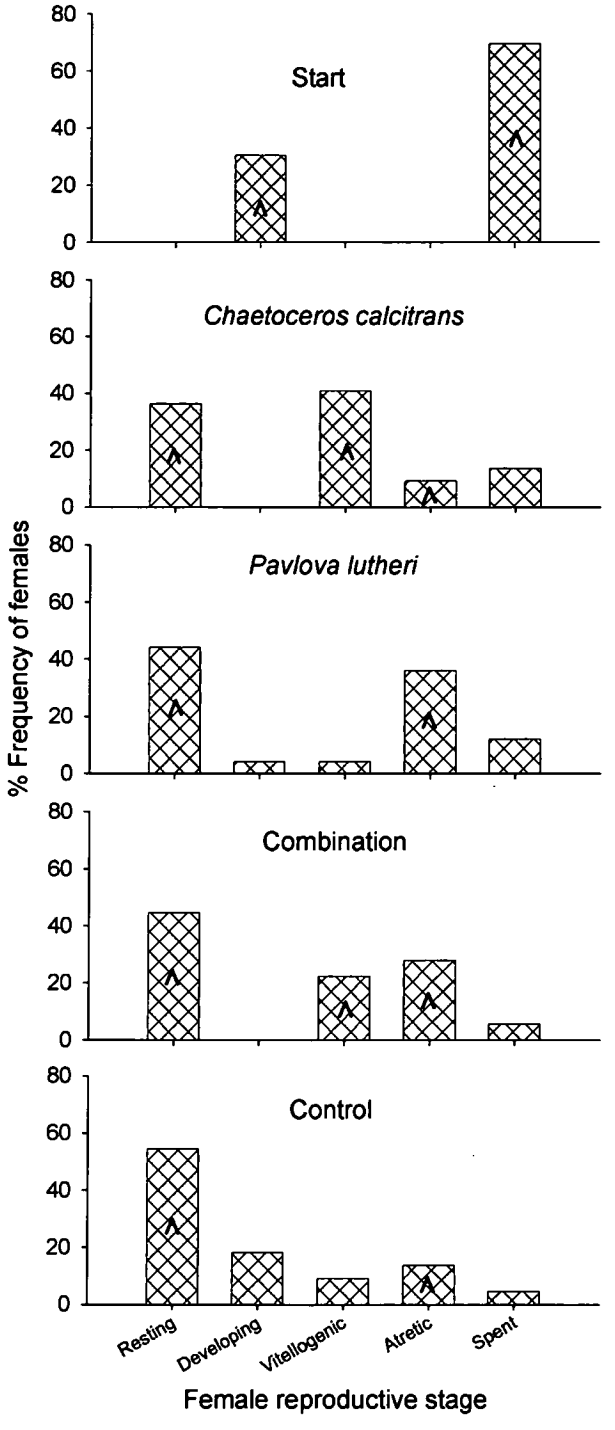


Figure 3.3. Frequency of reproductive stages of females held in four different feeding regimes over six weeks, compared to the reproductive stages at the start of the experiment. Arrows indicate a departure from expected frequencies on the assumption that reproductive stage is independent of diet (The arrows indicate observed values greater than expected ↑).

The frequency of female reproductive stages was significantly different between the start and the end of the experiment in each diet (Figure 3.3). The *C. calcitrans* and combination diets produced significantly greater proportions of vitellogenic, atretic and resting mussels (χ^2 = 135.069, df 5, p<0.001 and χ^2 = 156.295, df 5, p<0.001 respectively), while a significantly greater proportion of atretic and regressed mussels were produced by the *P. lutheri* and control diets (χ^2 = 165.115, df 4, p<0.001 and χ^2 = 198.376, df 4, p<0.001 respectively) compared to reproductive stages at the start of the experiment. Additionally the only difference between the combination diet and the *C. calcitrans* diet, was that the combination diet produced a significantly greater proportion of atretic mussels (χ^2 = 18.299, df 3, p<0.001), similarly, the *P. lutheri* diet produced a significantly greater proportion of atretic mussels than the control diet (χ^2 = 23.761, df 4, p<0.001).

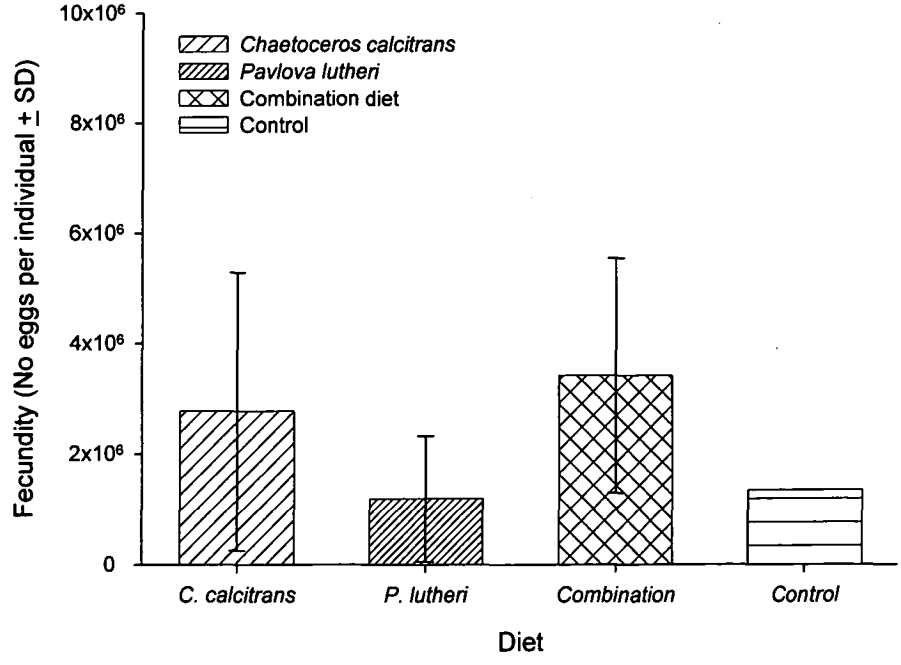


Figure 3.4. Average fecundity of female mussels (number oocytes per female ±SD) fed three different algal diets over six weeks, compared to a raw seawater control.

The fecundity of female mussels in each of the treatments was highly variable (Figure 3.4); no significant difference in fecundity among diets was detected ($F=1.79$, df 8,12, $p=0.176$). The average unfertilised oocyte diameter was 63.1 µm across all diets, and no difference was evident in oocyte diameter among diets ($F=0.53$, df 7,12, $p=0.800$). The production of D-veliger larvae from fertilised oocytes after 48 h, was 8% less than expected ($\chi^2=9.78$, df 2, $p=0.008$) from mussels fed the *P. lutheri* diet (Figure 3.5).

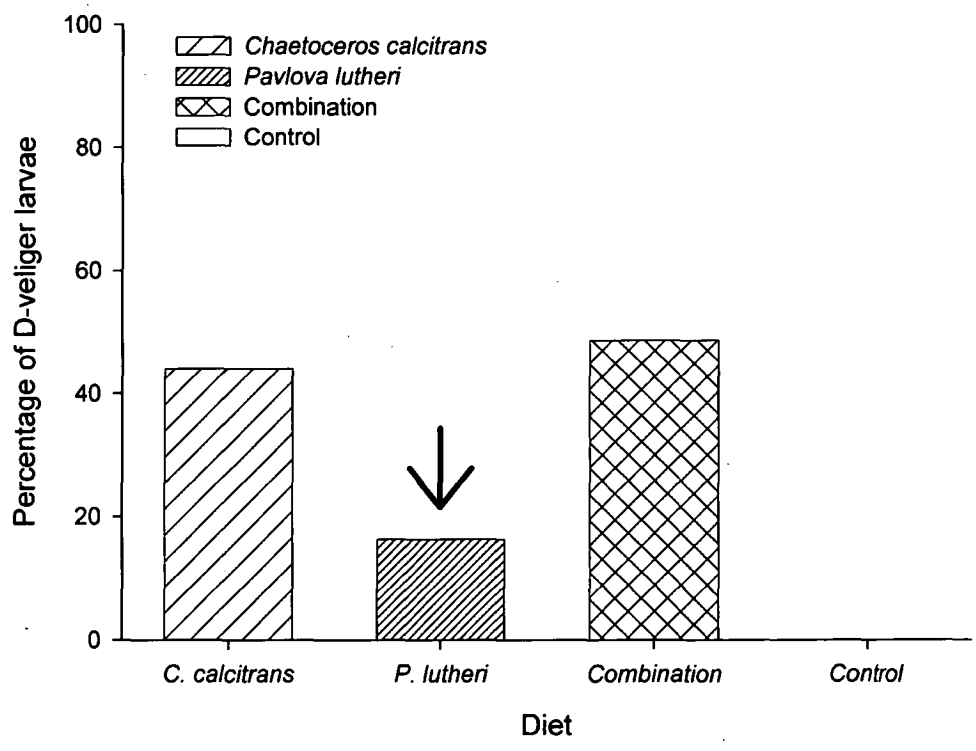


Figure 3.5. Percentage of live D-veliger larvae produced from fertilized oocytes from each treatment after 48 h. Arrows indicate departure from expected frequencies on the assumption that production of D-veliger larvae is independent of diet (The arrow indicates observed value less than expected ↓).

3.4 Discussion

The occurrence and rates of gametogenesis and glycogen storage were influenced by the biochemical composition of the broodstock diet in this study, as evidenced by the differences in reproductive effort among the single and combined supplemental diets. By manipulating the composition of the diet, using different algal species, an insight into the dietary requirements of adult mussels during gametogenesis has been provided.

The first priority for assimilated energy in mussels is the cost of metabolism, and any excess energy is then allocated to somatic growth (tissue and/or shell), stored glycogen, or reproduction; with stored glycogen accessible as an energy source for reproductive maturation (Bayne *et al.*, 1982). A diet providing a combination of both essential lipids and simple carbohydrates caused mussels to allocate energy to both reproduction, and either to storage or the increased energetic demands of reproductive maturation. At the end of this experiment the combination diet, containing both algal species, produced the greatest percentage of mussels that were able to spawn, and a significantly greater proportion of vitellogenic and atretic mussels. As samples were not taken during the experiment, it is not clear whether these mussels initially stored energy as glycogen and then used it for the energetic costs of reproduction, or whether gametogenesis was initiated at the start of the experiment and energy was used completely for the energetic costs of metabolism and reproduction. It is clear that gametogenesis requires a supply of essential lipids from the diet (DHA, EPA, sterols), along with sufficient energy resources (carbohydrates, lipids etc.).

The influence of diet on reproductive development of females was not immediately obvious, with all treatments producing greater proportions of resting females than at the start of the experiment. Considering 70% of the mussels had spawned at the start of the experiment, an increase in resting mussels, featuring adipogranular cells, is to be expected, as mussels rebuild from empty follicles to structural cells within the mantle. Additionally, when the reproductive stages of the *C. calcitrans* and combination diets at the end of the experiment were compared, the combination diet produced greater proportions of atretic mussels. Similarly, at the end of the experiment, the *P. lutheri* diet produced greater proportions of atretic mussels than the control diet. Considering the combination diet produced the greatest number of mussels that spawned, and *P. lutheri* diet produced more mussels that were able to spawn than the *C. calcitrans* diet, it appears that the presence of greater proportions of atretic mussels was associated with preparedness to spawn. The cause(s) of atresia in bivalves are unclear and may involve several factors; certainly limited nutritional requirements can cause atresia in bivalves (Galap *et al.*, 1997). Furthermore, the contents of oocytes remaining in the follicle after spawning are commonly reabsorbed through atresia (Suarez *et al.*, 2005). Alternatively, in many vertebrates programmed cell death is used to eliminate abnormal, diseased and superfluous oocytes during later stages of development (Schatten and Constantinescu, 2007). Several invertebrate species demonstrate an increase in the frequency of atretic oocytes late in vitellogenesis and at the start of spawning, (Gaudron *et al.*, 2008; Gosling, 2003; Le Pennec *et al.*, 1991). Atretic oocytes are present throughout the year in the scallop, *Pecten maximus*, and it is suggested that that oocyte lysis is linked to the reproductive strategy in this species (Pazos *et al.*,

1996). As atresia occurs in vitellogenic oocytes, the presence of atretic mussels indicates that gametogenesis has taken place previously, and may be indicative of a more advanced stage of maturation.

Given that few mussels fed the *Chaetoceros calcitrans* diet spawned at the end of the experiment, but showed a significant increase in glycogen concentration after 6 weeks, it appears that mussels in this treatment allocated excess energy preferentially to storage rather than gametogenesis. This may have been due to a diet relatively rich in sterols and simple sugars (glucose and galactose), and limited in DHA. As DHA has a structural role in the production of membranes (Marty *et al.*, 1992) the deficiency of DHA in the *C. calcitrans* diet may have impeded the generation of cell membranes required for gametogenesis. Furthermore, carbohydrates supplied in the diet increase growth rates in bivalve larvae (Enright *et al.*, 1986), and are a common respiratory substrate in adult scallops (Peirson, 1983); and it appears that they have an important role in catabolism for energy production, compared to lipids and protein (Whyte *et al.*, 1989). Glucose is the base molecule used for building glycogen, and glucose-1,6-phosphate is the molecule used to extract energy from carbohydrates (Berg *et al.*, 2007). Glucose is converted to glucose-1,6-phosphate by one step in the metabolic pathway, and galactose is converted to either glucose or glucose-1,6-phosphate in one step. Mannose, xylose, arabinose, and other monosaccharides present in algae require several energetic steps to be converted to glucose-1,6-phosphate (Berg *et al.*, 2007), therefore the transfer of glucose and galactose from the diet directly to stored glycogen, or use for the costs of metabolism, is likely to be the most efficient use of this energy source.

Considering this, the provision of a diet relatively rich in essential lipids (e.g. DHA, EPA and sterols), appears to have triggered mussels to allocate energy to reproduction. Evidence of this is seen in mussels fed the *Pavlova lutheri* diet, which produced greater proportions of mussels in the atretic stage, with a low glycogen concentration. Atresia and decreasing glycogen concentration in mussels from the *P. lutheri* diet suggests that gametogenesis occurred earlier in this treatment, at the expense of stored glycogen for metabolic costs. Increasing ambient water temperature during the experiment caused increased energy demands for metabolism. Thus the limited availability of energy from the diet, in the form of carbohydrates and sterols, and the increased energetic demands suggests that these mussels were unable to maintain gametogenesis, and that maturation was abandoned in some mussels. It is possible that the *P. lutheri* cells provided a combination of sufficient essential lipids (DHA and EPA), which triggered the onset of gametogenesis, but a lower availability of digestible carbohydrates and sterols made continued maturation unsustainable.

The preferred source of lipids for oocyte maturation in mussels is not well understood. Several studies show that lipids from the diet, and the ratios they are present in, are reflected in the oocytes of mature bivalves (Caers *et al.*, 1999; Soudant *et al.*, 1996b), and in scallops the digestive gland is able to store lipids (Napolitano and Ackman, 1992; Peirson, 1983). Conversely, adult scallops are shown to synthesise lipids 'de novo' for gametogenesis, using stored energy reserves (Barber and Blake, 1985; Napolitano and Ackman, 1993; Vassalo, 1973). As bivalves have a limited ability to elongate and desaturate fatty acids (Knauer and

Southgate, 1999; Utting and Millican, 1998), and a limited ability to synthesise sterols (Kanazawa, 2001), these particular types of lipid are considered essential in the diet and are often conferred directly to the developing oocytes (Soudant *et al.*, 1996a). Therefore when essential lipids are limiting in the diet, physiological processes, including gametogenesis, may be inhibited.

Larvae were less successful to D-veliger stage in the *Pavlova lutheri* diet, compared to the combination and *Chaetoceros calcitrans* diets. The reported requirements of bivalve larvae for lipids and carbohydrates vary, but both influence larval growth and survival (Knauer and Southgate, 1999; Whyte *et al.*, 1989; Wikfors *et al.*, 1984). The *P. lutheri* diet contained a higher proportion of essential fatty acids, and lower proportions of easily digestible carbohydrates and sterols, than the other diets in this experiment. Therefore, the poorer survival of larvae to D-veliger stage produced by females fed this diet suggests a potential energy deficiency in the oocytes, either in the form of sterols or carbohydrates, affecting larval development in the first 48 hours. Furthermore, the *C. calcitrans* diet contained lower proportions of DHA than the combination diet, yet the larval survival from females fed this diet was similar in both diets. This result supports the hypothesis that DHA and EPA are essential fatty acids for larval growth and development, and are actively distributed to oocytes during gametogenesis (Soudant *et al.*, 1996a).

The evidence presented in this study demonstrates the influence of energetic requirements and the requirements of specific nutrients for the success of gametogenesis. Furthermore, these responses to dietary quality suggest that mussels preferentially allocate energy to storage when gametogenesis is impeded by

nutritional deficiencies. The importance of lipid and carbohydrate in bivalves has been demonstrated, particularly components of the diet that are not biosynthesized in bivalves (Knauer and Southgate, 1999). However, the biochemical pathways used for allocation of dietary components to glycogen storage and gametogenesis are not well understood. This study presents the hypothesis that carbohydrate components of the diet are allocated to carbohydrate storage, while lipid components of the diet are allocated to gametogenesis. Further research examining the fate of individual carbohydrate and lipid components, from assimilation to anabolism or catabolism, during periods of gametogenic activity, would considerably enhance the current understanding of bivalve metabolism and nutrition in relation to reproduction.

Chapter 4

Warmer temperatures reduce rates of gametogenesis in temperate mussels, *Mytilus galloprovincialis*.

Part of this chapter has been published:

Fearman, J., Moltschaniwskyj, N.A. *Aquaculture* (in press)

4.1 Introduction

Ectothermic organisms are limited by an acclimated thermal tolerance; low temperatures cause slowed cellular activity, mostly due to protein inactivation, while high temperatures denature proteins and lead to death (Van Der Have, 2002). Consequently, O_2 binding and transport, respiration, excretion, organ function etc, are also positively correlated with increasing temperature, within the thermal tolerance range, resulting in an increase in the energetic cost of basal metabolism (Somero, 2002). This increase in energetic cost of metabolism may affect the energy balance in mussels, with reduced energy available to partition between somatic growth, energy storage and reproduction (Widdows and Johnson, 1988). However, feeding and filtration may also increase as temperatures increase (Kittner and Riisgard, 2005; Van Erkom Schurink and Griffiths, 1992), which may influence the energy intake at different temperatures. Varying evidence for the influence of temperature on energy intake in mussels is available; acclimation of filtration to temperature can occur over 14 days (Widdows and Bayne, 1971), but rates of filtration can also increase as temperature increases with no acclimation over three weeks (Kittner and Riisgard, 2005). Evidence for changes in the efficiency with which filtered food is assimilated is also mixed. No changes in assimilation efficiency may occur (Van Erkom Schurink and Griffiths, 1992), but in contrast mussels at 15 °C have a lower assimilation efficiency than mussels at 5 °C or 10 °C (Widdows and Bayne, 1971). Therefore, along with changes in energy demand, temperature can also influence the energy acquired, by altering the filtration and assimilation rates.

Temperature can substantially influence the process of gametogenesis (Giese and Pearse, 1974). For example, extremes in temperature in the natural environment elicit aseasonal reproduction in bivalves. Where water temperatures are relatively warm year round with minimal seasonal change, e.g. in the tropics, bivalves allocate excess energy to reproduction continuously throughout the year and display multiple spawning periods. This is seen in many tropical bivalve species, such as pearl oysters *Pinctada margaritifera* and *Pinctada albino sugillata* (O'Connor, 2002; Pouvreau *et al.*, 2000). Conversely, bivalves exposed to cool temperatures with minimal seasonal change, i.e. polar species, exhibit slow rates of gametogenesis, which in most species e.g. *Laternula elliptica* is greater than a year (Peck *et al.*, 2007). In less extreme environments, such as temperate regions, bivalves are subjected to seasonal changes in temperature and food, therefore reproduction is often strongly seasonal.

As a result temperature is often used in hatcheries to regulate the timing and rate of gametogenesis in many bivalve species, such as oysters, clams, mussels and scallops (Borcherding, 1995; Chavez-Villalba *et al.*, 2002; Han *et al.*, 2008; Heasman *et al.*, 1996; Honkoop *et al.*, 1999; Martinez and Perez, 2003; Martinez *et al.*, 2000; Uriarte *et al.*, 2004). Controlling the timing and the rate of gametogenesis in hatcheries allows for out-of-season reproductive conditioning of adults, and the resulting production of larvae and spat. In mussels, gametogenesis and energy storage occurs within the mantle tissue (Bayne *et al.*, 1982), within which a large shift in cell types is evident throughout an annual cycle. In Tasmania gametogenesis occurs in autumn and winter, while water temperature is decreasing from ~17 °C to 11 °C degrees, spawning follows in early spring, and spawning may be repeated in

late summer and early autumn (Dix and Ferguson, 1984). Mussels store energy as glycogen in adipogranular cells and vesicular connective tissue (referred to here as glycogen storage tissue) which are a dominant feature of the mantle tissue prior to gametogenesis; this decreases in the mantle tissue during gametogenesis and increases during resting periods (Bayne *et al.*, 1982). Cycles of glycogen storage and gametogenesis are well documented in many bivalve species, such as oysters (Honkoop, 2003; Ren *et al.*, 2003), scallops (Farias *et al.*, 1997), clams (Darriba *et al.*, 2005), and mussels (Zwaan and Zandee, 1972). As glycogen is used as an energy source for gametogenesis, the quantity of glycogen stored during the resting period may substantially influence individual fecundity, oocyte quality, and larval development.

With the expected increase in water temperature associated with climate change, a greater understanding of the responses of mussels, in terms of reproduction and energy storage, to a range of temperatures, is essential for understanding mussel population dynamics; and may provide a useful tool for controlling the timing and rate of reproductive maturation in a hatchery. Comparisons of warm versus cool temperatures provide limited information regarding the overall influence of temperature on mussels. Therefore the aim of this study is to examine changes in energy storage and reproduction in mussels over a range of temperatures that are beyond the natural seasonal changes in east coast Tasmanian waters.

4.2 Materials and Methods

4.2.1 Experimental Design

To determine the effect of temperature on rates of change in glycogen concentration and gametogenesis in *Mytilus galloprovincialis*, females were held in one of five temperature treatments; 7 °C, 10 °C, 13 °C, 16 °C, and 19 °C maintained at ±1 °C, with three replicate 50 L tanks in a flow through system at each temperature (Figure 4.1). Males were also held in the same tanks to provide sperm to fertilise eggs at the end of the experiment. Each tank was initially stocked with 38 mussels which were fed microalgae from an agricultural dripper, designed to provide algae at a constant rate of 1 L/h. The feeding ration was set at ~10% dry weight of algae per dry weight of mussel per day (~0.25 g). The mussels were fed a 0.3:1:3 cell count ratio of *Chaetoceros calcitrans*, *Isochrysis galbana* (Tahitian strain) and *Pavlova lutheri* based on commercial availability of algae. Algae was grown in batch culture with Walne nutritional medium, under continuous light, at 24 ± 1.5 °C, and the pH was maintained between 7.73 and 8.85 with addition of CO_2 at 1% (volume) in the supplied air. *Chaetoceros calcitrans* was grown in 17 L carboys with the additional of silicate, while *Pavlova lutheri* and *Isochrysis galbana* were grown in 500 L polypropylene bags. Algae was harvested during the late logarithmic phase and algal densities were determined daily through algal counts. Seawater was filtered to 20 µm and UV treated to remove incoming particles as a potential food source.

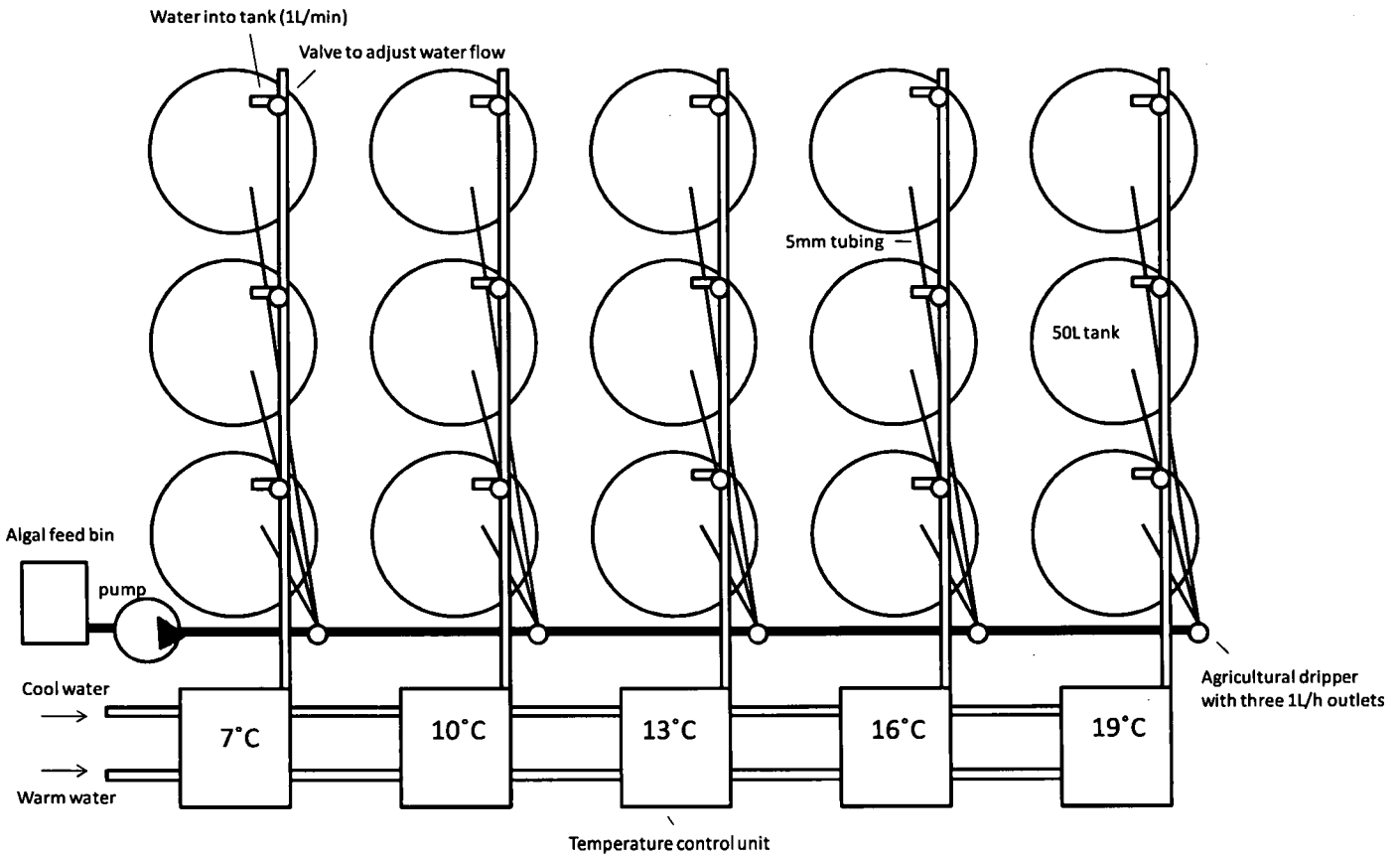


Figure 4.1 Experimental set-up.

A total of 590 mussels were collected from subsurface mussel culture lines in Mercury Passage, Tasmania, in March 2007 when water temperature was ~ 17 °C. Shell length was measured for each individual and ranged from 60 to 100 mm. Mussels were subjected to a spawning cue to ensure mature gametes were released before the experiment began, and prior to the experiment commencing an initial sample of 20 mussels (8 females) was selected randomly and shell length was measured, along with total weight, wet shell weight, and wet meat weight. The wet meat of the mussel was divided into two along the dorso-ventral margin; one half was fixed in FAACC (formalin, glacial acetic acid, and calcium chloride) for histological analysis, and the other frozen and stored at -80 °C for estimation of glycogen concentration. Histological examination of the ovary tissue of this initial sample showed that 75% of the female mussels had completely spawned, based on the observation of large empty follicles with degenerating structure in the mantle tissue, and the remaining 25% of mussels were in the resting state with no mature gametes. Mussels were held in experimental conditions for nine weeks and tanks were monitored daily for mortalities and evidence of spawning, which was the presence of remnant sperm or eggs in the water or on the bottom of the tank. A random sample of six mussels (2-3 females) was taken from each tank every three weeks, and processed in the same manner as the initial sample to assess changes in glycogen concentration and reproductive state.

4.2.2 Spawning and larval rearing

At the end of nine weeks a spawning cue was applied to the remaining mussels in each tank, and from each female that spawned fecundity and the

production of D-veliger larvae from fertilized oocytes was determined. A spawning cue was applied by cycling ~24 °C and ~18 °C water with 5 minutes air exposure between cycles for eight hours daily over three consecutive days. As individuals began spawning they were removed and placed in individual containers, allowed to spawn for one hour then relocated into new individual containers and allowed to continue spawning. Oocytes released during the first hour were fertilised using sperm from males held in the same temperature treatment. Fertilised oocytes from each female were pooled across the three replicate tanks at each temperature and placed in 1 L larval rearing container at a density of 40 eggs/mL. At 48 h post-fertilisation, counts of two 1 mL samples of D-veliger larvae were made using a Sedgewick-Rafter counting cell and a compound microscope and the total number of D-larvae in each larval rearing tank was calculated.

A 20 mL sample of oocytes released within the first hour and, and a 20 mL sample of oocytes released after the first hour were taken from each individual and fixed in formalin for later analysis. From each 20 mL sample of oocytes two 100 µL subsamples were counted as described above and total individual fecundity determined by combining the total from the first hour sample with the total from the sample taken after the first hour.

4.2.3 Determination of glycogen concentration

Frozen mussel samples were freeze-dried and weighed to estimate water content, then ground using a mortar and pestle and homogenised with 8 mL deionised water and 10 mL 0.6 M perchloric acid. Glycogen was enzymatically broken down into glucose units according to the method of Keppler and Decker

(1983). Glucose concentration (mM) was determined using an ANALOX GM7 Micro-Stat Analyser according to the manufacturers' instructions and protocols. The stored glycogen concentration was determined by subtracting the initial glucose concentration from the concentration produced by the breakdown of glycogen.

4.2.4 Determination of cellular mantle composition and oocyte size

From the fixed tissue a ~5 mm slice of tissue from the midsection of each mussel was embedded in paraffin, sectioned to 5 μm, and sections stained with Haemotoxylin and Eosin and mounted with DPX. Eight histology images from each female were haphazardly selected by moving the field of view along the section of tissue from left to right. Images were captured at 10 x magnification using a Leica DC300F camera mounted on an Olympus BH2 microscope. To determine the fraction of each cell type in the mantle tissue, a 3 cm (~60 μm at 10 x magnification) grid size was determined to be appropriate for vitellogenic, atretic and adipogranular cells, based on their average size, and was overlaid on the histology image and used for point counts (Weibel, 1979). Similarly a 1 cm (~20 μm at 10 x magnification) grid was used for previtellogenic oocytes and oogonia. Pedunculated vitellogenic and 'free' mature oocytes were both assigned to the vitellogenic oocyte stage, as their attachment to the follicle wall was difficult to determine using two-dimensional images. Based on stereological principles that point counts represent volume density (Weibel, 1979), the fraction of point counts for each cell type, from the total point counts, was used to represent the volume fraction of each cell type in the mantle tissue, an approach verified for mussels (Lowe *et al.*, 1982). Points that overlayed

empty space in the histology image were used as an indication of the extent of spawning.

As pedunculated vitellogenic oocytes and free mature oocytes were grouped together during the point counts, different stages of vitellogenesis could not be determined from point counts. Therefore, to assess the stage of maturation of vitellogenic oocytes after the nine week conditioning period, five females from each temperature were randomly selected, and the diameter of 100 vitellogenic oocytes from each female was measured. Measurements were made across the shortest diameter of the oocyte, to avoid measuring the stalk of pedunculated oocytes, and only oocytes containing a nucleus were measured.

4.2.5 Data analysis

In the week before the last sampling point technical issues resulted in the mussels in one replicate tank in temperatures 7 °C and 10 °C not being fed for one week; therefore mussels sampled from these tanks on the last sampling day were removed from all analyses. The removal of these tanks from the design resulted in an unbalanced design. The variability between tanks was assessed by including a random sample of replicates, to replace missing samples from the two tanks, and running a nested ANOVA. As the variability between tanks in each temperature at each date was not significant ($F=0.902$, df 28,80, $p=0.609$), the unbalanced design was dealt with by pooling tanks within each treatment. To determine the effect of temperature and sampling date on the concentration of glycogen a two-way ANCOVA (temperature and date orthogonal to one another, with wet meat weight and shell length as covariates) with a Tukeys post-hoc test was used. The inclusion of

a covariate in the analysis was only done if the assumptions of the ANCOVA were not violated. The effect of temperature and sampling date on the volume fraction of cell types in the mantle issue was examined using a two way MANOVA (temperature and date orthogonal to one another) and a canonical discriminant analysis (CDA), to graphically represent the data and determine where differences among levels of temperature and date were occurring. The effect of temperature on fecundity at the end of the experiment was determined using a one-way ANOVA with a Tukeys post-hoc test. Data used in ANOVAs were checked for equal variances using residual plots, and a square root transformation was used when the assumption of equal variance was violated. The frequency of vitellogenic oocyte diameters in females after nine weeks, and the frequency of D-veliger larvae produced from fertilised oocytes, from each temperature, were analysed using χ^2 test of independence. Data for D-veliger development was removed for 19 °C treatment as only one female spawned in this treatment.

4.3 Results

Mussels showed significant responses to temperature, with changes in glycogen concentration and reproductive effort during the nine week experiment. During the nine week experiment the concentration of glycogen in females declined significantly (F_{Time}=19.01, df 2,109, p>0.001). However, there was no evidence that the rate of decline was a function of the temperature that the females were held at ($F_{Time*Temperature}$=1.27, df 8,109, p=0.267) or that the concentration of glycogen differed among the different temperatures ($F_{Temperature}$=2.30, df 4,109, p=0.063). Glycogen concentration in female mussels declined by more than 50% within six

weeks and by nine weeks glycogen had declined by more than 70% (Figure 4.2). There was no evidence of growth in shell length amongst temperatures (F=1.52, df 4,50, p=0.210) or time (F=1.03, df 2,50, p=0.307).

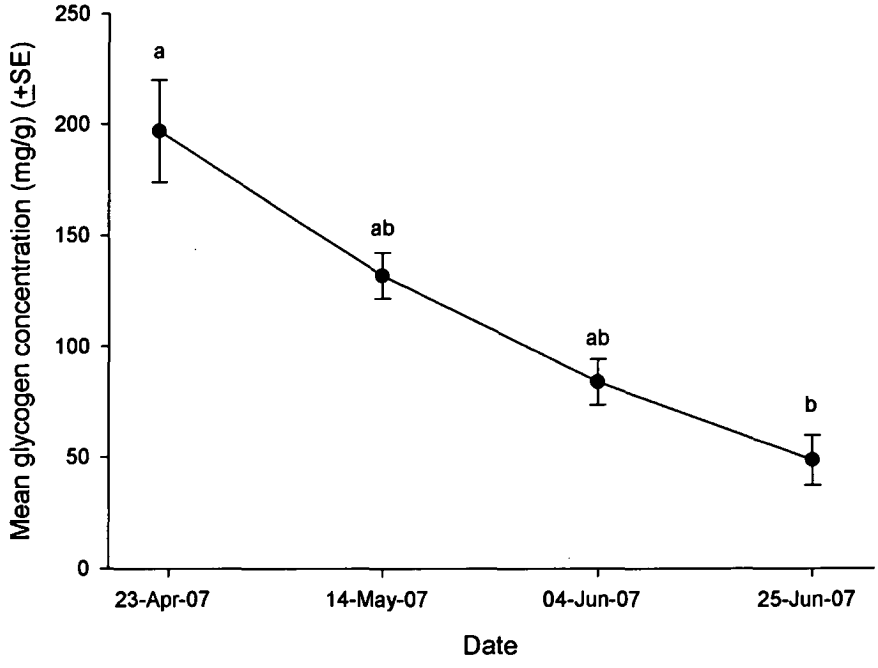


Figure 4.2. Mean glycogen concentration, adjusted for the covariates, over time. Means with the same letters are not significantly different from one another. n=124.

Changes in mean volume fraction of cell types in the mantle tissue of females can be explained by temperature (F=2.47, df 16,432, p=0.001) and time (F=5.29, df 8,212, p<0.001) independently of one another ($F_{Temperature*Time}$=1.13, df 32,432, p=0.286). The difference in the volume fractions of each cell type in the mantle tissue among temperatures was attributed to the differences in glycogen storage tissue and empty follicle space in the mantle tissue on the first discriminant function (Figure 4.3) ($R^2_{Storage}$=-0.693, R^2_{Space}=0.653), and vitellogenic oocytes (Figure 4.3) on the second dimension ($R^2_{Vitellogenic}$=-0.857). Glycogen storage tissue was 58%

more prevalent in the mantle tissue at 19°C than at 7 °C and 10 °C, while space in the mantle tissue was 34% greater at 7 °C than at 16 °C (Figure 4.4). At 13 °C and 10 °C vitellogenic oocytes were 51% greater in the mantle tissue (Figure 4.4) than at 19 °C. Through time, the volume fraction of glycogen storage tissue contributed the most to the difference ($R^2_{Storage}$=0.736) on the first discriminant function, and was 47% of the mantle tissue lower after six weeks than after three weeks.

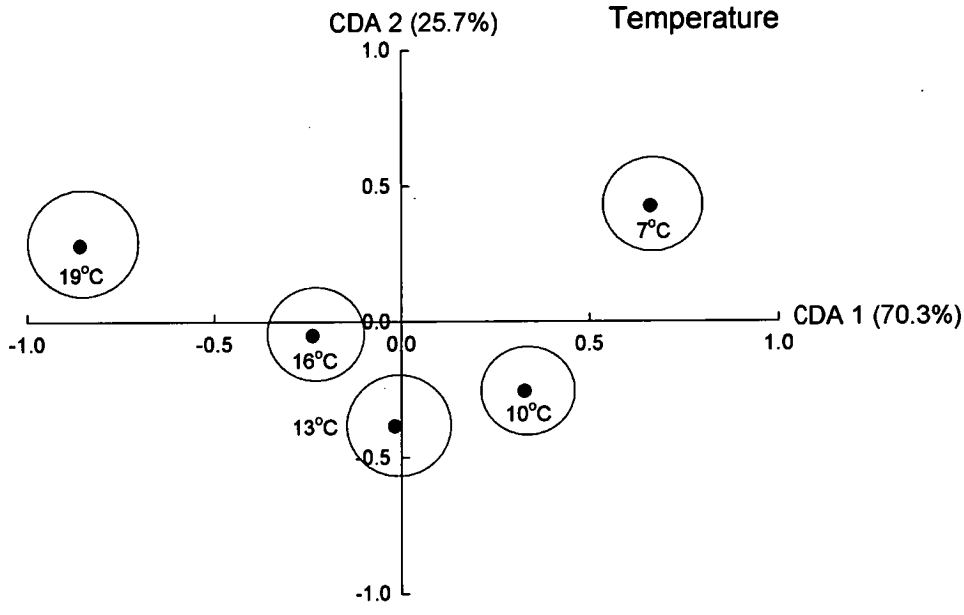


Figure 4.3. Plot of differences in temperatures compared amongst all cell types in the mantle tissue, based on Canonical Discriminant Analysis, in multi-dimensional space.

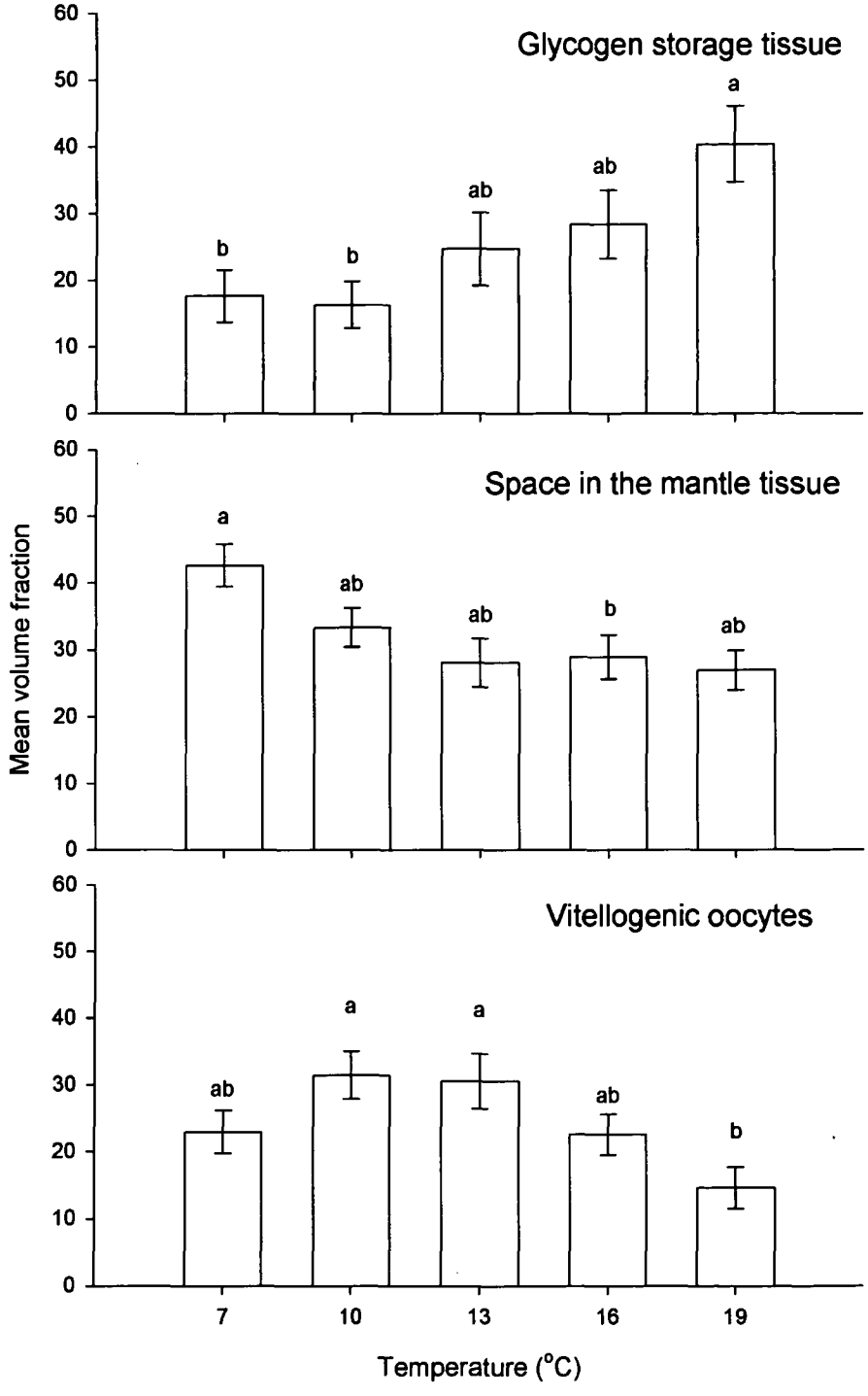


Figure 4.4. Mean volume fraction of mantle tissue occupied by specific cell types in each of the temperatures. Means with the same letters are not significantly different from one another. n=126.

Comparison of the frequencies of vitellogenic oocyte diameters in the mantle of females after the nine week conditioning period, across temperatures, showed differing frequencies as a function of temperature, with the most frequent oocyte size decreasing at warmer temperatures (χ^2 =187, df 20, p>0.001). Vitellogenic oocytes >55 µm were more frequent at 7 °C, 50-55 µm were more frequent at 7 °C and 10 °C, 40-45 µm were more frequent at 19 °C, and 35-40 µm were more frequent at 13 °C and 16 °C (Figure 4.5).

Individual fecundity of females that spawned ranged from 4900 - 22 million oocytes per female, but female fecundity was highly variable within temperatures (Figure 4.6) therefore no difference due to temperature was evident (F=0.936, df 3,30, p=0.436). No evidence of spawning was seen in the replicate tanks during the experiment. The temperature at which females were held during gametogenesis did not significantly affect the proportion of fertilised eggs that developed to the D-veliger stage (χ^2=6.122, df 3, p=0.106) with an average of 47% of fertilised oocytes developing to D-veliger stage.

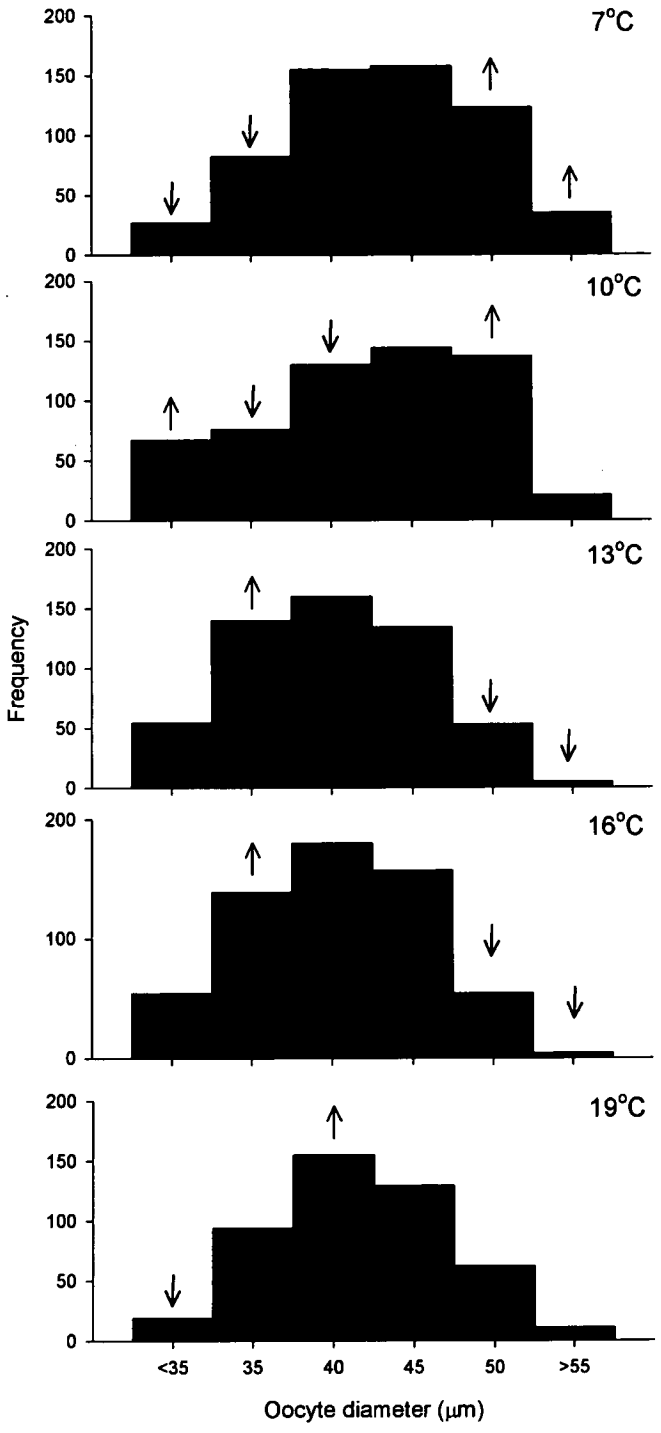


Figure 4.5. Frequency distribution of oocyte diameters in females after nine weeks, from each temperature. (Arrows indicate the direction of the departure of the observed frequencies from expected frequencies on the assumption that oocyte diameter is independent of temperature).

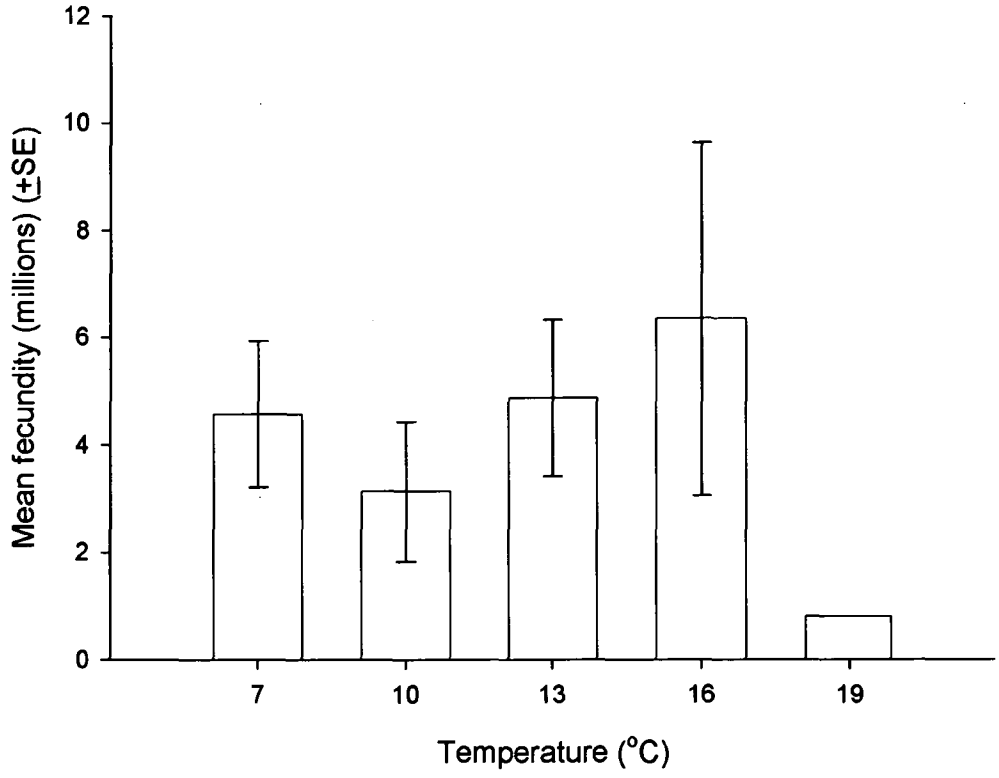


Figure 4.6. Average fecundity (number of oocytes per female ±SE) in each temperature.

4.4 Discussion

The rate of gametogenesis in the mantle tissue was substantially influenced by temperature in this study; it was faster at cool temperatures (7 °C and 10 °C), and slower at warm temperatures (19 °C). Temperature appears to indirectly affect the rate of gametogenesis in females by influencing energy allocation and metabolic activity within the mussel.

Table 4.1 Summary table of reproductive and glycogen results across temperatures.

	7°C	10°C	13°C	16°C	19°C
Glycogen concentration	↓	↓	↓	↓	↓
Oocyte diameter	↑	↑	↓	↓	↓
Space in the mantle tissue	↑			↓	
Glycogen storage tissue	↓	↓			↑
Vitellogenic oocytes		↑	↑		↓

The rate of gametogenesis appeared to decrease as temperature increased in this study (Table 4.1). The greater than expected frequency of vitellogenic oocytes >55 µm, and 50-55 µm, at 7 °C, along with the greater fraction of empty follicle space in the mantle tissue, suggests that females held at this temperature had undergone partial spawning before the end of the experiment at nine weeks, as oocyte diameter and space in the mantle increase with spawning (Kennedy, 1977). Similarly, the greater than expected frequency of vitellogenic oocytes of 50-55 µm at 10 °C, compared to greater than expected frequencies of oocytes of 35-40 µm at 13 °C and 16 °C suggests that the rate of increase of oocyte size over the nine week conditioning period was faster at 10 °C; and partial spawning may also have occurred at this temperature, but to a lesser extent. The combination of these results demonstrates the fastest rate of maturation occurring at 7 °C, and decreasing as temperature increases. The low temperature threshold for aerobic metabolism in *Mytilus* from temperate European populations is 6.7 °C (Jansen *et al.*, 2007b), therefore, on the assumption that temperate populations of *Mytilus* in the southern

hemisphere have a similar low temperature threshold, ~7 °C is the temperature in this study with the lowest aerobic metabolic rate, and potentially has the greatest residual energy available to allocate to growth and reproduction. However, this depends on the energy intake at different temperatures.

Energy intake may change with temperature through changes in filtration rate or assimilation efficiency. The evidence for temperature influencing filtration rates is varied, with evidence supporting both acclimation of filtration rates but also a lack of acclimation of filtration rates (Kittner and Riisgard, 2005; Van Erkom Schurink and Griffiths, 1992; Widdows and Bayne, 1971). If filtration rates increase with increasing temperature, then mussels held at 19 °C may have acquired more food than mussels held at 7 °C. However, *Mytilus edulis* filtered 60 mL per minute per individual when held at 8 °C after acclimation to 18 °C, and also when held at 6 °C degrees after acclimation to 11 °C (Kittner and Riisgard, 2005). Considering this, mussels in this experiment were taken from ~17 °C, so it is likely that at 7 °C filtration would be similar to those at 8 °C after 18 °C acclimation. Given the number of mussels in each tank, mussels at 7 °C would have filtered approximately 2.3 L per minute in each tank (38 mussels x 60 mL per minute per individual). The water exchange in each tank was provided at 1 L per minute, therefore it is likely that all food particles were consumed in each tank at the lowest temperature. Even if filtration increased at warmer temperatures, the overall ration provided to each tank of mussels was the same, so mussels at 19 °C would have acquired the same ration, but more rapidly. Therefore, scope for growth would decrease with increasing temperature due to the increased cost of metabolism in mussels provided with the

same ration. Additionally, there is mixed evidence for decreased absorption efficiency with increased temperature (Van Erkom Schurink and Griffiths, 1992; Widdows and Bayne, 1971). If absorption efficiency is decreased at increased temperature, then scope for growth would be further decreased at increased temperature due to decreased assimilation efficiency with increased metabolic costs.

Low temperatures have been suggested to delay gametogenesis (Loosanoff and Davis, 1951), which occurred in mussels at 5 °C (Bayne, 1965). However in populations at higher latitudes the rate of oocyte maturation is increased to ensure similar reproductive output at lower latitudes within a shorter season (Kokita, 2003; Yamahira and Conover, 2002). The results from this experiment suggest that at 7 °C and above energy partitioning, as an indirect effect of temperature, influences the rate of gametogenesis. A similar response was seen in oysters, where a group with higher energy reserves commenced gametogenesis at 10 °C, while a group with lower energy reserves also commenced gametogenesis at 10 °C, but at a slower rate (Chavez-Villalba *et al.*, 2003). Further support for this concept is seen in mussels in the Baltic Sea, where gametogenesis continues throughout winter, albeit slowly, when water temperatures are nearing 0 °C, and as the spring phytoplankton bloom occurs the rate of gametogenesis increases rapidly – even though water temperatures remain between 1 °C and 3 °C (Kautsky, 1982).

In warmer temperatures of this study, partitioning of energy appears to have limited the rate of maturation in mussels. This is particularly evident in mussels held at 19 °C, where the concentration of glycogen was depleted, yet the fraction of vitellogenic oocytes in the mantle tissue was less than in cooler temperatures, and

smaller vitellogenic oocytes 40-45 μm were more frequent than expected. Depletion of glycogen implies that energy derived from food was insufficient for the total energetic requirements of the mussel. Energy available within a mussel is partitioned between metabolism, growth, and reproduction, with the priority allocation of energy to metabolism (Ross and Nisbet, 1990). Therefore, the depletion of glycogen with no allocation of energy to growth, and lesser allocation of energy to vitellogenic oocytes compared to the other temperatures, provides evidence to suggest that there is little residual energy available for growth or reproduction. It is well understood that temperature influences metabolic rates in ectothermic animals, and that increased temperature results in increased metabolic rates (Somero, 2002), therefore reduced energy allocation to reproduction at warmer temperatures is likely due to increased energetic cost of metabolism at a fixed energy intake.

Furthermore, limited reproduction at warmer temperatures, even with the provision of a relatively high ration, provides possible evidence for temperature limitation of reproduction at 19 °C. Decreased rates of gametogenesis are also seen in scallops and oysters provided with excess food at high temperatures (Heasman *et al.*, 1996; Jeffs *et al.*, 2002; Martinez and Perez, 2003), indicating that food is not entirely the limiting factor at warm temperatures. *Mytilus* was found to have a 'breakpoint' threshold temperature of approximately 24 °C in northern European populations beyond which metabolism is deleteriously affected, which was only a few degrees above the natural environmental maximum (Jansen *et al.*, 2007). Furthermore, a breakdown in compensating mechanisms was observed in *Mytilus* above 20 °C, inhibiting acclimation of metabolic rates to temperature (Widdows,

1973). As reproduction was not successful at 19 °C, and 19 °C is greater than the environmental maximum usually experienced by these mussels, it is possible that this temperature is approaching a level where cellular function is deleteriously affected. For species that naturally reproduce during cooler winter months, such as mussels, warming ocean temperatures associated with climate change may have serious implications on the total reproductive effort and mortality of individuals. An example of this is seen in clams from the Netherlands, where long term studies have revealed substantial decreases in reproductive output during milder winters, along with a reduced ability to rebuild reserves during autumn, and therefore reduced survival during summer; all attributed to the energy balance within the bivalve (Beukema *et al.*, 2009).

Although temperature influenced the rate of gametogenesis in female mussels, it does not appear to influence fecundity or egg quality, as indicated by D-veliger production, as these parameters were not significantly different among treatments. Typically trade-offs occur between egg quality and fecundity; however food quality and quantity is generally found to influence these parameters, while temperature often influences the rate of maturation. Studies of temperature and diet on the scallop, *Argopecten purpuratus*, attribute greater D-larvae survival in scallops fed with a lipid enriched diet, while temperature influenced gonad recovery (Martinez *et al.*, 2000; Navarro *et al.*, 2000).

The evidence presented in this study supports the hypothesis that temperature is negatively correlated to rates of gametogenesis in mussels, and proposes the main driving factor for this as energy partitioning in relation to increased metabolic rates

with increased temperature. The results found in this study may be used to successfully control the timing and rate of maturation in females within a hatchery environment, and has serious implications for reproductive success and population dynamics in a warming climate.

Chapter 5

Does seasonal temperature or ration drive energy storage and reproduction in mussels, *Mytilus galloprovincialis*?

5.1 Introduction

Reproduction in mussels varies according to environmental conditions, including temperature and phytoplankton (Chapter 2). However, it is difficult to predict the most important environmental variable influencing reproduction in mussels, and what is driving reproductive strategies, particularly as changes in temperature and food are often correlated in the natural environment (Brockington and Clarke, 2001). Temperature can influence rates of metabolism (Widdows, 1973; 1976), and rates of maturation (Gaspar and Monteiro, 1999; Grubert and Ritar, 2005; Jeffs *et al.*, 2002; MacDonald and Thompson, 1985) in many animals, including mussels (Chapter 4). As temperature changes substantially across seasons in a predictable way in temperate regions, changing temperature may also act as an exogenous cue for maturation or spawning (Giese and Pearse, 1974).

The source of energy for reproduction in animals varies along a gradient between capital and income energetic investment, where capital reproductive strategies use stored energy for reproduction, while income reproductive strategies derive energy from food sources during production of offspring (Bonnet *et al.*, 1998; Jonsson, 1997). The extent of energetic investment into reproduction varies greatly among species, with some species giving birth to live, fully developed offspring and providing parental care, and others producing eggs and providing no further parental care. Energy investment into reproduction by broadcast spawning marine invertebrates is limited by the amount of energy that can be packaged into many small oocytes, which must provide energy for embryogenesis until larvae have the ability to feed (Gabbott, 1976). Therefore in these species, capital or income energy

strategies in the female are specific to the energy acquired prior to or during oocyte maturation.

The use of varying degrees of capital (conservative) reproduction and income (opportunistic) reproduction is common in many bivalve species (Gomez-Robles and Saucedo, 2009; Luna-Gonzalez *et al.*, 2000; Vite-Garcia and Saucedo, 2008). There are many examples of increased food supply (income) initiating greater reproductive effort, while a limited food supply induces gonad reabsorption or limits vitellogenesis and gonad development (Heasman *et al.*, 1996; Madrones-Ladja *et al.*, 2002; Utting and Millican, 1997; 1998). Capital (stored) energy also contributes to reproductive effort, and stored glycogen is depleted during gamete production in many marine bivalves (Barber and Blake, 1985; Bayne *et al.*, 1982; Berthelin *et al.*, 2000; Gabbott, 1976). However, the factors regulating the extent to which mussels rely on capital or income energy for reproduction are not well understood. What triggers mussels to accumulate glycogen for use in reproduction, or to use energy directly from food for reproduction? Is it purely a case of seasonal periods of excess energy availability, or do seasonal cycles in temperature trigger differences in strategies?

In species that favour capital reproduction, the potential for a threshold energy requirement for reproduction has been proposed (Bonnet *et al.*, 1998; Drent and Daan, 1980; Stearns, 1992). A minimum threshold body mass index (BMI) has been identified in the clam *Macoma balthica* where reproduction does not occur below a minimum BMI, however the energy content of this body mass is not discussed (Beukema *et al.*, 2001). In mussels the density of the soft tissue increases

with increased glycogen concentration, and condition (body mass) index increases as glycogen storage tissue increases, but there was no relationship between condition index and glycogen concentration (Chapter 2). Therefore, although a minimum threshold for reproduction in terms of soft tissue mass has been identified for one bivalve species, little information is available on possible energetic thresholds for reproduction in bivalves.

If seasonal changes act as a trigger for a shift in reproductive strategy, then the allocation of energy to reproduction or storage should be different under different seasonal conditions. Based on observations of capital reproduction in autumn and winter, and income reproduction in spring and summer (Chapter 2), energy for reproduction should be derived from food in summer and from glycogen stores in autumn. Furthermore, accumulation of glycogen should occur in summer temperatures to be used as capital for autumn reproduction. If mussels have a threshold energy requirement for reproduction in autumn, then accumulation of glycogen in summer should be the priority for energy allocation, and should occur prior to maturation of oocytes. Therefore the aim of this study is to test these predictions, and determine the influence of seasonal temperatures, and different supplemental rations, on the reproductive strategies of mussels.

5.2 Materials and Methods

5.2.1 Experimental design

To determine the influence of changing temperature and feed ration on the allocation of energy to reproduction or storage, mussels were held at one of two

rations, high (H) - 6% dry weight of algae to dry weight of mussel or low (L) - 3% dry weight of algae to dry weight of mussel; and one of three temperature changes, spring to early summer (12 – 15 °C, Spring), late summer (16 – 18 °C, Summer), or autumn (16 – 14 °C, Autumn), with three replicate tanks for each combination of temperature and ration. Mussels were held at Spring temperatures for five weeks, and then transferred to either Summer or Autumn temperatures for a further four weeks (Figure 5.1). All mussels were fed a 1:1:1 mixed ration of *Chaetoceros muelleri*, *Pavlova lutheri* and *Isochysis galbana* (Tahitian strain), and ration was increased, for mussels held at Spring and Summer temperatures, by 0.125% per week to account for increasing energetic demands as temperature increased. Food was delivered to tanks premixed with incoming water using an agricultural venturi device, and was grown in batch culture with Walne nutritional medium, under continuous light, at 24 $\pm$ 1.5 °C. The pH was maintained between 7.73 and 8.85 with addition of CO_2 at 1% (volume) in the supplied air. *Pavlova lutheri* and *Isochrysis galbana* were grown in 500 L polypropylene bags, as was *Chaetoceros muelleri* with the additional of silicate. Algal densities were determined daily through algal counts. Tanks were monitored daily for mortalities and evidence of spawning, which was the presence of remnant sperm or eggs in the water or on the bottom of the tank.

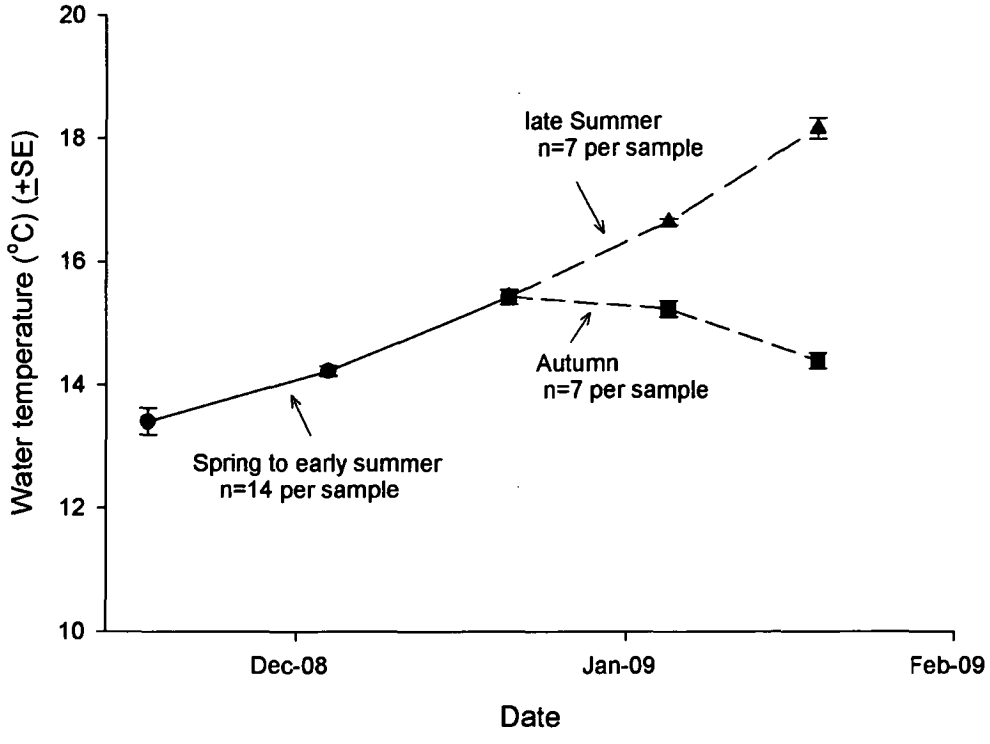


Figure 5.1. Temperature changes used to simulate Spring, Summer and Autumn.

Mussels were obtained from a farm in south-eastern Tasmania (Port Arthur) during late August 2008, subjected to a spawning cue (detailed below), and only females that spawned were retained for this experiment and placed directly into experimental tanks. A total of 660 pre-spawned females were randomly allocated to one of twelve 50 L experimental tanks, 55 mussels per tank, and provided with seawater filtered to 20 μm for two weeks and to 5 μm for two weeks to induce starvation and reduce stored glycogen.

A sample of seven mussels was taken from each tank at the start of the experiment, and every 17 days during the Spring period, and then every two weeks during the Summer and Autumn period. Shell length, total weight and wet meat

weight were measured. The mantle and the digestive gland were dissected out, and stored at -80 °C for estimation of glycogen concentration, while the remaining half of mantle tissue was fixed in FAACC (formalin, glacial acetic acid, and calcium chloride) for histological analysis.

5.2.2 Spawning and larval rearing

Mussels remaining in each tank, at the end of the Summer and Autumn temperature changes, were induced to spawn, to assess the number of mussels able to spawn. Spawning was induced by cycling water temperatures between ~24 °C and ~18 °C hourly with 5 min of air exposure between cycles for 8 h daily over two consecutive days. As individuals began spawning they were removed and placed in individual containers. A 20 mL sample of spawned eggs was taken from each individual and fixed in formalin. Two 100 µL subsamples from each 20 mL sample of eggs were counted using a Sedgewick-Rafter counting cell and a compound microscope, to determine total individual fecundity.

5.2.3 Determination of glycogen concentration

Frozen mussel samples were freeze-dried and weighed, ground using a mortar and pestle and homogenised with 5 mL of 50 mM sodium chloride solution. Glycogen was enzymatically broken down into glucose units according to the method of Keppler and Decker (1983). Glucose concentration (mM) was determined using an ANALOX GM7 Micro-Stat Analyser according to the manufacturers' instructions and protocols. The stored glycogen concentration was

determined by subtracting the initial glucose concentration from the concentration produced by the breakdown of glycogen.

5.2.4 Determination of lipid concentration

Aliquots of homogenised tissue, equivalent to 100 mg dry weight, were homogenised again in methanol and chloroform following the Bligh and Dyer method (1959), a 4 mL aliquot of the chloroform layer was allowed to evaporate on pre-weighed dishes, and the extracted lipid weighed to the nearest 10 µg determine total lipid concentration.

5.2.5 Determination of cellular mantle composition

From the fixed tissue a ~5 mm slice of tissue from the midsection of each mussel was embedded in paraffin, sectioned to 5 µm, and sections stained with Haemotoxylin and Eosin and mounted with DPX. Eight histology images from each female were haphazardly selected by moving the field of view along the section of tissue from left to right. Images were captured at 10 x magnification using a Leica DC300F camera mounted on an Olympus BH2 microscope. Reproductive maturity was determined based on the volume fraction of different cell types (previtellogenic oocytes, oogonia, vitellogenic oocytes, atretic oocytes, and adipogranular cells) in the mantle. To determine the fraction of vitellogenic oocytes, atretic oocytes, and adipogranular cells in the mantle tissue, based on their average size, a 3 cm (~60 µm at 10 x magnification) grid size was determined to be appropriate and was overlaid on the histology image and used for point counts (Weibel, 1979). Pedunculated vitellogenic and 'free' mature oocytes were both assigned to the vitellogenic oocyte

stage, as their attachment to the follicle wall was difficult to determine using two-dimensional images. To estimate the fraction of previtellogenic oocytes and oogonia a 1 cm (~20 μm at 10 x magnification) grid was used. Based on stereological principles that point counts represent volume density (Weibel, 1979), the fraction of point counts for each cell type, from the total point counts, was used to represent the volume fraction of each cell type in the mantle tissue, an approach verified for mussels (Lowe *et al.*, 1982), with points that overlayed empty space in the histology image indicative of empty space, which increases during spawning, in the follicle.

5.2.6 Data analysis

To determine the effect of ration, temperature, and time on the concentration of glycogen in the mantle tissue, digestive gland and total glycogen, a mixed model ANOVA (ration, temperature, and time as orthogonal factors of interest, and tanks nested within ration and temperature, and orthogonal to time), with Tukeys post-hoc tests, was used. A MANOVA was used to determine the effect of ration, temperature, and time on the average fraction of cells in the mantle tissue, (ration, temperature, and time as orthogonal factors of interest, and tanks nested within ration and temperature, and orthogonal to time), and a canonical discriminant function analysis and a Tukeys posthoc test were used to determine where the differences were. Fecundity of females that spawned at the end of the experiment was examined using a one way ANOVA with a Tukeys post-hoc test, and the frequency of females that spawned at the end of the experiment was compared using a Chi-squared test of independence. For all data used in analyses of variance, residual plots were used to

check for equal variances, and data was square root transformed when the assumption of equal variances was violated.

5.3 Results

Glycogen concentration in the digestive gland of female mussels changed differently in each temperature through time ($F_{Temperature*Time}$=7.19, df 4,37, p<0.001), while glycogen in the digestive gland of mussels fed a high ration was consistently greater than in mussels fed a low ration (F_{Ration}=6.3, df 1,37, p=0.017). In the mussels held at Autumn temperatures no change in the concentration of glycogen in the digestive gland was detected through time, while mussels held in Summer temperatures increased the concentration of glycogen in the digestive gland by 42% by the end of the experiment, and mussels held at Spring temperatures decreased in glycogen by 37% in the first two weeks (Figure 5.2).

The concentration of glycogen in the mantle tissue differed as a function of temperature and ration through time ($F_{Temperature*Ration*Time}$=2.93, df 4,37, p=0.033). Mussels held in Summer temperatures and fed a high ration had 56% more glycogen at the end of the experiment than mussels held at Autumn temperatures and fed a low ration (Figure 5.3). In mussels held at Spring temperatures, the concentration of glycogen in the mantle tissue was 50% greater, when fed the high ration rather than the low ration, after 17 days (Figure 5.3).

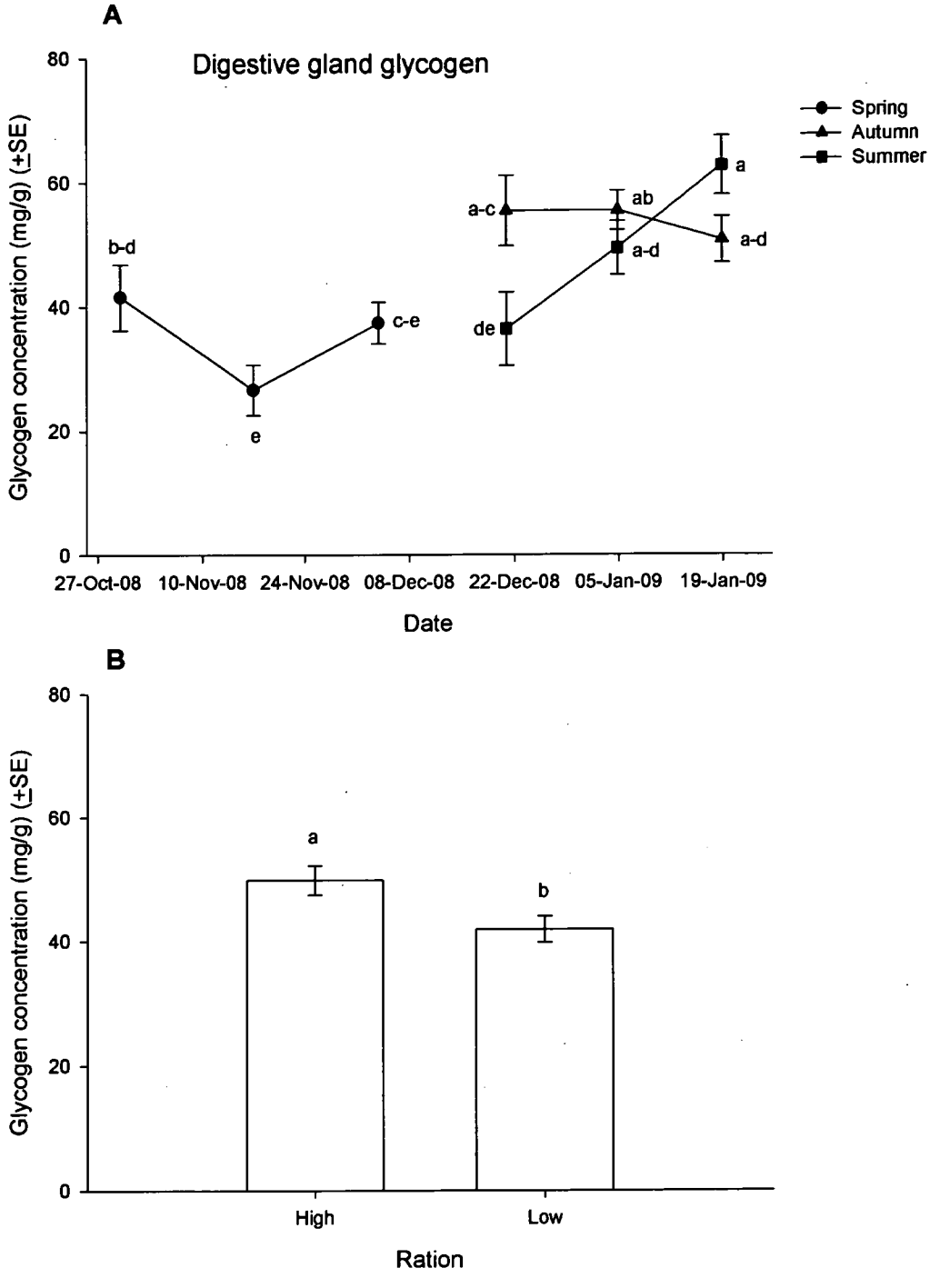


Figure 5.2. Glycogen concentration in the digestive gland A) at different temperatures over time, and B) at each ration. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different.

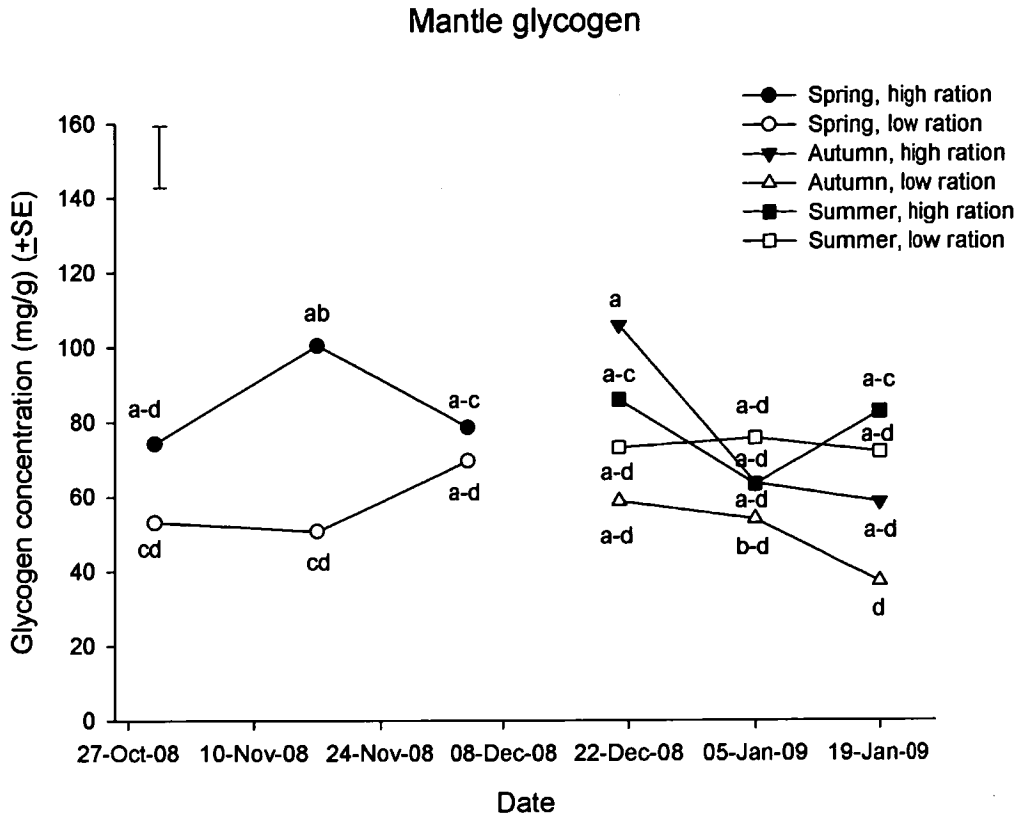


Figure 5.3. Change in glycogen concentration in the mantle tissue at different temperatures and rations over time. The average standard error across all the groups is provided in the top left corner. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different.

The total concentration of glycogen, mantle tissue and digestive gland combined, differed as a function of temperature over time ($F_{Temperature*Time}$=4.14, df 4,37, p=0.007), while total glycogen was greater in mussels fed the high ration than in mussels fed the low ration (F_{Ration}=22.78, df 1,37, p<0.001). The total concentration of glycogen in mussels held at Summer temperatures was 29% greater than in mussels held at Autumn temperatures by the end of the experiment, and at Spring temperatures after 17 days, and the total concentration of glycogen was 20% greater in mussel fed the high ration (Figure 5.4). The concentration of lipid in the digestive gland was 68% greater in mussels held at Summer and Autumn conditions,

than in mussels held at Spring conditions ($F_{Digestive\ gland}$ =9.38, df 2,17, p=0.002), but there was no difference in lipid concentration between temperatures in the mantle tissue (F_{Mantle}=0.638, df 2,15, p=0.542). There were also no differences in lipid, in the mantle or digestive gland, amongst ration (F_{Mantle}=0.625, df 1,15, p=0.441; $F_{Digestive\ gland}$=0.136, df 1,17, p=0.717) or time (F_{Mantle}=0.518, df 1,15, p=0.437; $F_{Digestive\ gland}$=0.366, df 1,17, p=0.553).

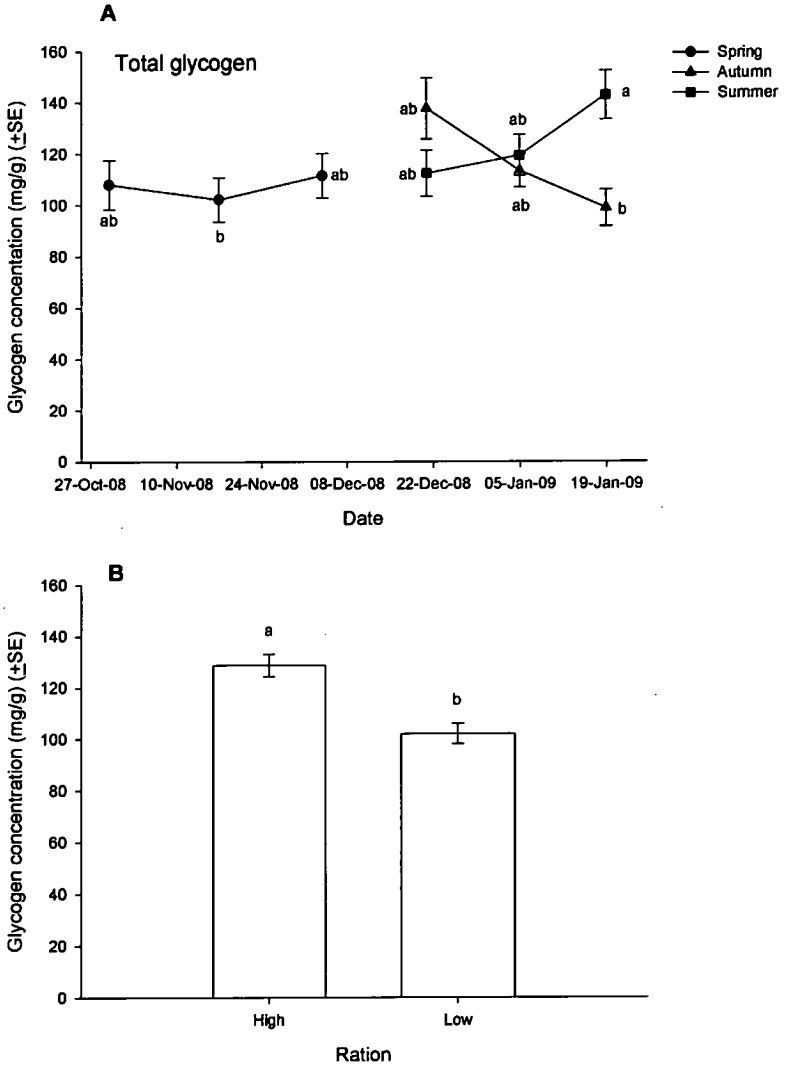


Figure 5.4. Total glycogen concentration A) at different temperatures over time, and B) at each ration. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different.

The differences amongst temperatures in the volume fraction of cell types in the mantle tissue changed over time ($F_{Temperature*Time}$=1.98, df 20,1128, p=0.012), and at different feeding rations ($F_{Temperature*Ration}$=2.0, df 8,560, p=0.045). Most of the variation in volume fraction of cell types (72%) among mussels in the different temperatures over time, was largely a function of differences in the fractional volume of space in the mantle tissue and vitellogenic oocytes (R^2_{Space}=0.75, $R^2_{Vitellogenic}$=-0.52). Space in the mantle tissue decreased by 30% during Spring (Figure 5.5 A), while vitellogenic oocytes were 48% greater after four weeks in Autumn temperatures than after four weeks in Summer temperatures (Figure 5.5B).

Most of the variability in cell types in the mussels held in the different combinations of temperatures and rations (75%) was explained by differences in space in the mantle and vitellogenic oocytes (R^2_{Space}=0.67 $R^2_{Vitellogenic}$=-0.63). Space in the mantle tissue was 26% greater in Spring at both rations, than Autumn and Summer at high rations (Figure 5.6 A). Vitellogenic oocytes were 38% greater in mussels held at Autumn temperatures and fed a high ration, than in mussels held at Summer and Spring temperatures and fed a low ration, and 57% greater than mussels held at Spring temperatures and fed a high ration (Figure 5.6 B). Mussels held at Summer temperatures and fed a high ration had the same proportion of vitellogenic oocytes in the mantle as mussels held at Autumn temperatures and fed a low ration (Figure 5.6 B).

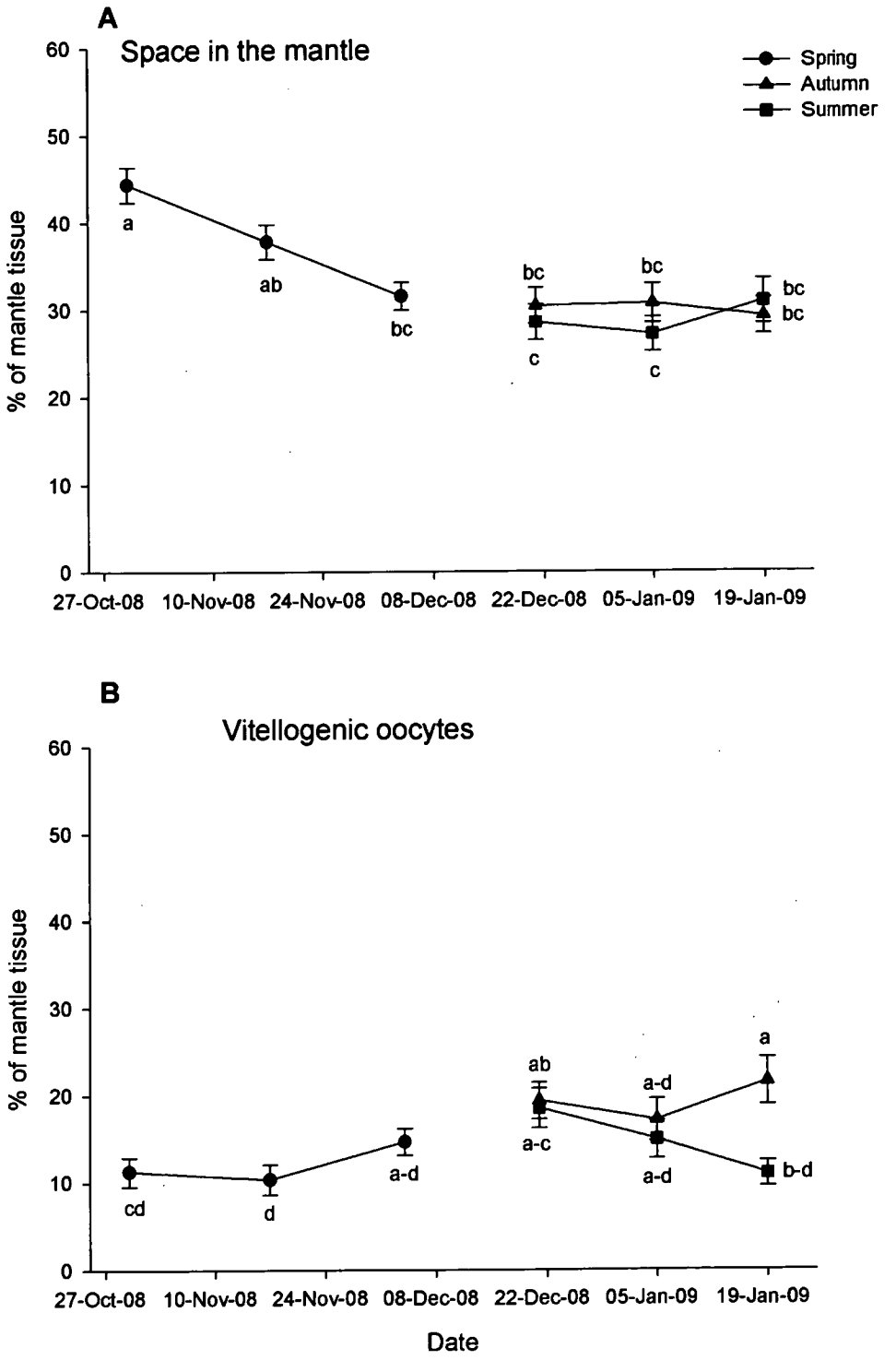


Figure 5.5. Changes in the mean volume fraction of A) Space in the mantle tissue and B) Vitellogenic oocytes at different temperatures over time. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different.

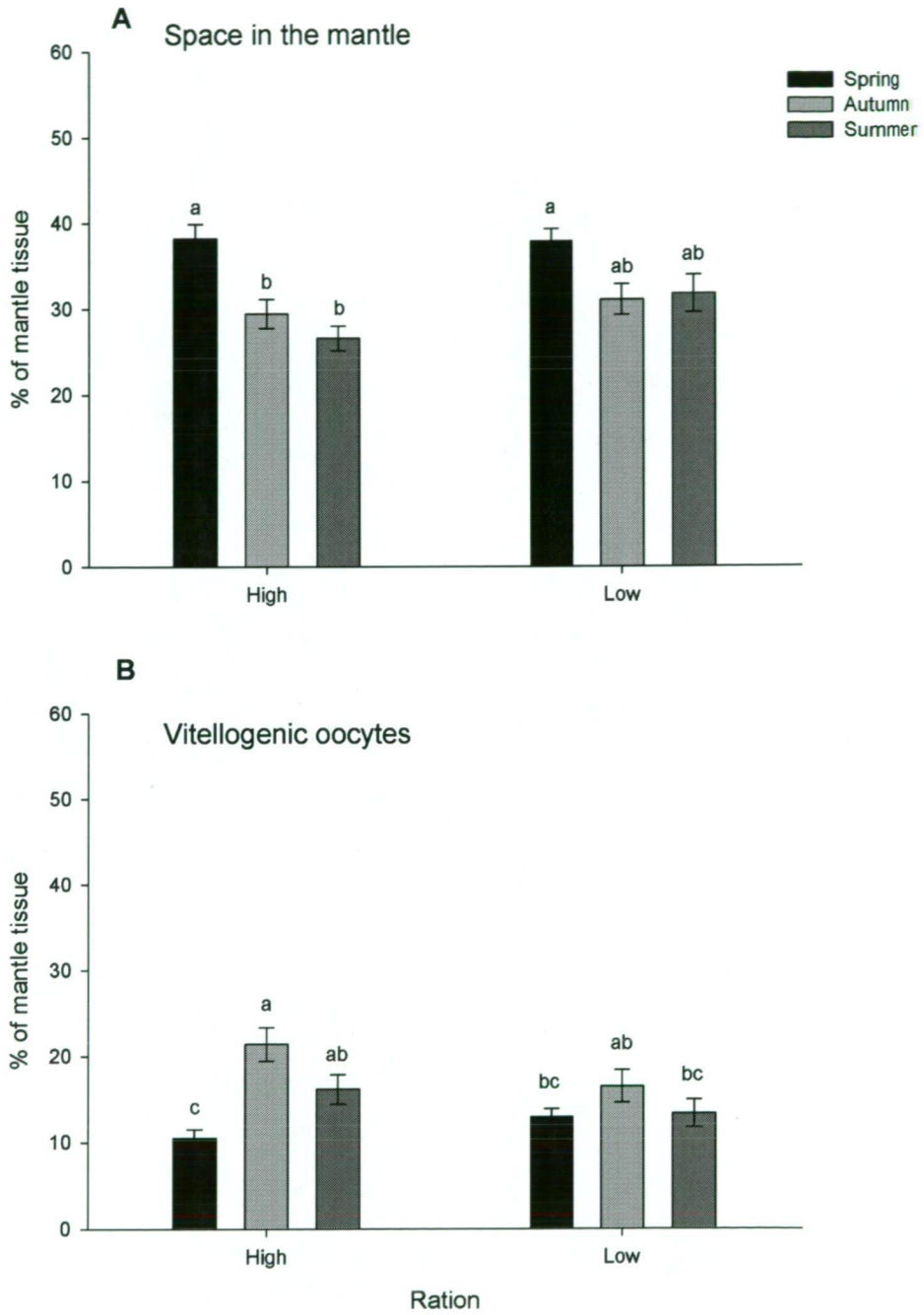


Figure 5.6. Differences in the mean volume fraction of A) Space in the mantle tissue and B) Vitellogenic oocytes at different temperatures and rations. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different.

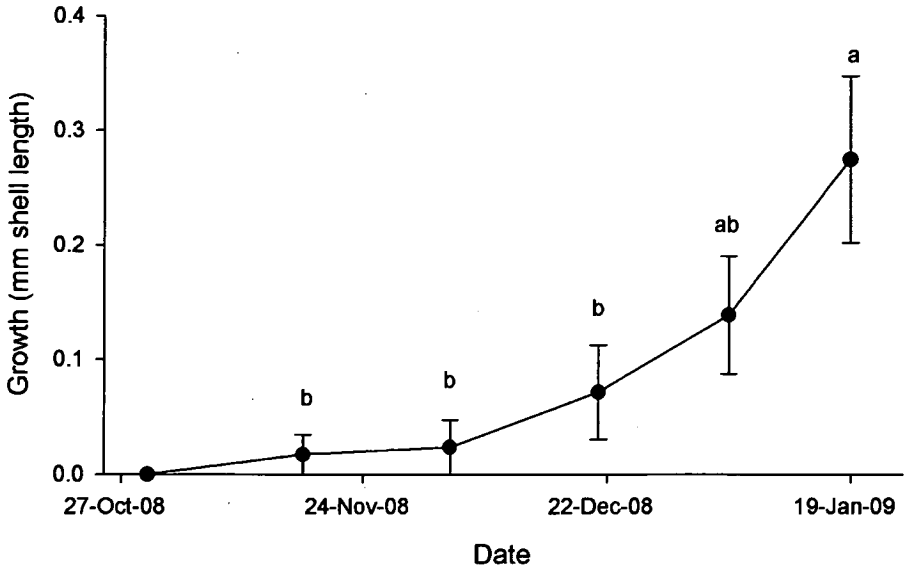


Figure 5.7. Difference in shell length from the start of the experiment at each of the sampling dates. Groups of letters with dashes (-) indicate all letters between the first and last are included, and means with the same letters are not significantly different.

Shell growth was not significantly different amongst Summer and Autumn treatments, but increased with time ($F=3.42$, df 2,328, $p=0.034$), shell length increased by 26% in the last four weeks of the experiment (Figure 5.7).

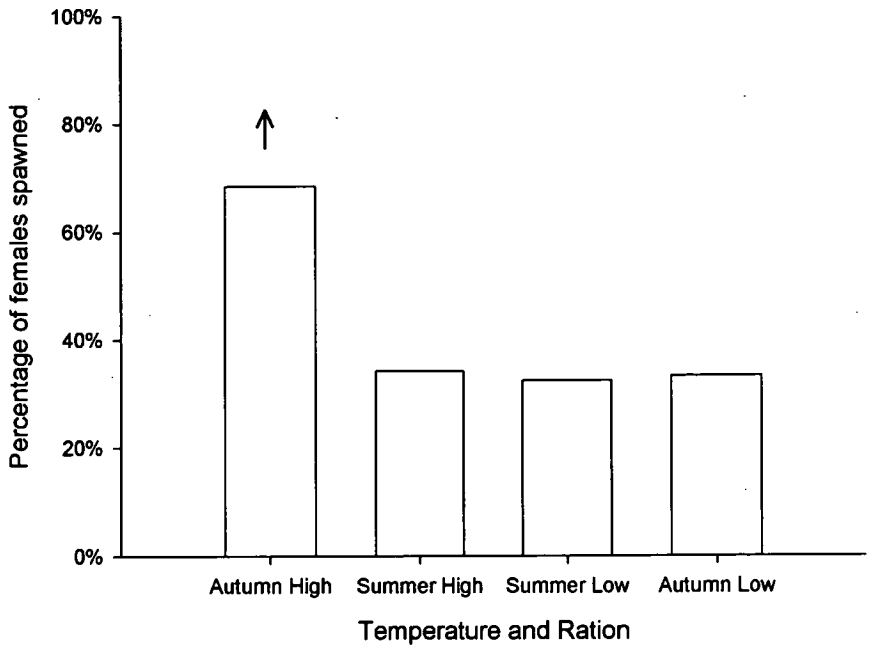


Figure 5.8. Percentage of mussels that spawned at the end of the experiment. (Arrows indicate the direction of the departure of observed values from expected frequencies on the assumptions that spawning is independent of temperature and ration).

Autumn temperatures and high ration produced 59% more females that were able to spawn at the end of the experiment (Figure 5.8) than in other treatments (χ^2 14.22, df 3, p=0.003), and no spawning was observed in the replicate tanks during the experiment. The mean fecundity ranged from 0.17 to 9.825 million oocytes per female, and was not significantly different amongst temperatures (F=0.019, df 1,61, p=0.889) or rations (F=0.958, df 1,61, p=0.332) (Figure 5.9).

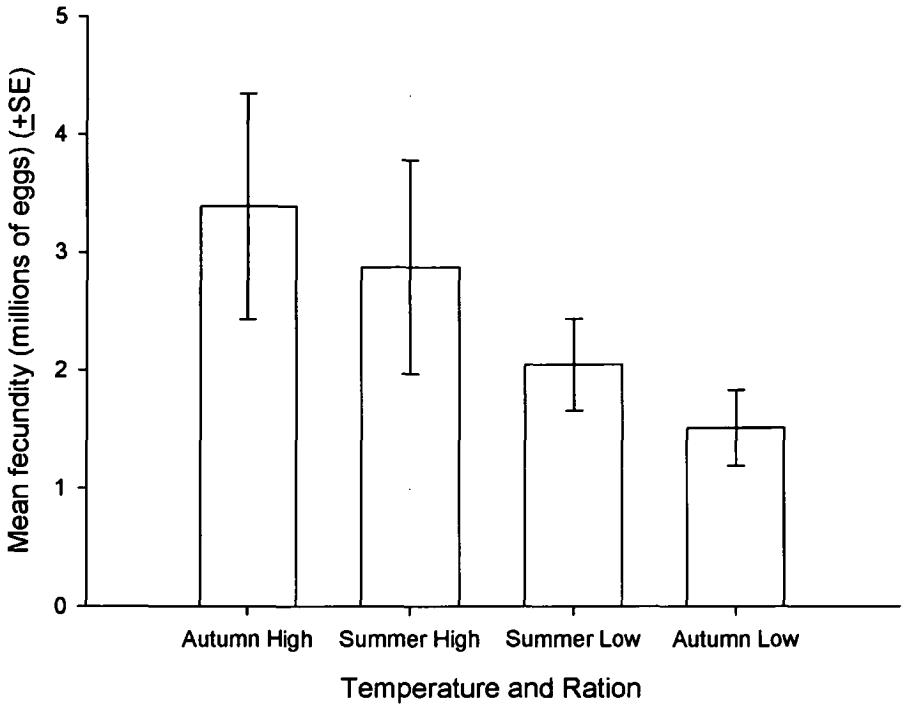


Figure 5.9. Mean fecundity of females (number of oocytes per female ±SE) in each treatment at the end of the experiment.

5.4 Discussion

This study provides evidence of seasonal adjustment of reproduction and energy dynamics in relation to reproduction. Summer temperatures triggered allocation of energy to glycogen and reproduction while Autumn temperatures triggered allocation of energy to reproduction. A combination of capital and income energy sources for reproduction was observed, and results suggest that energy for reproduction is derived directly from food (income) in most seasons, but is supplemented by stored energy (capital) during autumn when a greater proportion of vitellogenic oocytes are produced in the mantle.

Seasonal adjustment of reproductive maturation was observed during Autumn temperatures, where mussels produced greater proportions of vitellogenic oocytes (Table 5.1). This is similar to the seasonal cycle of reproduction exhibited by natural populations of mussels (Chapter 2), where the proportion of vitellogenic oocytes increases during autumn, maturation slows during winter, and major spawning occurs in spring (eg. Seed, 1976; Suarez *et al.*, 2005; Villalba, 1995). Responses to experimental seasonal temperatures were also observed in scallops, where winter conditions favoured somatic growth and spring conditions favoured gonad growth in *Pecten maximus* (Saout *et al.*, 1999). In oysters, seasonal patterns of temperature change regulated the reproductive pattern, with accelerated seasonal temperature change resulting in accelerated gonad development (Chavez-Villalba *et al.*, 2002; Fabioux *et al.*, 2005). Therefore seasonal temperatures have a direct influence on the proportion of vitellogenic oocytes produced.

Spawning synchrony amongst females was also influenced by seasonal temperature change and ration, and was greater in the combination of Autumn temperatures and high food, compared to Autumn temperatures with low food, and Summer temperatures with high and low food. Many species initiate spawning when environmental conditions are favourable for offspring survival. Therefore, maturation in autumn and winter may be a response to predictable spring phytoplankton blooms in temperate environments (Harris *et al.*, 1987).

Table 5.1. Summary of results. Larger arrows indicate the treatments that showed the greatest change and smaller arrows indicate a lesser change, with the direction of the arrow indicating the direction of change. N/A indicates that spawning did not occur in this treatment, and a dash (-) indicates that there were no differences.

	Spring High	Spring Low	Autumn High	Autumn Low	Summer High	Summer Low
D. gland glycogen	decreased then increased		no change		↑	↑
Mantle glycogen	no change		no change	↓	↑	no change
Total glycogen	no change		↓	↓	↑	↑
D. gland lipid	less		greater		greater	
Vitellogenic oocytes	↓	↓	↑	↑	↑	↓
Space	decreased		no change		no change	
% spawned	N/A	N/A	↑	-	-	-

Seasonal adjustment of energy dynamics in relation to reproduction was also observed in this study. Mussels appeared to adjust the extent to which they relied on capital and income energy for reproduction, according to the seasonal temperature change they experienced. Evidence of income reproduction (energy derived from food) is seen in mussels held at Spring and Summer temperatures, where vitellogenic oocytes were produced without using stored energy reserves, as stored glycogen did not change in Spring and increased during Summer (Table 5.1). Evidence of income reproduction was also observed in mussels held at Autumn temperatures as the total glycogen concentration was reduced by less in mussels fed a high ration compared to mussels fed a low ration. This suggests that extra energy from food in the high ration

was used for oocyte maturation, allowing less stored glycogen to be used for the same amount of vitellogenic oocytes at the two rations. Allocation of energy from food directly to reproduction has been observed in mussels fed radio-labelled food (Bayne *et al.*, 1975), and in scallops, maturation of oocytes from energy derived directly from food has been suggested as the preferred strategy (MacDonald and Thompson, 1985; Maldonado-Amparo *et al.*, 2004). The simultaneous use of capital and income energy for reproduction is also observed in scallops (Barber and Blake, 1985).

Evidence of capital reproduction is found in the mussels held at Autumn temperatures, where total glycogen and digestive gland glycogen were reduced, and the proportion of vitellogenic oocytes increased compared to mussels held at Summer temperatures. Also, in mussels held at Summer temperatures glycogen was accumulated, suggesting storage of capital resources for the following reproductive period. Capital reproduction is observed in many bivalve species, including oysters (Honkoop, 2003; Ren *et al.*, 2003), scallops (Farias *et al.*, 1997), clams (Darriba *et al.*, 2005), and mussels (Zwaan and Zandee, 1972), that all store energy, in the form of glycogen or lipid, during one season and then use it at a later date for reproduction.

The provision of a greater ration resulted in greater allocation of energy to storage. This is seen in total and digestive gland glycogen, where glycogen was higher in all mussels fed greater amounts of food. The scope for growth model suggests that where energy exceeds the requirement of metabolism, capacity for storage or reproduction is increased (Griffiths and Griffiths, 1987). Therefore at

higher ration, scope for growth is greater, allowing mussels to allocate more energy to reproduction or storage. However, the production of vitellogenic oocytes did not increase with greater amounts of food. This result suggests that the extent of reproduction is regulated by other factors and, when sufficient food is provided, is not influenced by ration. Similar responses are seen in scallops, where the egg production rate and GSI were reduced at high temperatures, despite excess food (Heasman *et al.*, 1996; 2002; Martinez and Perez, 2003).

The results from this experiment show that energy dynamics and reproduction in mussels are influenced by seasonal temperature change and ration, and may highlight the mechanism that shifts strategies between income and capital reproduction. Mussels held in Summer temperatures and fed a high ration allocated the same proportion of the mantle tissue to vitellogenic oocytes as mussels held in Autumn temperatures and fed a low ration, and they also accumulated glycogen. Concurrent maturation of oocytes and storage of glycogen rules out a threshold energy requirement for reproduction in mussels. Evidence for a lower proportion of vitellogenic oocytes in the mantle at warmer temperatures is demonstrated both in this experiment and the temperature experiment (Chapter 4). Therefore, perhaps glycogen is stored in mussels due to a combination of reduced reproduction at warmer summer temperatures and greater availability of food; rather than as a specific strategy for energy acquisition prior to maturation. The allocation of energy from food directly to oocytes is greater in mussels fed a low ration (Bayne *et al.*, 1975), suggesting that reproduction has a higher priority for energy than storage. Further investigation of the priority energy allocation between reproduction and

storage, using finer time scales after energy is assimilated, may reveal whether accumulation of glycogen is part of a conservative (capital) reproductive strategy by mussels, or whether it is simply a consequence of excess energy availability during periods of reduced reproductive capacity.

Chapter 6

General Discussion

6.1 Summary

Changes in the environment have a strong influence on reproduction in marine invertebrates in temperate regions; temperature triggers synchrony in maturation of gametes (Giese, 1959) while food influences the energy budget of an animal (Griffiths and Griffiths, 1987), and often temperature and food (phytoplankton abundance) are correlated in the natural environment. Stored energy also contributes to the energy budget of an animal, and may therefore buffer temporal periods of limited food availability. The use of energy stores for reproduction is referred to as capital reproduction, while energy from food used for reproduction is known as income reproduction (Jonsson, 1997). The synergistic influence of temperature and food on cycles of reproduction in mussels, and their reliance on capital and income energy, is evident in this study. The effect of seasonal temperature is seen in the reduced maturation of oocytes at warmer temperatures coinciding with energy storage, and the increased production of oocytes at cooler temperatures along with depletion of energy reserves. Seasonal changes in the availability of food also strongly influence reproduction, with energy being used directly for reproduction and stored as glycogen during periods of limited oocyte maturation and high food, or being used directly for reproduction and supplemented from energy stores during periods of maximal oocyte maturation. Warm temperatures with high food may drive mussels to store energy (Figure 6.1), while cool temperatures with low food would require mussels to use stored (capital) energy for reproduction (Figure 6.1). Cool temperatures with consistently high food may be ideal for continual income

reproduction rather than a reliance on capital energy stores, and may facilitate the production of successive batches of oocytes in mussels.

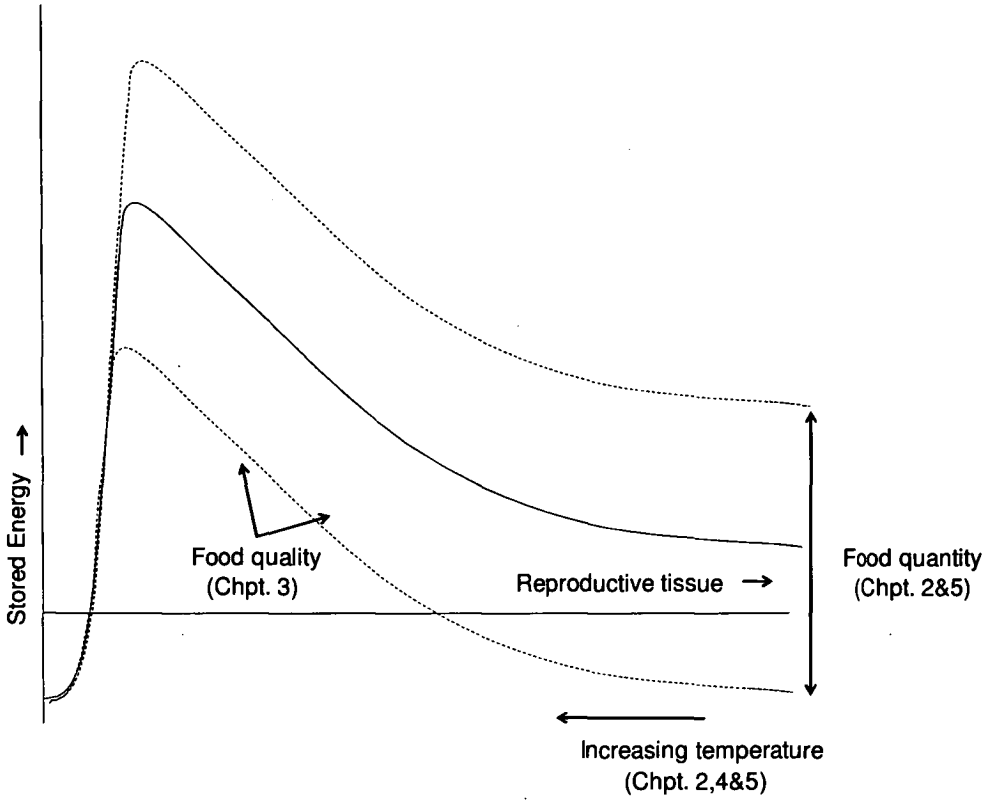


Figure 6.1. Proposed relationship between energy storage and reproduction within mussels, and the influence of temperature and food on energy allocation.

At constant temperatures and in simulated summer temperatures, warmer water reduced the rate and number of vitellogenic oocytes produced (Figure 6.1– Chpt. 4 & 5). Greater proportions of vitellogenic oocytes were produced more rapidly by mussels held at temperatures between 7°C and 13°C at constant temperature (Chpt. 4), 16 °C to 14 °C in decreasing autumn temperatures (Chpt. 5), and increasing from 12 °C to 15 °C when fed a combination diet (Chpt. 3), which reflects the range of water temperatures experienced by mussels during natural periods of oocyte

maturation in autumn (Chpt. 2). However, the production of vitellogenic oocytes in the mantle tissue was not increased by increased food (Figure 6.1– Chpt. 5). This suggests that the production of oocytes is regulated by temperature. Cool temperatures inhibit vitellogenesis in scallops, but not the production of new oocytes (Sastry, 1968). In contrast, a period of exposure to cold water is required for oocyte maturation in barnacles (Crisp, 1957). Therefore, just as scallops have a low temperature threshold for vitellogenesis, mussels in this population may have high temperature threshold for vitellogenesis. The concept of an ideal temperature window for reproduction has long been proposed (Orton 1920; in Olive, 1995) and support for this theory is provided in this study, however, only when food, or other variables, are not limiting. If food is a limiting factor then the effects of increasing temperature are confounded by the limited energy from food along with increased demand for metabolic energy at higher temperatures. Therefore food limitation may have a much more pronounced effect on reproduction than temperature (Olive, 1995).

Fecundity was highly variable among females in all experiments and differences in batch fecundity could not be attributed to temperature or food in mussels, even though the number of maturing oocytes in the mantle was related to these factors. Spawning was delayed, or potentially stopped completely, at 5 °C in temperate mussels (Bayne, 1965), and can be induced prior to the timing of natural spawning by various methods such as rapid temperature changes, rough handling, injection with potassium chloride, provision of algae and other methods (Bayne, 1965; Chipperfield, 1953; Seed, 1976). This suggests that the timing and extent of

spawning is not entirely regulated by the rate of maturation of the oocytes, and the final stages of maturation may be rapidly induced within the mussel (Chipperfield, 1953). Mussels spawn oocytes in the prophase I stage of oocyte maturation (Longo and Anderson, 1969), however evidence of metaphase I oocytes in the mantle tissue (Chpt. 2) suggests that oocytes can be spawned at either stage of maturation, therefore the initiation of spawning may be temporally flexible. This flexibility may allow mussels to time spawning events to coincide with favourable environmental conditions. Further investigation of the synergy of endogenous and exogenous cues for spawning, and the spawning of gametes at prophase I or metaphase I, is required to understand the timing and extent of spawning in mussels.

Significant increases in glycogen only occurred when mussels were held at increasing temperatures and provided sufficient energy from food (Chpt. 3 & 5). The accumulation of glycogen was greatest in the experiment where mussels were supplementally fed in addition to raw seawater (Chpt. 3). Particulate organic matter (non-phytoplankton) provides up to 80% of the energy acquired by some scallops (Hawkins *et al.*, 2002; Myasnikov and Zgurovsky, 1995), therefore provision of raw seawater, rather than filtered seawater, most likely provided additional food, which was filtered out for further experiments to allow for comparisons of controlled rations. The provision of more food facilitated maintenance of stored energy in mussels, i.e. glycogen was less depleted when more food was provided during oocyte maturation, and glycogen was stored when more food was provided and maturation of oocytes was limited (Figure 6.1– Chpt. 5). This is consistent with the scope for growth model, where greater energy availability reduces the partitioning between

competing energy demands such as reproduction and growth (Griffiths and Griffiths, 1987).

6.1.1 Conclusions

The evidence throughout this study demonstrates that seasonal temperature change and food availability results in cycles of energy storage and reproduction fueled from income and capital energy stores. Temperature and food availability both influence the extent to which mussels produce oocytes and rely on capital energy reserves to fuel reproduction, and a cool aseasonal environment with adequate food is likely to result in the faster production of more oocytes, fueled by income energy.

6.2 Feed delivery systems

Mussels in this study were fed using three different supplemental feeding approaches. Many authors use batch feeding for bivalves (Caers *et al.*, 2003; Farias *et al.*, 2003; Ferreiro *et al.*, 1990; Uriarte *et al.*, 2004), and some authors do not specify the feeding method (Martinez *et al.*, 2000). Mussels in the first experiment were batch fed, however continuous feeding was used for the second experiment as it produced greater gonadosomatic indices and heavier dry weight of spawned eggs in scallops (Racotta *et al.*, 1998; Ramirez *et al.*, 1999). Issues with the continuous feeding system becoming blocked in the constant temperature experiment (Chpt. 4) made it unreliable and unsuitable for long experiments, and more recent research found that batch feeding produced greater proportions of oysters with vitellogenic oocytes (Hurtado *et al.*, 2009), therefore extended batch feeding was used in the seasonal temperature experiment (Chpt. 5).

With each method the ration was fixed, and therefore interpretations can be made based on the overall ration. The estimated metabolic cost of filtration (feeding) in mussels is insignificant (< 3% in juveniles), while the cost of digestion and assimilation can be up to 17% (Jorgensen *et al.*, 1986; Widdows and Hawkins, 1989), therefore the differences in metabolic cost for filtration of different concentrations of algae, associated with the different feeding methods, are not likely to be substantial. Psuedofaeces are produced by mussels above a threshold algal concentration (Widdows *et al.*, 1979), therefore the concentration of algal cells in the experimental tanks was kept below the concentration where pseudofaeces are produced, and no pseudofaeces were observed during feeding. Also, the filtration rate substantially decreases when the concentration of algal cells falls below 100 cells.mL^{-1} (Riisgard *et al.*, 2003), therefore the concentration of algal cells was maintained above this level. In scallops the percentage of particles filtered from the water column decreased as the food ration (phytoplankton concentration) increased (Utting and Millican, 1998), and in mussels and scallops the absorption efficiency decreased as the quality of the feed increased (Cranford, 1995; Cranford and Hill, 1999). Widdows (1978) found that the filtration rate of mussels did not change with particle concentration, but absorption efficiency did. Therefore the number of particles, or the exact ration consumed by mussels, cannot be directly measured in this study, and interpretation of results is based on the assumption that the cost of filtration was low, and that all mussels consumed and assimilated the algae they were provided with.

A greater understanding of the energetic costs associated with feeding, under different algal concentrations but at fixed overall rations, would provide valuable insight into the most efficient method for supplemental feeding of mussels. If the metabolic cost of filtration is increased when feeding occurs over an extended period, then the experiment where mussels were held at constant temperatures (Chpt. 4) would have had the highest metabolic cost of filtration, and the diet and seasonal temperature experiments (Chpt. 3 & 5) would have had lower metabolic cost of filtration. This may be part of the explanation for the decreased glycogen during the constant temperature experiment, and the increase in glycogen in the diet and seasonal temperature experiments. However, this may be counteracted by increased absorption efficiencies at low particle concentration (Widdows, 1978).

6.3 Future studies

The results of this research have led to the hypothesis that a cool aseasonal environment is likely to increase reliance on income energy for reproduction and therefore increase the successive batches of oocytes that can be produced by mussels, while seasonal environments increase reliance on capital energy reserves for reproduction. Spawning was delayed in temperate *Mytilus edulis* at 5 °C, however maturation of oocytes increased at 0°C in arctic mussels when phytoplankton availability increased (Bayne, 1965; Kautsky, 1982). Further experiments on reproduction and changes in energy stores at cool aseasonal temperatures, with adequate food, over extended time periods, would provide greater insight into the potential for continuous income reproduction in temperate mussels.

The allocation of energy to reproduction and storage differed according to the biochemical composition of the diet in this study (Chpt. 3). This raises the question as to whether specific biochemical components of the diet are used for specific purposes; for example, carbohydrates are used more for energy production and respiration in scallops (Peirson, 1983; Whyte *et al.*, 1989). Labeled algal cells have been traced through to oocytes and biochemical fractions in scallops and mussels (Barber and Blake, 1985; Bayne *et al.*, 1975), and glucose has been traced to glycogen incorporation in oysters (Berthelin *et al.*, 2003), however, specific components in the diet have not been traced through to the final cellular destination and biochemical form. Further investigation of the fate, both the location and form, of specific biochemical components would allow for the provision of a diet that was formulated specifically for maturation of oocytes. In natural populations the biochemical components of the natural seston may also vary seasonally according to blooms of different phytoplankton species. This is worth further investigation, as it may influence the allocation of energy to storage or reproduction, and may also be involved in temporal switches from energy storage to reproduction.

Evidence of metaphase I oocytes in the mantle tissue (Chpt. 2) means that mussels are able to spawn oocytes in both the prophase I and metaphase I stage of oocyte maturation. However, it is not known what effect this has on the success of fertilisation, embryogenesis, and larval growth and survival. A comparison of these characteristics between eggs spawned at the two different stages of maturation may provide valuable information for hatchery production of larvae.

Variability in glycogen concentration, oocyte production and fecundity amongst individual females was high in this study. Considering the global distribution of this species, and its adaptation to many environmental conditions, genetic and phenotypic factors may contribute significantly to variability in this population, and should be investigated. Increased growth in small animals, and reproduction in larger animals has been attributed to greater heterozygosity in mussels and scallops (Bayne and Hawkins, 1997; Volckaert and Zouros, 1989), due to decreased energy requirements for maintenance (Hawkins *et al.*, 1986). Inherited susceptibility to summer mortality in oysters was associated with greater investment into reproduction (Samain *et al.*, 2007), and mortality was greater in oysters fed a higher ration and susceptible to summer mortality (Delaporte *et al.*, 2007). Selection of genetic traits that increase the scope for growth in mussels would be advantageous for broodstock conditioning, and the effect of temperature and food on the expression of these genes would also be of considerable interest.

Variation in the proportions of cell types in the mantle tissue was due to the use of different individuals in each sample. Tracking reproduction in the same individuals through time provides a better understanding of reproductive cycles and the environmental factors that influence them, and biopsies have been used to assess cellular changes in the same animal over time; however, the damage caused by biopsies may confound these results (Giese and Pearse, 1974). An alternative method for measuring reproductive condition has been developed for oysters using MRI, where the development of the gonad can be measured without opening the shell (Pouvreau *et al.*, 2006). However, the expense of this technology means that only

small numbers can be analysed, and still only provides an external assessment of reproduction, rather than a histological examination. The development of a non-destructive method to assess reproductive stage based on cellular information would be extremely advantageous.

Various other environmental parameters may also influence reproduction in mussels, such as salinity, photoperiod, etc (Sastry, 1979; Seed, 1976). It has been suggested that the phase shift in photoperiod and temperature associated with climate change may influence the timing of reproduction (Olive, 1995). Therefore, an understanding of the direct influence of photoperiod and other parameters, and potential interactions with other variables, may reveal more about the synchronisation of reproduction in mussels.

6.4 Implications of this Research for Aquaculture and Wild Populations

6.4.1 Hatchery

The greatest spawning synchrony was achieved using mussels that have spawned prior to conditioning, therefore the best approach to conditioning adult mussels using the information gained through this research would be to keep mussels that have spawned out from the major natural spawning event in late winter (usually September); hold them at 7°C and feed them between 6 and 10% dry weight of algae to dry weight of mussel per day, with food provided pre-mixed into the incoming water and fed over several hours. Based on an average of 2.5g dry weight of mussel, and using a mixed diet of *Pavlova lutheri*, *Chaetoceros mueleri* and *Isochrysis galbana*, this would equate to between 8.52×10^9 and 1.42×10^{10} algal cells per

mussel per day. Using *C. calcitrans* this ration would equate to between 1.97 x 10^{10} and 3.28 x 10^{10} cells per mussel per day. The number of cells provided here is lower than provided for other mussel broodstock experiments which were 3.0 x 10^{10} cells per mussel per day in the negative control treatments, which only achieved 17% spawning (Nevejan *et al.*, 2008; Pronker *et al.*, 2007), however this ration achieved up to 70% spawning in the current study. Therefore, if feasible, the provision of greater quantities of food than 6% to 10% may increase spawning success. If the temperature cannot be maintained at 7°C, the number of oocytes produced by each female may be reduced, and the time taken to mature oocytes may increase. The difference between steady cool water temperature and decreasing water temperature has not been tested, but both were successful in producing mature females for spawning.

The variability between tanks was not significant in any of the experiments, therefore mussels provided with the same amount of food should be in a similar state in each broodstock conditioning tank. However, the number of oocytes produced by each female varied between 4900 and 22 x 10^6 oocytes, averaging 4.4 x 10^6 oocytes. As fecundity was so variable amongst females, a larger number of female broodstock will be required to meet necessary numbers for a successful larval run.

Overall, summer temperatures limited oocyte maturation while autumn/winter temperatures increased oocyte maturation, and more food with mixed species composition provided greater scope for growth, which may allow for glycogen to be maintained or increased depending on the temperature.

Collection of broodstock in summer will provide animals with more glycogen, and exposure to cooling temperatures may induce oocyte maturation. Provision of more food to these animals may allow them to retain some energy reserves during oocyte maturation, and survival post-spawning may be increased. However, adults may contain some mature gametes during summer, and spawning synchrony was greatest using female mussels that were spawned had no stored energy or oocytes that could be reabsorbed for energy. Therefore collection of broodstock after the major spawning event in spring may result in greater synchrony among individuals.

Supplemental food that is limited in essential fatty acids or potentially other essential nutrients, but with more energy, may result in reduced oocyte maturation and increased energy storage, while a combination of essential fatty acids and greater energy stores facilitates successful maturation of oocytes and spawning.

The density of larvae in the water column, for wild collection of spat, is likely to be greatest during spring/summer; however this may be influenced by the availability of food during spring, and the concentration of glycogen stored over summer. Years with strong EAC influence on the Tasmanian coast may result in delayed maturation of oocytes and failure of spring spawning.

6.4.2 Climate change

Warmer temperatures decreased the production of vitellogenic oocytes in mussels in this study, therefore long term increases in water temperature associated with climate change, during summer or winter, may result in long term decreases in reproductive effort, and/or changes in reproductive strategies. Long term data

showed that increased winter temperatures in the spring spawning clam, *Macoma balthica*, resulted in reduced reproductive output and recruitment, and warmer than average summers resulted in decreased survival to the next reproductive season (Beukema *et al.*, 2009). Warming temperature has resulted in a poleward shift in the biogeographical range of mussel populations in the US (Jones *et al.*, 2009). Therefore warming ocean temperatures coupled with less productive waters from the East Australian Current may substantially influence recruitment, maturation and survival in this mussel population.

References

Ackman, R.G., 1981. Fatty acid metabolism of bivalves, Second international conference on aquaculture nutrition: Biochemical and physiological approach to shellfish nutrition. World Mariculture Society, Rehoboth Beach, Delaware, pp. 358 - 374.

Alfaro, A.C., Jeffs, A.G., Hooker, S.H., 2001. Reproductive behavior of the green-lipped mussel, *Perna canaliculus*, in northern New Zealand. *Bulletin of Marine Science* **69**, 1095-1108.

Barber, B.J., Blake, N.J., 1985. Intra-organ biochemical transformation associated with oogenesis in the bay scallop, *Argopecten irradians concentricus* (Say), as indicated by 14C incorporation. *Biological Bulletin* **168**, 39 - 49.

Bayne, B.L., 1965. Growth and the delay of metamorphosis of the larvae in *Mytilus edulis* (L.). *Ophelia* **2**, 1 - 47.

Bayne, B.L., Hawkins, A.J.S., 1997. Protein metabolism, the costs of growth, and genomic heterozygosity: Experiments with the mussel *Mytilus galloprovincialis* Lmk. *Physiological and Biochemical Zoology* **70**, 391-402.

Bayne, B.L., Gabbott, P.A., Widdows, J., 1975. Some effects of stress in the adults on the eggs and larvae of *Mytilus edulis* L. *Journal of the Marine Biological Association of the United Kingdom* **55**, 675 - 689.

Bayne, B.L., Thompson, R.J., Widdows, J., 1976. Physiology I. In: Bayne, B.L. (Ed.), Marine mussels: their ecology and physiology. Cambridge University Press, Cambridge, pp. 121 - 206.

Bayne, B.L., Bubel, A., Gabbott, P.A., Livingstone, D.R., Lowe, D.M., Moore, M.N., 1982. Glycogen utilisation and gametogenesis in *Mytilus edulis* L. *Marine Biology Letters* **3**, 89 - 105.

Berg, J.M., Tymoczko, J.L., Stryer, L., 2007. Biochemistry. W.H. Freeman and Company, New York

Berthelin, C., Kellner, K., Mathieu, M., 2000. Storage metabolism in the Pacific oyster (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (West Coast of France). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **125**, 359 - 369.

Berthelin, C.H., Fievet, B., Leclerc, G., Germain, P., Kellner, K., Mathieu, M., 2003. In vivo and in vitro approaches to the analysis of glycogen metabolism in the Pacific oyster, *Crassostrea gigas*. *Journal of Shellfish Research* **22**, 715-720.

Beukema, J.J., Drent, J., Honkoop, P.J.C., 2001. Maximizing lifetime egg production in a Wadden Sea population of the tellinid bivalve *Macoma balthica*: a trade-

off between immediate and future reproductive outputs. *Marine Ecology-Progress Series* **209**, 119-129.

Beukema, J.J., Dekker, R., Jansen, J.M., 2009. Some like it cold: populations of the tellinid bivalve *Macoma balthica* (L.) suffer in various ways from a warming climate. *Marine Ecology Progress Series* **384**, 135-145.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911 - 917.

Bonnet, X., Bradshaw, D., Shine, R., 1998. Capital versus Income Breeding: An Ectothermic Perspective. *OIKOS* **83**, 333-342.

Borcherding, J., 1995. Laboratory experiments on the influence of food availability, temperature and photoperiod on gonad development in the fresh water mussel *Dreissena polymorpha*. *Malacologia* **36**, 15-27.

Brockington, S., Clarke, A., 2001. The relative influence of temperature and food on the metabolism of a marine invertebrate. *Journal of Experimental Marine Biology and Ecology* **258**, 87-99.

Brokordt, K., Guderley, H., 2004. Energetic requirements during gonad maturation and spawning in scallops: sex differences in *Chlamys islandica* (Muller 1776). *Journal of Shellfish Research* **23**, 25 - 32.

Brown, M.R., 1991. The amino-acid and sugar composition of 16 species of microalgae used in mariculture. *Journal of Experimental Marine Biology and Ecology* **145**, 79 - 99.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., Dunstan, G.A., 1997. Nutritional properties of microalgae for mariculture. *Aquaculture* **151**, 315 - 331

Caceres-Martinez, J., Figueras, A., 1998. Long-term survey on wild and cultured mussels (*Mytilus galloprovincialis* Lmk) reproductive cycles in the Ria de Vigo (NW Spain). *Aquaculture* **162**, 141-156.

Caers, M., Coutteau, P., Sorgeloos, P., Gajardo, G., 2003. Impact of algal diets and emulsions on the fatty acid composition and content of selected tissues of adult broodstock of the Chilean scallop *Argopecten purpuratus* (Lamark, 1819). *Aquaculture* **217**, 437 - 452.

Caers, M., Coutteau, P., Cure, K., Morales, V., Gajardo, G., Sorgeloos, P., 1999. The Chilean scallop *Argopecten purpuratus* (Lamarck, 1819): II. Manipulation of the fatty acid composition and lipid content of the eggs via lipid supplementation of the broodstock diet. *Comparative Biochemistry and Physiology Part B* **123**, 97 - 103.

Charnov, E.L., Schaffer, W.M., 1973. Life-History Consequences of Natural Selection: Cole's Result Revisited. *The American Naturalist* **107**, 791-793.

Chavez-Villalba, J., Barret, J., Mingant, C., Cochard, J.C., Le Pennec, M., 2002. Autumn conditioning of the oyster *Crassostrea gigas*: A new approach. *Aquaculture* **210**, 171-186.

Chavez-Villalba, J., Cochard, J.C., Le Pennec, M., Barret, J., Enriquez-Diaz, M., Caceres-Martinez, C., 2003. Effects of temperature and feeding regimes on gametogenesis and larval production in the oyster *Crassostrea gigas*. *Journal of Shellfish Research* **22**, 721-731.

Cheshuk, B.W., 2001. Integrated open-water mussel and salmon culture. PhD Thesis.

Cheshuk, B.W., Purser, G.J., Quintana, R., 2003. Integrated open-water mussel (*Mytilus planulatus*) and Atlantic salmon (*Salmo salar*) culture in Tasmania, Australia. *Aquaculture* **218**, 357-378.

Chipperfield, P.N.J., 1953. Observations on the breeding and settlement of *Mytilus edulis* (L.) in British waters. *Journal of the Marine Biological Association of the United Kingdom* **32**, 449 - 476.

Cranford, P.J., 1995. Relationships between food quantity and quality and absorption efficiency in sea scallops *Placopecten magellanicus* (Gmelin). *Journal of Experimental Marine Biology and Ecology* **189**, 123 - 142.

Cranford, P.J., Hill, P.S., 1999. Seasonal variation in food utilization by the suspension feeding bivalve molluscs *Mytilus edulis* and *Placopecten magellanicus*. *Marine Ecology-Progress Series* **190**, 223-239.

Crisp, D.J., 1957. Effect of low temperature on the breeding of marine animals. *Nature* **179**, 1138-1139.

Cuomo, A., Di Cristo, C., Paolucci, M., Di Cosmo, A., Tosti, E., 2005. Calcium currents correlate with oocyte maturation during the reproductive cycle in *Octopus vulgaris*. *Journal of Experimental Zoology Part A: Comparative Experimental Biology* **303**, 193-202.

Curiel-Ramirez, S., Caceres-Martinez, J., 2004. Reproductive cycle of coexisting mussels, *Mytilus californianus* and *Mytilus galloprovincialis*, in Baja California, New Mexico. *Journal of Shellfish Research* **23**, 515-520.

Darriba, S., San Juan, F., Guerra, A., 2005. Energy storage and utilization in relation to the reproductive cycle in the razor clam *Ensis arcuatus* (Jefferys 1865). *ICES Journal of Marine Science* **62**, 886 - 896.

Delaporte, M., Soudant, P., Lambert, C., Jegaden, M., Moal, J., Pouvreau, S., Degremont, L., Boudry, P., Samain, J.F., 2007. Characterisation of physiological and immunological differences between Pacific oysters

(*Crassostrea gigas*) genetically selected for high or low survival to summer mortalities and fed different rations under controlled conditions. *Journal of Experimental Marine Biology and Ecology* **353**, 45-57.

Dix, T.G., Ferguson, A., 1984. Cycles of reproduction and condition in Tasmanian blue mussels, *Mytilus edulis planulatus*. *Australian Journal of Marine and Freshwater Research* **35**, 307-313.

Dohmen, M.R., 1983. Gametogenesis. In: Verdonk, N.H., van den Biggelaar, J.A.M., Tompa, A.S. (Eds.), The Mollusca. Academic Press, New York, pp. 352.

Drent, R.H., Daan, S., 1980. The prudent parent: Energetic adjustments in avian breeding. *Ardea* **68**, 225-252.

Drummond, L., Mulcahy, M., Culloty, S., 2006. The reproductive biology of the Manila clam, *Ruditapes philippinarum*, from the north-west of Ireland. *Aquaculture* **254**, 326 - 340.

Duinker, A., Haland, L., Hovgaard, P., Mortensen, S., 2008. Gonad development and spawning in one and two year old mussels (*Mytilus edulis*) from Western Norway. *Journal of the Marine Biological Association of the United Kingdom* **88**, 1465-1473.

Enright, C.T., Newkirk, G.F., Craigie, J.S., Castell, J.D., 1986. Growth of juvenile *Ostrea edulis* (L.) fed *Chaetoceros gracilis* Schutt of varied chemical composition. . *Journal of Experimental Marine Biology and Ecology* **96**, 15 - 26.

Epifanio, C.E., 1979. Growth in bivalve molluscs: nutritional effects of two or more species of algae in diets fed to the American oyster *Crassostrea virginica* (Gmelin) and the hard clam *Mercenaria mercenaria* (L.). *Aquaculture* **18**, 1 - 12.

F.A.O, 2009. Fisheries and Aquaculture Statistics. http://www.fao.org/figis/servlet/SQServlet?file=/usr/local/tomcat/FI/5.5.23/figis/webapps/figis/temp/hqp_53996.xml&outtype=html

Fabioux, C., Huvet, A., Le Souchu, P., Le Pennec, M., Pouvreau, S., 2005. Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. *Aquaculture* **250**, 458-470.

Farias, A., Uriarte, I., Varas, P., 1997. Nutritional study on the broodstock conditioning of the Chilean scallop, *Argopecten purpuratus* (Lamarck, 1819). *Revista de Biologia Marina y Oceanografia* **32**, 127-136.

Farias, A., Bell, J.G., Uriarte, I., Sargent, J.R., 2003. Polyunsaturated fatty acids in total lipid and phospholipids in chilean scallop *Argopecten purpuratus* (L.) larvae: effects of diet and temperature. *Aquaculture* **228**, 289 - 305.

Ferreiro, M.J., Perez-Camacho, A., Labarta, U., Beiras, R., Planas, M., Fernandez-Reiriz, M.J., 1990. Changes in the biochemical composition of *Ostrea edulis* larvae fed on different food regimens. *Marine Biology* **106**, 395-401.

Gabbott, P.A., 1976. Energy metabolism. In: Bayne, B.L. (Ed.), Marine mussels: their ecology and physiology. Cambridge University Press, Plymouth, pp. 293 - 356.

Gabbott, P.A., Peek, K., 1991. Cellular biochemistry of the mantle tissue of the mussel *Mytilus edulis* -L. *Aquaculture* **94**, 165-176.

Galap, C., Leboulenger, F., Grillot, J.P., 1997. Seasonal variations in biochemical constituents during the reproductive cycle of the female dog cockle *Glycymeris glycymeris*. *Marine Biology* **129**, 625-634.

Gallager, S.M., Mann, R., 1986. Growth and survival of larvae of *Mercenaria mercenaria* (L) and *Crassostrea virginica* (Gmelin) relative to broodstock conditioning and lipid content of eggs. *Aquaculture* **56**, 105-121.

Gardner, J.P.A., 1992. *Mytilus galloprovincialis* (Lmk) (Bivalvia, Mollusca) - the taxonomic status of the mediterranean mussel. *Ophelia* **35**, 219-243.

Gardner, J.P.A., 2004. A historical perspective of the genus *Mytilus* (Bivalvia: Mollusca) in New Zealand: multivariate morphometric analyses of fossil, midden and contemporary blue mussels. *Biological Journal of the Linnean Society* **82**, 329-344.

Gaspar, M.B., Monteiro, C.C., 1999. Gametogenesis and spawning in the subtidal white clam *Spisula solida*, in relation to temperature. *Journal of the Marine Biological Association of the United Kingdom* **79**, 753-755.

Gaudron, S.M., Kohler, S.A., Conand, C., 2008. Reproduction of the sea cucumber *Holothuria leucospilota* in the Western Indian Ocean: Biological and ecological aspects. *Invertebrate Reproduction and Development* **51**, 19-31.

Giese, A.C., 1959. Comparative physiology: annual reproductive cycles of marine invertebrates. *Annual Review of Physiology* **21**, 547-576.

Giese, A.C., Pearse, J.S., 1974. Reproduction of marine invertebrates. Academic Press, New York

Gomez-Robles, E., Saucedo, P.E., 2009. Evaluation of quality indices of the gonad and somatic tissues involved in reproduction of the pearl oyster *Pinctada mazatlanica* with histochemistry and digital image analysis. *Journal of Shellfish Research* **28**, 329-335.

Gosling, E.M., 2003. Bivalve molluscs: biology, ecology and culture. Blackwell, Oxford

Griffiths, C.L., Griffiths, R.J., 1987. Bivalvia. In: Pandian, T.J., Vernberg, F.J. (Eds.), Animal energetics. Academic Press, New York, pp. 631.

Grubert, M.A., Ritar, A.J., 2005. The effect of temperature and conditioning interval on the spawning success of wild-caught blacklip (*Haliotis rubra*, Leach 1814) and greenlip (*H. laevigata*, Donovan 1808) abalone. *Aquaculture Research* **36**, 654-665.

Han, K.N., Lee, S.W., Wang, S.Y., 2008. The effect of temperature on the energy budget of the Manila clam, *Ruditapes philippinarum*. *Aquaculture International* **16**, 143-152.

Harris, G., Nilsson, C., Clementson, L., Thomas, D., 1987. The water masses of the east coast of Tasmania: Seasonal and interannual variability and the influence on phytoplankton biomass and productivity. *Australian Journal of Marine and Freshwater Research* **38**, 569 - 590.

Hasle, G.R., 1978. The inverted-microscope method. In: Sournia, A. (Ed.), Phytoplankton manual. Monographs on oceanographic methodology 6. UNESCO, Paris, pp. 88-96.

Hawkins, A.J.S., Bayne, B.L., Day, A.J., 1986. Protein turnover, physiological energetics and heterozygosity in the blue mussel, *Mytilus edulis* - the basis of variable age-specific growth. *Proceedings of the Royal Society of London Series B-Biological Sciences* **229**, 161-176.

Hawkins, A.J.S., Duarte, P., Fang, J.G., Pascoe, P.L., Zhang, J.H., Zhang, X.L., Zhu, M.Y., 2002. A functional model of responsive suspension-feeding and growth in bivalve shellfish, configured and validated for the scallop *Chlamys farreri* during culture in China. *Journal of Experimental Marine Biology and Ecology* **281**, 13-40.

Heasman, M.P., O'Connor, W.A., Frazer, A.W., 1996. Temperature and nutrition as factors in conditioning broodstock for the commercial scallop *Pecten fumatus* Reeve. *Aquaculture* **143**, 75 - 90.

Hendriks, I.E., van Duren, L.A., Herman, P.M.J., 2003. Effect of dietary polyunsaturated fatty acids on reproductive output and larval growth of bivalves. *Journal of Experimental Marine Biology and Ecology* **296**, 199-213.

Honkoop, P.J.C., 2003. Physiological cost of reproduction in the Sydney rock oyster *Saccostrea glomerata*. *Oecologia* **135**, 176 - 183.

Honkoop, P.J.C., Luttikhuizen, P.C., Piersma, T., 1999. Experimentally extending the spawning season of a marine bivalve using temperature change and fluoxetine as synergistic triggers. *Marine Ecology Progress Series* **180**, 297-300.

Hurtado, M.A., Ramirez, J.L., Rodriguez-Jaramillo, C., Tovar, D., Ibarra, A.M., Soudant, P., Palacios, E., 2009. Comparison of continuous and batch feeding systems on maturation, biochemical composition and immune variables of the oyster *Crassostrea corteziensis* (Hertlein 1951). *Aquaculture Research* **40**, 464-472.

IMOS, 2009. Integrated Marine Observing System. http://www.marine.csiro.au/remotesensing/imos/aggregator.html

Jansen, J.M., Wendelaar Bonga, S., Hummel, H., 2007b. Differential cold-shock resistance among acclimatised European mussel populations. *Journal of Marine and Freshwater Behaviour and Physiology* **40**, 233 - 245.

Jansen, J.M., Pronker, A.E., Kube, S., Sokolowski, A., Sola, J.C., Marquiegui, M.A., Schiedek, D., Wendelaar Bonga, S., Wolowicz, M., Hummel, H., 2007. Geographic and seasonal patterns and limits on the adaptive response to temperature of European *Mytilus* spp. and *Macoma balthica* populations. *Oecologia* **154**, 23-34.

Jeffs, A.G., Dunphy, B.J., Wells, R.M.G., 2002. Experimental effects of water temperature on the gametogenic development of broodstock in the oyster, *Ostrea chilensis*. *Journal of Shellfish Research* **21**, 743-747.

Jones, S.J., Mieszkowska, N., Wethey, D.S., 2009. Linking thermal tolerances and biogeography: *Mytilus edulis* (L.) at its southern limit on the East Coast of the United States. *Biological Bulletin* **217**, 73-85.

Jonsson, K.I., 1997. Capital and Income Breeding as Alternative Tactics of Resource Use in Reproduction. *OIKOS* **78**, 57-66.

Jordan, A.R., Pullen, G., Marshall, J., Williams, H., 1995. Temporal and spatial patterns of spawning in Jack Mackerel *Trachurus declivis* (Pices: Carangidae) during 1988-91 in Eastern Tasmanian Waters. *Australian Journal of Marine and Freshwater Research* **46**, 831-842.

Jorgensen, C.B., Mohlenberg, F., Sten-Knudsen, O., 1986. Nature of relation between ventilation and oxygen consumption in filter feeders. *Marine Ecology Progress Series* **29**, 73-88.

Kanazawa, A., 2001. Sterols in marine invertebrates. *Fisheries Science* **67**, 997 - 1007.

Kautsky, N., 1982. Quantitative studies on gonad cycle, fecundity, reproductive output and recruitment in a Baltic *Mytilus edulis* population. *Marine Biology* **68**, 143-160.

Kennedy, V.S., 1977. Reproduction in *Mytilus edulis aoteanus* and *Aulacomya maoriana* (Mollusca-Bivalvia) from Taylors Mistake, New-Zealand. *New Zealand Journal of Marine and Freshwater Research* **11**, 255-267.

Keppler, D., Decker, K., 1983. Glycogen determination with amyloglucosidase. In: Bergmeyer, H.U. (Ed.), Methods of enzymatic analysis. Academic Press, London, pp. 1127 - 1131.

Kimball, D.M., McElroy, A.E., 1993. Characterizing the annual reproductive cycle of *Mytilus edulis* from Boston Harbor and Cape Cod Bay - a comparison by means of stereology and condition indexes. *Marine Environmental Research* **35**, 189-196.

King, P.A., McGrath, D., Gosling, E.M., 1989. Reproduction and settlement of *Mytilus edulis* on an exposed rocky shore in Galway Bay, West-Coast of Ireland. *Journal of the Marine Biological Association of the United Kingdom* **69**, 355-365.

Kittner, C., Riisgard, H.U., 2005. Effect of temperature on filtration rate in the mussel *Mytilus edulis*: no evidence for temperature compensation. *Marine Ecology-Progress Series* **305**, 147-152.

Knauer, J., Southgate, P.C., 1999. A review of the nutritional requirements of bivalves and the development of alternative and artificial diets for bivalve aquaculture. *Reviews in Fisheries Science* **7**, 241-280.

Kokita, T., 2003. Potential latitudinal variation in egg size and number of a geographically widespread reef fish, revealed by common environment experiments. *Marine Biology* **143**, 593 - 601.

Le Pennec, M., Beninger, P.G., Dorange, G., Paulet, Y.-M., 1991. Trophic sources and pathways to the developing gametes of *Pecten maximus* (Bivalvia: Pectinidae). *Journal of the Marine Biological Association of the United Kingdom* **71**, 451 - 463.

Longo, F.J., Anderson, E., 1969. Cytological aspects of fertilisation in the lamellibranch, *Mytilus edulis*. Polar body formation and development of the female pronucleus. *Journal of Experimental Zoology* **172**, 69 - 96.

Loosanoff, V.L., Davis, H.C., 1951. Delaying spawning of lamellibranchs by low temperature. *Journal of Marine Research* **10**, 197-202.

Lowe, D.M., Moore, M.N., Bayne, B.L., 1982. Aspects of gametogenesis in the marine mussel *Mytilus edulis* -L. *Journal of the Marine Biological Association of the United Kingdom* **62**, 133-145.

Luna-Gonzalez, A., Caceres-Martinez, C., Zuniga-Pacheco, C., Lopez-Lopez, S., Ceballos-Vasquez, B.P., 2000. Reproductive cycle of *Argopecten ventricosus* (Sowerby 1842) (Bivalvia : Pectinidae) in the Rada Del Puerto de Pichilingue, B.C.S., Mexico and its relation to temperature, salinity and food. *Journal of Shellfish Research* **19**, 107-112.

MacDonald, B.A., Thompson, R.J., 1985. Influence of temperature and food availability on the ecological energetics of the giant scallop *Placopecten magellanicus*. II. Reproductive output and total production. *Marine Ecology Progress Series* **25**, 295 - 303.

MacDonald, B.A., Bayne, B.L., 1993. Food availability and resource allocation in senescent *Placopecten magellanicus*: evidence from field populations. *Functional Ecology* **7**, 40-46.

MacIntyre, R.J., Murray, P.J., Pregezner, C., Stoddart, H.E., 1977. Experimental mussel culture in Australia. *Proceedings of the World Mariculture Society* **8**, 411-425.

Mackie, G.L., 1984. Bivalves. In: Tompa, A.S. (Ed.), The Mollusca. Academic Press, New York, pp. 486.

Maclean, J.L., 1972. Mussel culture: methods and prospects, Australian Fisheries Papers. Fisheries Division, Department of Primary Industries, Canberra, pp. 13.

Madrones-Ladja, J.A., dela Pena, M.R., Parami, N.P., 2002. The effect of micro algal diet and rearing condition on gonad maturity, fecundity and embryonic development of the window-pane shell, *Placuna placenta* Linnaeus. *Aquaculture* **206**, 313 - 321.

Maldonado-Amparo, R., Ramirez, J.L., Avila, S., Ibarra, A.M., 2004. Triploid lion-paw scallop (*Nodipecten subnodosus* Sowerby); growth, gametogenesis, and gametic cell frequencies when grown at a high food availability site. *Aquaculture* **235**, 185-205.

Maloy, A.P., Barber, B.J., Rawson, P.D., 2003. Gametogenesis in a sympatric population of blue mussels, *Mytilus edulis* and *Mytilus trossulus*, from Cobscook Bay (USA). *Journal of Shellfish Research* **22**, 119-123.

Martinez, G., Perez, H., 2003. Effect of different temperature regimes on reproductive conditioning in the scallop *Argopecten purpuratus*. *Aquaculture* **228**, 153-167.

Martinez, G., Aguilera, C., Mettifogo, L., 2000. Interactive effects of diet and temperature on reproductive conditioning of *Argopecten purpuratus* broodstock. *Aquaculture* **183**, 149-159.

Marty, Y., Delaunay, F., Moal, J., Samain, J.F., 1992. Changes in the fatty acid composition of *Pecten maximus* (L.) during larval development. *Journal of Experimental Marine Biology and Ecology* **163**, 221 - 234.

McDonald, J.H., Seed, R., Koehn, R.K., 1991. Allozymes and morphometric characters of three species of *Mytilus* in the Northern and Southern Hemispheres. *Marine Biology* **111**, 323-333.

Milke, L.M., Bricelj, V.M., Parrish, C.C., 2004. Growth of postlarval sea scallops, *Placopecten magellanicus*, on microalgal diets, with emphasis on the nutritional role of lipids and fatty acids. *Aquaculture* **234**, 293 - 317.

Milke, L.M., Bricelj, V.M., Parrish, C.C., 2008. Biochemical characterization and nutritional value of three *Pavlova* spp. in unialgal and mixed diets with *Chaetoceros muelleri* for postlarval sea scallops, *Placopecten magellanicus*. *Aquaculture* **276**, 130-142.

Murphy, G.I., 1968. Pattern in Life History and the Environment. *The American Naturalist* **102**, 391-403.

Myasnikov, V.G., Zgurovsky, K.A., 1995. Distribution and biology of commercial scallops (*Chlamys*) in the Northwest Pacific. *ICES Marine Science Symposia* **199**, 40-46.

Myrand, B., Guderley, H., Himmelman, J.H., 2000. Reproduction and summer mortality of blue mussels *Mytilus edulis* in the Magdalen Islands, southern Gulf of St. Lawrence. *Marine Ecology-Progress Series* **197**, 193-207.

Napolitano, G.E., Ackman, R.G., 1992. Anatomical distributions and temporal variations of lipid classes in sea scallops *Placopecten magellanicus* (Gmelin, 1791) from Georges Bank (Nova Scotia). *Comparative Biochemistry and Physiology* **103B**, 645 - 650.

Napolitano, G.E., Ackman, R.G., 1993. Fatty acid dynamics in sea scallops *Placopecten magellanicus* (Gmelin) from Georges Bank, Nova Scotia. *Journal of Shellfish Research* **12**, 267 - 277.

Natrah, F.M.I., Yusoff, F.M., Shariff, M., Abas, F., Mariana, N.S., 2007. Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. *Journal of Applied Phycology* **19**, 711-718.

Navarro, J.M., Leiva, G.E., Martinez, G., Aguilera, C., 2000. Interactive effects of diet and temperature on the scope for growth of the scallop *Argopecten purpuratus* during reproductive conditioning. *Journal of Experimental Marine Biology and Ecology* **247**, 67-83.

Nevejan, N., Pronker, A.E., Peene, F., 2008. Hatchery broodstock conditioning of the blue mussel *Mytilus edulis* (Linnaeus, 1758). Part II. New formulated feeds offer new perspectives to commercial hatcheries. *Aquaculture International* **16**, 483-495.

Newell, R.I.E., Hilbish, T.J., Koehn, R.K., Newell, C.J., 1982. Temporal variation in the reproductive cycle of *Mytilus edulis* -L (Bivalvia, Mytilidae) from localities on the east coast of the United States. *Biological Bulletin* **162**, 299-310.

O'Connor, W.A., 2002. Latitudinal variation in reproductive behavior in the pearl oyster, *Pinctada albina sugillata*. *Aquaculture* **209**, 333-345.

Olive, P.J.W., 1995. Annual breeding cycles in marine invertebrates and environmental temperature: Probing the proximate and ultimate causes of reproductive synchrony. *Journal of Thermal Biology* **20**, 79-90.

Orton, J.H., 1920. Sea temperature, breeding and distribution in marine animals. *Journal of the Marine Biological Association of the United Kingdom* **12**, 339-336.

Page, H.M., Hubbard, D.M., 1987. Temporal and spatial patterns of growth in mussels *Mytilus edulis* on an Offshore Platform - Relationships to water temperature and food availability. *Journal of Experimental Marine Biology and Ecology* **111**, 159-179.

Page, H.M., Ricard, Y.O., 1990. Food availability as a limiting factor to mussel *Mytilus edulis* growth in California coastal waters. *Fishery Bulletin* **88**, 677-686.

Pazos, A.J., Roman, G., Acosta, C.P., Abad, M., Sanchez, J.L., 1996. Stereological studies on the gametogenic cycle of the scallop, *Pecten maximus*, in suspended culture in Ria de Arousa (Galicia, NW Spain). *Aquaculture* **142**, 119-135.

Peck, L.S., Powell, D.K., Tyler, P.A., 2007. Very slow development in two Antarctic bivalve molluscs, the infaunal clam *Laternula elliptica* and the scallop *Adamussium colbecki*. *Marine Biology* **150**, 1191-1197.

Peirson, W.M., 1983. Utilization of eight algal species by the Bay scallop, *Argopecten irradians concentricus* (Say). *Journal of Experimental Marine Biology and Ecology* **68**, 1 - 11.

Phleger, C.F., 1998. Buoyancy in marine fishes: direct and indirect role of lipids. *American Zoologist* **38**, 321-330.

Pieters, H., Kluytmans, J.H., Zandee, D.I., Cadee, G.C., 1980. Tissue composition and reproduction of *Mytilus edulis* in relation to food availability. *Netherlands Journal of Sea Research* **14**, 349-361.

Pipe, R.K., 1987. Oogenesis in the marine mussel *Mytilus edulis*: an ultrastructural study. *Marine Biology* **95**, 405-414.

Ponis, E., Parisi, G., Le Coz, J.R., Robert, R., Zittelli, G.C., Tredici, M.R., 2006. Effect of the culture system and culture technique on biochemical characteristics of *Pavlova lutheri* and its nutritional value for *Crassostrea gigas* larvae. *Aquaculture Nutrition* **12**, 322-329.

Pouvreau, S., Rambeau, M., Cochard, J.C., Robert, R., 2006. Investigation of marine bivalve morphology by in vivo MR imaging: First anatomical results of a promising technique. *Aquaculture* **259**, 415-423.

Pouvreau, S., Gangnery, A., Tiapari, J., Lagarde, F., Garnier, M., Bodoy, A., 2000. Gametogenic cycle and reproductive effort of the tropical blacklip pearl oyster, *Pinctada margaritifera* (Bivalvia: Pteriidae), cultivated in Takapoto atoll (French Polynesia). *Aquatic Living Resources* **13**, 37 - 48.

Pronker, A.E., Nevejan, N., Peene, F., Geijsen, P., Sorgeloos, P., 2007. Hatchery broodstock conditioning of the blue mussel *Mytilus edulis* (Linnaeus 1758). Part I. Impact of different micro-algae mixtures on broodstock performance. *Aquaculture International.*

Quinn, G.P., Keough, M.J., 2002. Experimental design and data analysis for biologists. Cambridge University Press, New York

Racotta, I.S., Ramirez, J.L., Avila, S., Ibarra, A.M., 1998. Biochemical composition of gonad and muscle in the catarina scallop, *Argopecten ventricosus*, after reproductive conditioning under two feeding systems. *Aquaculture* **163**, 111-122.

Ramirez, J.L., Avila, S., Ibarra, A.M., 1999. Optimization of forage in two food-filtering organisms with the use of a continuous, low-food concentration, agricultural drip system. *Aquacultural Engineering* **20**, 175-189.

Ramirez Llodra, E., 2002. Fecundity and life-history strategy in marine invertebrates. In: Southward, A.J. (Ed.), Advances in Marine Biology. Academic Press, London, pp. 87 - 170.

Ren, J.S., Marsden, I.D., Ross, A.H., Schiel, D.R., 2003. Seasonal variation in the reproductive activity and biochemical composition of the Pacific oyster (*Crassostrea gigas*) from the Marlborough Sounds, New Zealand. *New Zealand Journal of Marine and Freshwater Research* **37**, 171-182.

Rico-Villa, B., Le Coz, J.R., Mignant, C., Robert, R., 2006. Influence of phytoplankton diet mixtures on microalgae consumption, larval development and settlement of the Pacific oyster *Crassostrea gigas* (Thunberg). *Aquaculture* **256**, 377 - 388.

Riisgard, H.U., Kittner, C., Seerup, D.F., 2003. Regulation of opening state and filtration rate in filter-feeding bivalves (*Cardium edule, Mytilus edulis, Mya arenaria*) in response to low algal concentration. *Journal of Experimental Marine Biology and Ecology* **284**, 105-127.

Rodriguez-Astudillo, S., Villalejo-Fuerte, M., Garcia-Dominguez, F., Guerrero-Caballero, R., 2005. Biochemical composition and its relationship with the

gonadal index of the black oyster *Hyotissa hyotis* (Linnaeus, 1758) at Espiritu Santo Gulf of California. *Journal of Shellfish Research* **24**, 975-978.

Ross, A.H., Nisbet, R.M., 1990. Dynamic models of growth and reproduction of the mussel *Mytilus edulis* -L. *Functional Ecology* **4**, 777-787.

Salkeld, P.N., 1995. Aspects of reproduction associated with the use of a segmented regression to describe the relationship between body-weight and shell length of *Mytilus edulis*. *Marine Ecology-Progress Series* **124**, 117-128.

Samain, J.F., Degremont, L., Soletchnik, P., Haure, J., Bedier, E., Ropert, M., Moal, J., Huvet, A., Bacca, H., Van Wormhoudt, A., Delaporte, M., Costil, K., Pouvreau, S., Lambert, C., Boulo, V., Soudant, P., Nicolas, J.L., Le Roux, F., Renault, T., Gagnaire, B., Geret, F., Boutet, I., Burgeot, T., Boudry, P., 2007. Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. *Aquaculture* **268**, 227-243.

Saout, C., Quere, C., Donval, A., Paulet, Y.-M., Samain, J.F., 1999. An experimental study of the combined effects of temperature and photoperiod on reproductive physiology of *Pecten maximus* from the Bay of Brest (France). *Aquaculture* **172**, 301 - 314.

Sastry, A.N., 1968. The relationships among food, temperature, and gonad development of the bay scallops *Aequipecten irradians* Lamark. *Physiological Zoology* **41**, 44 - 53.

Sastry, A.N., 1979. Pelecypoda (excluding Ostreidae). In: Giese, A.C., Pearse, J.S. (Eds.), Reproduction of marine invertebrates. Academic Press, New York, pp. 113-292.

Schatten, H., Constantinescu, G.M., 2007. Comparative Reproductive Biology. Blackwell Publishing, Ames

Seed, R., 1976. Ecology. In: Bayne, B.L. (Ed.), Marine mussels: their ecology and physiology. Cambridge Universtiy Press, Cambridge, pp. 13 - 65.

Shields, J., Barnes, P., Heath, D., 2008. Growth and survival differences among native, introduced and hybrid blue mussels (*Mytilus* spp.): genotype, environment and interaction effects. *Marine Biology* **154**, 919-928.

Slater, J., 2005. Spawning of king scallops, *Pecten maximus* (L.) in Mulroy Bay and the relationship with spatfall intensity. *Journal of Shellfish Research* **24**, 951-958.

Somero, G.N., 2002. Thermal physiology and vertical zonation of intertidal animals: optima, limits and costs of living. *Integrative and Comparative Biology* **42**, 780 - 789.

Soudant, P., Marty, Y., Moal, J., Samain, J.F., 1996a. Fatty acids and egg quality in great scallop. *Aquaculture International* **4**, 191 - 200.

Soudant, P., Le Coz, J.R., Marty, Y., Moal, J., Robert, R., 1998. Incorporation of microalgae sterols by scallop *Pecten maximus* (L.) larvae. *Comparative Biochemistry and Physiology A-Physiology* **119**, 451 - 457.

Soudant, P., Marty, Y., Moal, J., Robert, R., Quere, C., Le Coz, J.R., Samain, J.F., 1996b. Effect of food fatty acid content and sterol quality on *Pecten maximus* gonad composition and reproduction process. *Aquaculture* **143**, 361 - 378.

Stearns, S.C., 1992. The evolution of life histories. Oxford University Press, New York, 249.

Suarez, M.P., Alvarez, C., Molist, P., San Juan, F., 2005. Particular aspects of gonadal cycle and seasonal distribution of gametogenic stages of *Mytilus galloprovincialis* cultured in the estuary of Vigo. *Journal of Shellfish Research* **24**, 531-540.

Thorarinsdottir, G.G., Gunnarsson, K., 2003. Reproductive cycles of *Mytilus edulis* L. on the west and east coasts of Iceland. *Polar Research* **22**, 217-223.

Tsitsa-Tzardis, E., Patterson, G.W., Wikfors, G.H., Gladu, P.K., Harrison, 1993. Sterols of *Chaetoceros* and *Skeletonema. Lipids* **28**, 465 - 467.

Uriarte, I., Farias, A., Hernandez, J., Schafer, C., Sorgeloos, P., 2004. Reproductive conditioning of Chilean scallop (*Argopecten purpuratus*) and the Pacific oyster (*Crassostrea gigas*): Effects of enriched diets. *Aquaculture* **230**, 349-357.

Utting, S.D., Millican, P.F., 1997. Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability. *Aquaculture* **155**, 45-54.

Utting, S.D., Millican, P.F., 1998. The role of diet in hatchery conditioning of *Pecten maximus* L.: A review. *Aquaculture* **165**, 167-178.

Van Der Have, T.M., 2002. A proximate model for thermal tolerance in ectotherms. *OIKOS* **98**, 141-155.

van Erkom Schurink, C., Griffiths, C.L., 1991. A comparison of reproductive cycles and reproductive output in 4 southern African mussel species. *Marine Ecology-Progress Series* **76**, 123-134.

Van Erkom Schurink, C., Griffiths, C.L., 1992. Physiological energetics of four South African mussel species in relation to body size, ration and temperature. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **101**, 779-789.

Vassalo, M.T., 1973. Lipid storage and transfer in the scallop *Chlamys heredica* (Gould). *Comparative Biochemistry and Physiology* **44A**, 1169 - 1175.

Villalba, A., 1995. Gametogenic cycle of cultured mussel, *Mytilus galloprovincialis*, in the Bays of Galicia (Nw Spain). *Aquaculture* **130**, 269-277.

Vite-Garcia, N., Saucedo, P.E., 2008. Energy storage and allocation during reproduction of Pacific winged pearl oyster *Pteria sterna* at Bahis De La Paz, Baja California Sur, Mexico. *Journal of Shellfish Research* **27**, 375-383.

Volckaert, F., Zouros, E., 1989. Allozyme and physiological variation in the scallop *Placopecten magellanicus* and a general model for the effects of heterozygosity on fitness in marine molluscs. *Marine Biology* **103**, 51-61.

Volkman, J.K., Jeffrey, S.W., Nichols, P.D., Rogers, G.I., Garland, C.D., 1989. Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *Journal of Experimental Marine Biology and Ecology* **128**, 219 - 240.

Waite, A.M., Suthers, I.M., 2007. Open water: Plankton ecology. In: Connell, S.D., Gillanders, B.M. (Eds.), Marine Ecology. Oxford Unviersity Press, New York, pp. 279-312.

Weibel, E.R., 1979. Stereological Methods. Academic Press, London

West, G., 1990. Methods of assessing ovarian development in fishes: a review. *Marine and Freshwater Research* **41**, 199 - 222.

Whyte, J.N.C., 1987. Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. *Aquaculture* **60**, 231 - 241.

Whyte, J.N.C., Bourne, C., Hodgson, C.A., 1989. Influence of algal diets on biochemical composition and energy reserves in *Patinopecten yessoensis* (Jay) larvae. *Aquaculture* **78**, 333 - 347.

Widdows, J., 1973. The effects of temperature on the metabolism and activity of *Mytilus edulis*. *Netherlands Journal of Sea Research* **7**, 387 - 398.

Widdows, J., 1976. Physiological adaptation of *Mytilus edulis* to cyclic temperatures. *Journal of Comparative Physiology* **105**, 115 - 128.

Widdows, J., 1978. Combined effects of body size, food concentration and seston on the physiology of *Mytilus edulis*. *Journal of the Marine Biological Association of the United Kingdom* **58**, 109-124.

Widdows, J., Bayne, B.L., 1971. Temperature acclimation of *Mytilus edulis* with reference to its energy budget. *Journal of the Marine Biological Association of the United Kingdom* **51**, 827-843.

Widdows, J., Johnson, D., 1988. Physiological energetics of *Mytilus edulis* - Scope for growth. *Marine Ecology-Progress Series* **46**, 113-121.

Widdows, J., Hawkins, A.J.S., 1989. Partitioning of rate of heat dissipation by *Mytilus edulis* into maintenance, feeding, and growth components. *Physiological Zoology* **62**, 764-784.

Widdows, J., Fieth, P., Worrall, C.M., 1979. Relationships between seston, available food and feeding activity in the common mussel *Mytilus edulis*. *Marine Biology* **50**, 195-207.

Wikfors, G.H., Twarog, J.W.J., Ukeles, R., 1984. Influence of biochemical composition of algal food sources on growth of juvenile oysters, *Crassostrea virginica*. *Biological Bulletin* **167**, 251 - 263.

Wikfors, G.H., Ferris, G.E., Smith, B.C., 1992. The relationship between gross biochemical composition of cultured algal foods and growth of the hard clam, *Mercenaria mercenaria* (L.). *Aquaculture* **108**, 135 - 154.

Wilson, B.R., Hodgkin, E.P., 1967. A comparative account of the reproductive cycles of five species of marine mussels (Bivalvia:Mytilidae) in the vicinity of Fremantle, Western Australia. *Australian Journal of Marine and Freshwater Research* **18**, 175-203.

Wisely, B., 1964. Apsects of reproduction, settling, and growth in the mussel *Mytilus edulis planulatus* Lamarck. *Journal of the Malacological Society of Australia* **8**, 25 - 30.

Yamahira, K., Conover, D.O., 2002. Intra- vs. interspecific latitudinal variation in growth: Adaptation to temperature or seasonality? *Ecology* **83**, 1252-1262.

Yonge, C.M., 1976. The 'mussel' form and habit. In: Bayne, B.L. (Ed.), Marine mussels: their ecology and physiology. Cambridge University Press, Cambridge, pp. 1-13.

Zardi, G.I., McQuaid, C.D., Nicastro, K.R., 2007. Balancing survival and reproduction: Seasonality of wave action, attachment strength and reproductive output in indigenous *Perna perna* and invasive *Mytilus galloprovincialis* mussels. *Marine Ecology Progress Series* **334**, 155-163.

Zwaan, A.D., Zandee, D.I., 1972. Body distribution and seasonal changes in glycogen content of common sea mussel *Mytilus edulis*. *Comparative Biochemistry and Physiology* **43**, 53-58.