Effect of diet and sex-sorting on growth and gonad development in farmed South African abalone, *Haliotis midae*

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ABSTRACT

Abalone, *Haliotis midae*, farmers in South Africa that feed formulated diets reported a periodic drop in abalone growth during periods of increased gonad development. A large drop in abalone biomass was noticed after presumed spawning events. This study was aimed to determine the effect of diet and sex-sorting on gonad development in abalone. Experiments were conducted on a commercial abalone farm from July 2012 to the end of June 2013. Isonitrogenous and isoenergetic diets were formulated with two protein sources. A fishmeal and soybean meal (S-diet) diet and a fishmeal only (F-diet) diet were fed to abalone (50 - 70 g abalone^{-1}) over 12 months. Weight and length gain, gonad bulk index (GBI), visceral index (%) and meat mass index (%) were determined monthly and seasonally. A histological study on the female gonads was conducted. This study also included an experiment to test the effect of sex-sorting (70 - 80 g abalone^{-1}) on growth and body composition with treatments including males (M), females (F) and equal numbers of males and females (MF).

Weight gain and length gain were faster in S-diet-fed abalone (RM-ANOVA, $F_{(1, 16)} = 7.77$, $p = 0.01$; $F_{(1, 69)} = 49.9$, $p < 0.001$, respectively). Gonad development was significantly affected by the inclusion of soybean meal with S-diet-fed abalone showing higher GBI-values than F-diet-fed abalone (RM-ANOVA, $F_{(1, 33)} = 16.22$, $p = 0.0003$). Male abalone had higher GBI-values than females (RM-ANOVA, $F_{(1, 33)} = 39.87$, $p < 0.0001$). There was no significant difference in average feed conversion ratio (FCR) between diets over time (RM-ANOVA, $F_{(1, 21)} = 0.008$, $p = 0.97$). However, average FCR-values were significantly highest between November 2012 and March 2013, the presumed spawning season. The visceral mass (gut and gonad) as a proportion of whole mass (visceral index, %) was significantly higher in abalone fed the S-diet (RM-ANOVA; $F_{(1, 69)} = 68.06$, $p < 0.0001$). There was no difference in meat mass index (%) between diets for both male and female abalone (RM-ANOVA; $F_{(7, 248)} =$

0.80, p = 0.60; $F_{(7, 241)} = 1.7$, p = 0.11, respectively). Meat mass index significantly decreased from September 2012 to February 2013 coinciding with the period of high GBI-values.

The distribution of oocyte maturity stages differed between diets. The majority of oocytes within S-diet-fed abalone were fully mature stage 8 oocytes compared to a majority of stage 7 oocytes in F-diet-fed abalone. Histology corroborated peaks in GBI-values for abalone fed both diets.

There was no significant difference in growth, GBI, visceral index (%) and meat mass index (%) between abalone sorted into monosex and mixed-sex populations. Thus, the presence of the opposite sex did not have an effect on growth and gonad mass in *H. midae.*

The phytoestrogens daidzin, glycitin, genistin, daidzein, glycitein and genistein were present in soybean meal and only traceable amounts were found in the F-diet.

This study provided evidence that soybean meal present in formulated feed affected growth and gonad development in *H. midae*. The difference in the distribution of the maturity stages of oocytes was affected by diet. Sex-sorting abalone into monosex and mixed-sex populations had no influence on weight and length gain and gonad development.

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CHAPTER 1

GENERAL INTRODUCTION

The South African abalone, *Haliotis midae* is a large gastropod found in shallow sublittoral reefs from the Western Cape Province to Port St Johns (Barkai and Griffiths 1988, Hecht 1994). The distribution of *H. midae* is affected by temperature ranging from 12ºC in the Atlantic off the Western Cape to 21ºC in the Indian Ocean off the Eastern Cape (Sales and Britz 2001). Commercial exploitation of this species was driven by the export market which began in the 1950s (Tarr 1992). Rapid development of abalone aquaculture took place in the 1990s and is now widespread throughout many countries such as USA, Mexico, South Africa, Australia, New Zealand, Japan, China, Taiwan, Ireland and Iceland (Troell *et al.* 2006; Cook and Gordon 2010). The effort invested into research and development in South Africa began in 1990 when the University of Cape Town, the Council for Scientific and Industrial Research and Rhodes University initiated programmes to develop abalone aquaculture (Sales and Britz 2001). Since then, 12 abalone farms have been established ranging from Port Nolloth in the Western cape to East London with an overall investment of roughly US$ 12 million (Sales and Britz 2001). Most farms have a flow-through design and pump seawater into tanks on land. Some farms have both a hatchery and on-growing facilities while others rely on purchasing juveniles (Troell *et al.* 2006). The South African aquaculture industry is the second largest producer alongside Asia (Troell *et al.* 2006). This industry is economically important to South Africa fetching prices of about $34 to $40 per kilogram of live abalone, thus generating foreign income (Ten-Doeschate and Coyne 2008). The availability of abalone on the world market has rapidly increased from 20 000 metric tonnes

in the 1970s to an estimated 97 000 metric tonnes in 2012 (Gordon and Cook 2013). One of the major factors that has influenced the world market is the increase in illegal catches and the growth in farmed abalone production in China (Cook and Gordon 2010).

Abalone are dioecious broadcast spawners with a high fecundity. They release their gametes into the water through respiratory pores (Newman 1967). Eggs are fertilised externally followed by the development of a lecithotrophic larval stage (Wood and Buxton 1996). The ability to disperse is a major factor that affects the potential of abalone to colonise areas and the duration of the pelagic stages in gastropods can vary from two to 14 days. (Prince *et al.* 1987). Histological examinations and gonad bulk indices (Wood and Buxton 1996) showed that wild *H. midae* are iteroparous, asynchronous spawners, and the breeding season extends from March to October with spawning peaking between April and June. Natural spawning in molluscs is unpredictable however, a number of authors have identified water temperature (Webber and Giese 1969, Young and De Martini 1970), photoperiod (Wayne 2001), food availability (Shepard and Laws 1974) and sex pheromones (Counihan *et al.* 2001, Susswein and Nagle 2004, Cummins *et al.* 2005, Nhan *et al.* 2010) as cues. Factors that directly synchronise natural gamete release in abalone are not known due to the unpredictable spawning nature of haliotids. Induced spawning in abalone aquaculture has been successful using ultra-violet (UV) irradiated seawater, temperature shock, desiccation and hydrogen peroxide (Hahn 1989a). The structure of the ovary and testis, and the process of gametogenesis are typical of other haliotid species (Wood and Buxton 1996). Sexual maturity of *H. midae* is first attained at 20-25 mm shell width, and the size at 50% sexual maturity is 35 mm shell width (Wood and Buxton 1996). The gonad is cream-coloured in males, green in females and it surrounds the digestive gland. It makes up the bulk of the visceral mass (gut and gonad) and is found to the right side of the body around the right posterior margin of the

adductor muscle (Newman 1967). The gonad comprises of a lumen filled with a trabeculae of connective tissue bounded by the germinal epithelium (Newman 1967).

The techniques used for assessing gonad maturation and spawning periodicity include gonad indices, monitoring changes in gonad histology (descriptive) and analysing data from changes in oocyte development (maturity and size) over time (Gurney and Mundy 2004). Gonad indices are commonly used in many reproductive studies which involve measuring the mass of the gonads as a percentage of animal mass. More recently gonad indices have been based on cross-sectional measurements of the gonad digestive gland complex and differ in their complexity and accuracy (Grubert and Ritar 2004). The modified gonad bulk index (GBI) used by Tutschulte and Connell (1981) uses linear measurements from both the exterior and interior dimensions of gonad and digestive gland tissue in a cross section of the conical appendage to produce an estimated gonad volume (EGV). The EGV is then divided by the soft tissue mass of the abalone to give the GBI. The use of gonad bulk indices to study reproductive cycles involves assumptions (Gurney and Mundy 2004). These are that 1) a spent gonad is small and a ripe gonad is large, and that a decrease in mean gonad index (within a population) is an indication of spawning, 2) the gonad size is proportional to the animal's size (Hahn 1989a) and 3) the digestive gland size does not vary with season. Histological studies on the gonad development for annual reproductive cycles, cytological structures of maturing germ cells and oocyte morphometrics have been conducted on various abalone species (Newman 1967, Tutschulte and Connell 1981, Martin *et al.* 1983, Hahn 1994, Wood and Buxton 1996, Capinpin *et al.* 1998, Grubert and Ritar 2004, Najmudeen and Victor 2004, Fukazawa 2007, Najmudeen 2008, Bilbao *et al.* 2010, Roux *et al.* 2013). In descriptive histology, the state of maturation and reproductive activity is assigned a developmental stage to estimate reproductive development. The stage is defined by the

predominant characteristics of developing cells (Hahn 1989a). *Haliotis* species have complex spawning cycles where multiple spawning events occur, however, not all individuals spawn in a single event and asynchronous spawning patterns decrease the intensity of a spawning event (Gurney and Mundy 2004). For this reason, an extended decline over time in gonad indices indicates partial/incomplete (asynchronous) spawning compared to a significant drop and rise typical of a synchronous spawning event (Gurney and Mundy 2004).

One of the major drawbacks in abalone farming is the slow growth of the animal. Abalone under aquaculture conditions grow much faster compared to natural populations but they require four to five years to reach a market size of 80 mm (Ten-Doeschate and Coyne 2008). If this time period were to be shortened it would greatly reduce production costs and increase the annual turnover (Ten-Doeschate and Coyne 2008). As feed makes up the major component of operational costs, research into optimising these diets is essential (Britz *et al.* 1997). Although abalone are herbivorous, the industry is dependent on a fishmeal-based formulated diet and dependency will increase as the market grows and as availability harvested kelp becomes a limiting factor (Troell *et al.* 2006). South Africa was ranked the thirteenth largest consumer in the world in 2004 using approximately 101 000 tonnes of fishmeal (FIN 2005). This large consumption of fishmeal was largely dominated by the abalone industry (Hecht and Jones 2009). Due to logistical and accessibility problems, the supply of fresh macroalgae is limited. Therefore, many farms use formulated practical diets (Britz 1996b). Abalone are generalists and opportunistic herbivores that readily accept a wide range of diets. In the wild they prefer specific seaweeds, and a number of abalone species have been reported to prefer red algae (Barkai and Griffiths 1988, Troell *et al.* 2006). The South African industry used approximately 5900 t of kelp and about 180–200 tonnes of Abfeed® in 2004. Thus, research into alternative protein sources is important (Troell *et al.*

2006). Abfeed® (Marifeed Pty Ltd., Hermanus, South Africa) is a formulated feed that comprises of fishmeal, soybean meal, starch, vitamins and minerals. Abfeed® consists of approximately 35% protein, 43% carbohydrates, 5% fat, 1% crude fibre, 6% ash and 10% moisture (Marifeed (Pty) Ltd. pers. comm 2013). With kelp now approaching limits of sustainable harvesting, abalone feed development needs to match the growing demand for formulated diets (Troell *et al.* 2006). Commercially grown abalone grow well on Abfeed® until they reach 50 mm shell length and most of the farmers use this diet in the early stages of abalone growth (Francis *et al.* 2008).

One of the ingredients in Abfeed® is soybean meal which is widely used as a major protein source for aquaculture feeds because of its high protein content and essential amino acid profile (Robaina *et al.* 1995). However, soybean meal contains a number of different antinutrients such as protease inhibitors, lectins, phytic acid, saponins and phytoestrogens (Francis *et al.* 2001). Phytoestrogens are naturally occurring plant-based chemicals that can cause estrogenic and/or antiestrogenic effects due to their structural similarities to the hormone estradiol (Yildiz 2006). Phytoestrogens belong to a large group of substituted phenolic compounds known as flavonoids of which several groups have estrogenic properties (Yildiz 2006). The estrogenic potency of phytoestrogens is difficult to determine but *in vitro* and *in vivo* studies have shown that coumestans and isoflavones illustrate the greatest estrogenic properties with the most prevalent isoflavones being genistein, daidzein and glycitein (Yildiz 2006). Little information is known on the effect that phytoestrogens in formulated feeds have on aquaculture species but, phytoestrogens have been used in aquaculture and the amount of research conducted on this topic is greatly increasing. For example, genistein-based diets fed to rainbow trout, *Oncorhynchus mykiss* have shown to increase testicular development in males and plasma vitellogenin concentrations in females

(Bennetau-Pelissero *et al.* 2001). Plasma vitellogenin levels significantly increased in Siberian sturgeon, *Acipenser baeri* fed a soybean-based diet (Pelissero *et al.* 1991) and genistein-treated diets have been used for sex reversal in Mozambique tilapia, *Oreochromis mossambicus* (El-Sayed *et al.* 2012).

The worldwide decline of ocean fisheries stocks has given rise to growth in the aquaculture industry, however, it has questioned the environmental sustainability of ocean fisheries (Naylor *et al.* 2000). Approximately 1.9 kg of wild fish are required for each 1 kg of farmed production, and for flounder, sole, cod, sea bass and tuna more than 5 kg of wild fish were required (Naylor *et al.* 2000). The World Wildlife Fund for Nature (WWF) and the Southern African Sustainable Seafood Initiative (SASSI) promotes awareness to reduce the amount of wild fish caught for fishmeal in the aquaculture industry by largely focussing on retailers, restaurants and consumers (WWF 2011). The future of aquaculture feeds could largely depend upon lower-grade raw materials that may be further improved by processing and biotechnological transformation to provide a consistent nutrient source for farmed fish species (Costa-Pierce *et al.* 2012). Changing the farm's feed to a diet with less fishmeal, which does not reduce production and meat quality but still has enough protein to sustain optimal growth can greatly increase farm profits. Producing abalone using less fishmeal also creates a low forage fish efficiency ratio which has been proposed by the WWF to reduce fishing pressure on wild forage fish stocks (WWF 2011).

Abalone farmers in South Africa that rely on locally produced formulated feed, i.e. Abfeed®, reported a periodic drop in growth. This reduced rate of growth appeared to occur at certain times of year just after winter with presumed links to the production and release of gametes, and a large drop in the overall farm tonnage was noticed after spawning peaks or when environmental cues initiated mass spawning (Naylor pers. comm 2013). It also only affected

abalone of a certain age and it did not appear to affect abalone that were fed kelp. Abalone are slow growing and the infrastructure required for the land-based culture of these animals is expensive. Furthermore, the production costs are high due to the electrical energy required to pump seawater and the labour-intensive nature of these farming operations. Therefore, the increased time taken to reach market size, due to the periodic reduction in growth is costing the local abalone industry millions of Rand every year. This information was based on data collected by the farmers. Riddin (2013) conducted a study to find further evidence. Abalone fed only formulated feeds showed increased gonadal growth, and it was thus suggested that artificial abalone feeds can be manipulated to reduce gonad development (Riddin 2013). However, Riddin (2013) did not study gonad histology, thus, gonad development could not be explained.

This study evaluated the effects of diet and sex-sorting on growth and gonad development in farmed abalone. This was conducted by running farm-based experiments to compare two diets. Diets were formulated to be isonitrogenous and isoenergetic with the important difference being the protein source. One diet contained fishmeal and soybean meal as protein source and the other had only fishmeal as the protein source. This ensured that any differences observed in variables tested would be due to the difference in protein source. Overall growth, meat and gonad growth were measured to explain the effects that diets had on tissue composition. Riddin (2013) focussed on the effect of environmental conditions, dietary protein sources and energy levels on growth and gonad size in farmed *H. midae*. From the outcomes of this, the present study required the need to isolate the dietary ingredient, soybean meal and evaluate the effect this had on growth and gonad development. An assessment of the reproductive investment was conducted using a combination of growth, gonad bulk indices and histology. Furthermore, increased gonad development could be due to

the possibility of pheromones given off by opposite sexes. Therefore abalone were separated into monosex and mixed-sex populations to evaluate the effect the presence of the opposite sex had on growth and gonad development. The trade-off between growth and gonad development in farmed abalone during spawning season created a foundation for this research that was aimed at answering and manipulating this phenomenon.

AIM AND OBJECTIVES

The aim of the study was to explain the drop in growth that has been reported on commercial abalone farms in Hermanus, South Africa. This reduction in growth coincided with a period of increased gonad development. Thus, this research was aimed to determine the effect of diet and sex-sorting on gonad development in growing abalone. The research objectives of this study were to:

- Explain which ingredient in the formulated abalone feed was responsible for gonad development.
- Describe and validate when this drop in growth occurs.
- Provide reasons for increased gonad development by including a histological examination of the gonads.
- Determine whether sex-sorting abalone has an effect on growth and gonad development.

CHAPTER 2

EFFECT OF DIET ON GROWTH AND GONAD DEVELOPMENT

INTRODUCTION

Formulated feeds make up the majority of production costs on many of the abalone farms in South Africa (Britz and Hecht 1997, Sales 2001). With this in mind, and the global decreasing supply of fishmeal many studies have focused on alternative protein sources for abalone feeds (Britz 1996a, Naylor *et al.* 2000, Tung and Alfaro 2012, Troell *et al.* 2006, Shipton and Britz 2001). Formulated feeds result in higher growth rates compared with natural diets and abalone fed formulated diets had reduced weight loss during processing in comparison with those fed algal diets (Britz 1996b, Green *et al.* 2011). Formulated feed has been used in abalone culture in Japan, USA, and Australia, and feeding experiments in Taiwan showed that growth of juvenile abalone fed formulated diets was 65 % greater than in juveniles fed their natural diet of macroalgae (Bautista-Teruel *et al.* 2003). Formulated feed for abalone must contain a sufficient amount of protein and essential amino acids in order to satisfy their nutrient requirements and the most common ingredients used in abalone diets are fishmeal, defatted soybean meal and casein (Guzman and Viana 1998). Fishmeal is a protein source which supports good growth in abalone fed a formulated feed and, used in combination with other sources such as soybean meal, it can significantly reduce feed costs. This study evaluated the effect of the dietary ingredient soybean meal on growth and gonad development, by formulating two isonitrogenous and isoenergetic diets to exclude other potentially confounding factors such as energy and protein ratios.

The inclusion of soybean meal into formulated feeds had an effect on gonad development in *Haliotis midae*, however, the reasons for this were not understood (Riddin 2013). Soybean meal has been included in many formulated abalone diets without a significant effect on overall growth (Shipton and Britz 2001). Farmers have reported highly variable growth during spawning season with presumed links to the production and release of gametes, and a large drop in the farm production was noticed after spawning peaks (Naylor pers. comm 2013). Furthermore, soybean meal contains antinutrients such as phytoestrogens that may affect reproductive development (El-Sayed *et al.* 2012). The majority of studies on phytoestrogens have reported isoflavones in soybean products. However, the isoflavone concentration in soybean is affected by variety, environmental conditions, the genotype and interaction between these factors (Wang and Murphy 1994). Phytoestrogens may have the same effects as estrogen or have estrogen-blocking effects. However, this depends on the ratio of phytoestrogens to endogenous estrogens, enzyme activity, animal species, reproductive status, length of the exposure and method of administration (Bennetau-Pelissero *et al.* 2001, El-Sayed *et al.* 2012, Pollack *et al.* 2003). This study will demonstrate the effects of diet on reproduction in *H. midae* with the addition of testing the phytoestrogen content in the dietary ingredient, soybean meal.

Haliotis midae is long-lived, however, the proportion of energy allocated to somatic growth differs between juveniles and mature abalone (Barkai and Griffiths 1988). All energy is allocated to somatic growth in juveniles. Conversely, when maturity is attained an increasing proportion of energy is allocated to reproductive output (Barkai and Griffiths 1988). Furthermore, there is a difference between the dietary protein requirements of juvenile and young adult abalone (Shipton and Britz 2001). It is very difficult to quantify and make comparisons of fecundity estimations and reproductive output due to the many variations in research methodology and physiological complexity among *Haliotis* species (Bilbao *et al.*

2010). Some studies are based on artificial spawning inductions and some are based on natural populations and counting eggs during natural spawning (Capinpin *et al.* 1998, Leaf *et al.* 2008). Furthermore, other reproduction studies are approached by identifying mature oocytes according to size or morphological features and calculating the fecundity based on gonad mass or volume (Tutschulte and Connell 1981, Wood and Buxton 1996). This chapter looked at similar reproductive assessments by describing reproductive output according to size or morphological features based on weight and volume calculations. Gonad bulk indices (GBI) were used to quantify reproductive investment based on methods used by Tutschulte and Connell (1981), using linear measurements from both the exterior and interior (the dimensions of each tissue in cross section) of the gonad and digestive gland to produce an estimate of gonad volume (EGV) and calculate GBI. In many abalone species there is no resting phase in the reproductive cycle and gametogenesis is initiated immediately after each spawning event allowing for assessment of reproductive development all year round rather than restricting observations to periods during the spawning seasons (Webber 1970).

The research aims of this study were to evaluate the effect of the dietary ingredient, soybean meal and its effect on growth and gonad development in farmed *H. midae.* The aim was addressed by quantifying morphological features such as meat and visceral mass (gut and gonad) based on weight and volume calculations to assess the effect of soybean meal. Other factors such as water loss in tissues and change in average mass and length were also evaluated. Phytoestrogen concentrations in soya were measured in order to obtain reference values for this and other studies. This lead to the following hypotheses:

H_{01}: The formulated abalone feed ingredient, soybean meal, has no effect on growth and gonad development in farmed South African abalone, *Haliotis midae.*

H_{a1}: Growth and gonad development in farmed South African abalone, *Haliotis midae* are affected by the addition of soybean meal to the formulated diet.

METHODS AND MATERIALS

Experimental system

The experiments were conducted at HIK Abalone Farm (Pty) Ltd. in Hermanus, Western Cape (34º26'04.35"S; 19º13'12.51"E) from July 2012 to June 2013. HIK Abalone Farm (Pty) Ltd. pumped water directly from the sea into a header tank where it was filtered by micro-screen drum filters (100 µm). Water was then gravity-fed into the farm's canvas tanks (3.95 X 1.75 X 0.75 m), which were all supported by wooden frames. Each canvas tank held 12 'oyster mesh' baskets (75 X 50 X 65 cm) that contained seven vertical acrylonitrile butadiene styrene plastic plates with a surface area of 3.2 m^2, and a horizontal feeder plate that was submerged 10 cm under the water. The water flow rate to the farm tanks was maintained to achieve 1.5 exchanges per hour. All tanks on the farm were aerated through 20 mm polyvinyl chloride (PVC) tubing which ran along the bottom length of the tank. Each tank was cleaned once a week by moving baskets across to clean open tanks as per standard rotation procedures used on the farm.

Diet preparation

Two isonitrogenous and isoenergetic diets were formulated with fishmeal and soybean meal as the main protein sources (Table 2.1). The soya/fishmeal (S-diet) diet had a combination of soybean meal and fishmeal and the other diet (F-diet) consisted of only fishmeal as the protein source. These diets were manufactured at Marifeed (Pty) Ltd. in Hermanus. Abalone were fed their respective diet to apparent satiation at 16h00 every day for 12 months. Feed was given to each basket that had less than 15 pellets on the feeder plate from a cup that contained 66.6 ± 3.2 g of pellets, a method to minimise feed waste (Britz *et al.* 1997). Nine

tanks and 108 baskets were used for each diet. Each tank was allocated a bucket of the respective diet of which the remaining food was weighed before the bucket was refilled to obtain the data needed to calculate feed conversion ratio (FCR) (Equation 1). The feed conversion ratio (dry weight fed / wet weight gain) was calculated at the end of every four months after farm size-grading procedures when every basket was weighed.

$$FCR = \frac{Feed\ consumed\ (g)}{Mass\ gained\ (g)} \quad [1]$$

Proximate analysis was done to obtain moisture, ash, crude protein, gross energy and lipid content from the feed evaluation unit at the University of Kwazulu Natal using standard methods of the AOAC (2003) (Table 2.1). Six samples were taken from each diet. Crude protein was determined using the Dumas combustion method in a LECO FP2000 Nitrogen analyser (AOAC 2003, method 990.03). Lipid was extracted from samples by solvent petroleum ether (Soxhlett procedure) using a Buchi 810 Soxhlett fat extractor. The lipid percentage was then calculated by gravimetric analysis (AOAC 2003, method 920.39). Ash content was determined by placing the sample in a furnace for four hours at 550 °C (AOAC, method 942.05). The samples were dried in an air circulated oven at 95°C for 72 h to determine the moisture content (AOAC 2003, method 934.01). Energy was calculated using a bomb calorimeter (DDS isothermal CP500, Digital Systems, Johannesburg).

Table 2.1: Proximate analysis of crude protein, moisture, fat, gross energy and ash for each diet (mean ± standard deviation). The S-diet (n = 6) and F-diet (n = 6) diets were fed from July 2012 to June 2013. The S-diet diet consisted of soybean meal and fishmeal as main protein source and F-diet had only fishmeal as the main protein source.

	S-diet	F-diet
Fishmeal / soybean meal ratio:		
Fishmeal	0.68	1
Soybean meal	0.32	0
Crude protein (%)	35.98 ± 0.64	35.45 ± 0.85
Moisture (%)	8.09 ± 0.37	8.00 ± 0.58
Fat (%)	3.93 ± 0.56	4.65 ± 0.86
Gross energy (MJ/kg)	18.98 ± 0.59	18.70 ± 0.74
Ash (%)	7.07 ± 1.38	9.03 ± 2.3

Both experimental diets and all feed ingredients were sent to Covance Laboratories Inc. Philadelphia, United States of America for isoflavone analysis (AOAC 2000). The samples were extracted using a solution of hydrochloric acid and reagent alcohol heated on steam baths or hot plates. The extract was brought to volume, diluted, and centrifuged. An aliquot of the supernatant was placed onto a C18 solid-phase extraction column where unwanted components of the matrix were rinsed off with 20 % methanol and then isoflavones were eluted with 80 % methanol. The samples were analyzed on a high-performance liquid chromatography (HPLC) system with ultraviolet (UV) spectrophotometric detection and compared against an external standard curve of known standards (AOAC 2000).

Experimental abalone and acclimation

Haliotis midae spawned by several females in November 2009 on HIK Abalone Farm (Pty) Ltd. were kept aside. Prior to this, these abalone were fed a formulated abalone diet (34.7 % protein, 2.4 % lipid, 57.3 % carbohydrate, 1.6 % fibre, 5.6 % ash, Abfeed® S34, Marifeed (Pty) Ltd, Hermanus, South Africa) daily. Mature abalone were fed their respective experimental diets for one month to acclimate the animals to each diet before the start of the experiments. After the acclimation period the abalone were allocated to 18 standard farm tanks (nine tanks per diet) by grading them into a 50 - 70 g abalone^{-1} weight range. These animals were stocked at 18 % of the available surface area with 161.2 ± 4.5 abalone basket^{-1}. This stocking density was maintained for 12 months as abalone were graded every four months using standard farm size-grading procedures. At each size-grading, 10 abalone from each basket were weighed (0.01 g) using an electronic scale (Kern PLS 4200-2F, serial number: WIC1200486) and length was measured (0.01 mm) using vernier callipers (n = 1080 for each diet).

Environmental parameters

Temperature, pH, dissolved oxygen concentration (mg L^{-1}), percent oxygen concentration and the concentration of total ammonia nitrogen (TAN; µg L^{-1}) were measured every second week from the effluent water of each tank using a water quality meter (YSI Inc. Pro Plus Multi-parameter meter, Yellow Springs, Ohio, USA). Average pH was calculated using log-transformed values. Samples were obtained in acid-washed glassware between 08h00 and 10h00 every morning as this was the most reliable time of day to do water quality analysis due to the nocturnal activity of abalone (Yearsley *et al.* 2009). The TAN values were calculated by using the phenol hypochlorite method of Solorzano (1969). After the reagents (Merck (Pty) Ltd, South Africa) were added, the samples were kept dark for one hour. Colour

absorbance readings were then read on a spectrophotometer (Prim Light, Secomam, 30319, Ales, France) at a wavelength of 640 nm. These readings were converted into concentration of TAN using linear regression standard curves derived from using known concentrations of ammonium chloride (Appendix). Free ammonia nitrogen (FAN) was then calculated using TAN values, pH, temperature and salinity (Bower and Bidwell 1978).

Experimental design

Dry weights

On the first Monday of every month three male and three female abalone were randomly selected from six baskets in every tank (n = 108) and transferred into labelled cotton mesh bags. These bags were then packed immediately into boxes and taken to the processing plant (SPP Canning (Pty) Ltd. Hermanus, South Africa). The abalone were left in a chilling room (± -10ºC) before being shucked. Prior to this, all experimental abalone were not fed for two days to purge their gut. This method of purging, compared to moving the animals to purging tanks for three days, reduced the amount of handling on the animal and therefore reduced the chances of an unwanted spawning event that may be triggered by handling stress. Each abalone was shucked, weighed (0.01 g) using an electronic scale (Kern PLS 4200-2F, serial number: WIC1200486) and length was measured (0.01 mm) using vernier callipers. The total soft tissue was separated into meat and visceral complex (gut and gonad), which was cut from the foot of the abalone. The meat and visceral complex were then weighed (0.01 g). The meat section was cut into quarters and two quarter pieces were weighed (0.01 g), placed into tins and dried at 50 ºC in a drying oven for 96 hours. The visceral complex and shells were placed in separate tins and dried in the same oven. The whole piece of meat was too large to dry and therefore the dried samples of meat were used to calculate the total meat dry mass by factoring in a moisture loss ratio (Equation 2). The dried pieces of meat, viscera and shell

were weighed (0.01 g) and the water loss from viscera and meat were calculated (Equations 3 and 4, respectively).

$$M_D = M_T - \left[M_T \times \left(\frac{(M_{WS} - M_{DS})}{M_{WS}} \times 100\right)\right] \quad [2]$$

Total dry meat (M_D) was calculated using Equation 2 where M_T is the total wet mass of the meat (g), M_{WS} is the wet mass of the wet meat mass sample (g) and M_{DS} is the dry mass of the meat mass sample (g).

$$Water\ loss\ from\ viscera\ (\%) = \left[\left(\frac{V_M - V_D}{V_M}\right) \times 100\right] \quad [3],$$

where V_M is the total wet mass of the viscera (g), V_D is the total dry mass of the viscera (g)

$$Water\ loss\ from\ meat\ (\%) = \left[\left(\frac{M_T - M_D}{M_T}\right) \times 100\right] \quad [4],$$

M_T is the total wet mass of the meat (g) and M_D is the total dry mass of the meat (g).

Gonad bulk index

The gonad bulk index (GBI) was used to represent the volume of the gonad as a proportion of the total body weight. The GBI used by Tutschulte and Connell (1981) used linear measurements from both the exterior and interior (the dimensions of each tissue in cross section) of the gonad and digestive gland to produce an estimate of gonad volume (EGV). The EGV was then divided by the total wet weight of the animal to give the GBI. On the first Tuesday of every month three male and three female abalone were randomly selected from

six baskets in every tank (n = 108) and transferred into labelled cotton mesh bags. These bags were then packed immediately and taken to the processing plant (SPP Canning (Pty) Ltd.). The animals were then left in a chilling room (± -10ºC) before being shucked. Each abalone was shucked, weighed (0.01 g) and measured (0.01 mm) using vernier callipers. The total soft tissue was then separated into meat and visceral complex (gut and gonad), which was cut from the foot of the abalone. The meat and visceral complex were then weighed (0.01 g) and a meat mass index and visceral index was calculated (Equations 3 and 4, respectively). The removed visceral mass from each animal was placed in separate 40-ml labelled plastic vials with Davidson's fixative (20 % formalin, 10 % glycerol, 10 % glacial acetic acid, 30 % absolute ethanol and 30 % seawater). These gonads were stored until needed to calculate the GBI in the laboratory. Meat mass index was calculated using Equation 5:

$$Meat\ mass\ index\ (\%) = \frac{M_m}{W_m} \times 100 \quad [5],$$

where meat mass index is the percentage of meat that makes up the total abalone mass, M_m is the wet meat mass (g) and W_m is the whole abalone mass (g). Visceral index was calculated using Equation 6:

$$Visceral\ index\ (\%) = \frac{V_m}{W_m} \times 100 \quad [6],$$

where visceral index is the percentage of viscera that makes up the total abalone mass, V_m is the wet visceral mass (g) and W_m is the whole abalone mass (g).

The gonads were placed onto a calibrated labelled grid and a photographed (Finepix XP20, Fujifilm, Japan). A section was then cut through the midpoint of the gonad and placed back onto the same grid where another photograph was taken. The length and width of the cross-sectional view of the gonad and digestive gland were measured using a measuring software programme SIGMASCAN® PRO 5 (Systat Software, San Jose, CA, USA). These measurements were then used to calculate the effective gonad volume (EGV) according to Equation 7 (Tutschulte and Connell 1981), where L_{ca} is the curved length of the conical appendage, *a, b, x* and *y* are the length and breadth of the gonad and digestive gland, respectively (Figure 2.1 and Figure 2.2).

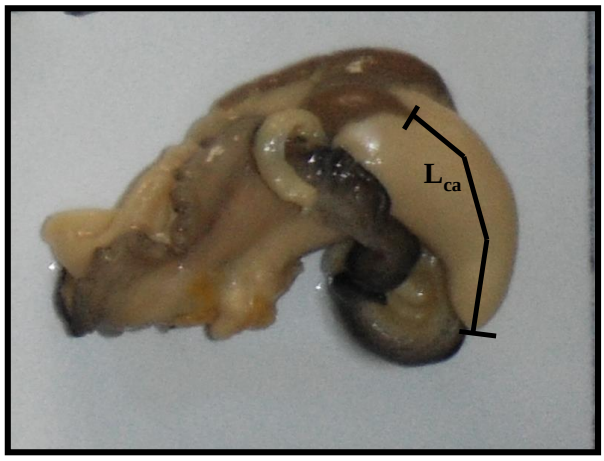


Figure 2.1: Photograph of a male viscera illustrating the curved length of the conical appendage (L_{ca}).

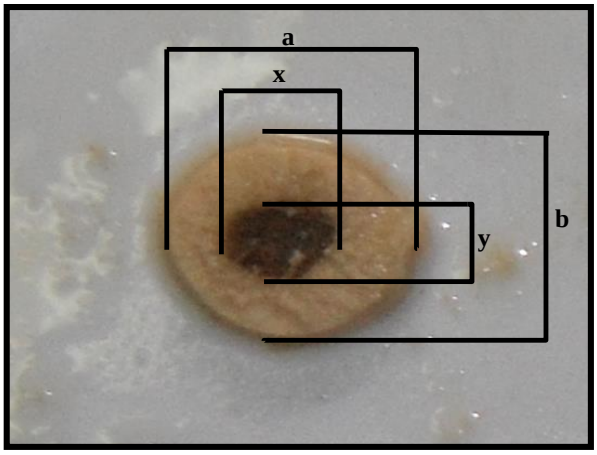


Figure 2.2: Photograph of a section from the midpoint of the curved length from a male viscera. Linear dimensions of the gonad (a and b) and digestive gland (x and y) are used in the calculation of EGV (Equation 7).

$$EGV = \frac{\text{Lca}\,\pi}{96}\left[8(a+b)^2 - \frac{(x+y+a+b)^3}{(a+b)}\right] \quad [7]$$

This EGV of each abalone was divided by its whole body weight to calculate gonad size as a proportion of the abalone soft tissue (Equation 8), where GBI is the gonad bulk index, EGV is effective gonad volume and W_s is the shucked weight (g) of the abalone.

$$GBI = \frac{EGV}{W_s} \quad [8]$$

The DG index is the percentage of the cross sectional area of the digestive gland tissue relative to gonad tissue where linear dimensions of the gonad (a and b) and digestive gland (x and y) are used in the calculation (Equation 9).

$$DG\ index\ (\%) = \frac{(x \times y)}{(a \times b)} \times 100 \quad [9]$$

The condition factor (CF) was calculated from abalone taken every four months, where weight is the mass of the animal (g) and length is the length (mm) of the shell (Equation 10; Britz 1996a). Ten animals from each basket were randomly sampled, weighed (0.01 g) and measured using vernier callipers to calculate CF.

$$CF = \left(\frac{weight}{length^{2.99}}\right) \times 5575 \quad [10]$$

Statistical analysis

The treatment means for each of the dependent variables were compared using a repeated measures analysis of variance (RM-ANOVA) at an error level of 5 % ($p \leq 0.05$). The assumptions that were met included homogeneity of variance (Levene 1960) and normal distribution of the residuals (Shapiro and Wilk 1965). If sphericity was not met the p-values and degrees of freedom were adjusted using the Greenhouse and Geisser (G-G) method (Greenhouse and Geisser 1959). Data for meat mass index (Equation 5) and visceral index (Equation 6) were pooled for both sampling days (i.e. dry weight data from Monday and GBI data from Tuesday every month). Tukey's HSD post-hoc tests were used to compare treatment means. Linear regression analysis was carried out to determine linear relationships between variables. All analyses were performed using Statistica 11 software (Copyright © 1984-2012 StatSoft, Inc.).

RESULTS

Growth

The average mass at the start of the experiment was 62.9 ± 0.71 g abalone $^{-1}$ and by the end of the trial this had increased to 107.1 ± 7.27 g abalone $^{-1}$. Change in average mass was significantly faster in S-diet-fed abalone compared to F-diet fed abalone (RM-ANOVA, $F_{(1,}$

$_{16)}$ = 7.77, p = 0.01, Figure 2.3). There was a significant interaction between diet and time in average abalone mass (RM-ANOVA, $F_{(2, 28)}$ = 8.35, p = 0.002, G-G adjusted, Figure 2.3). There was no difference in mass between treatments between July 2012 and March 2013 (Tukey's post-hoc; p > 0.05). However, there was a significant difference in average abalone mass between diet treatments in June 2013 (Tukey's post-hoc; p < 0.0001).

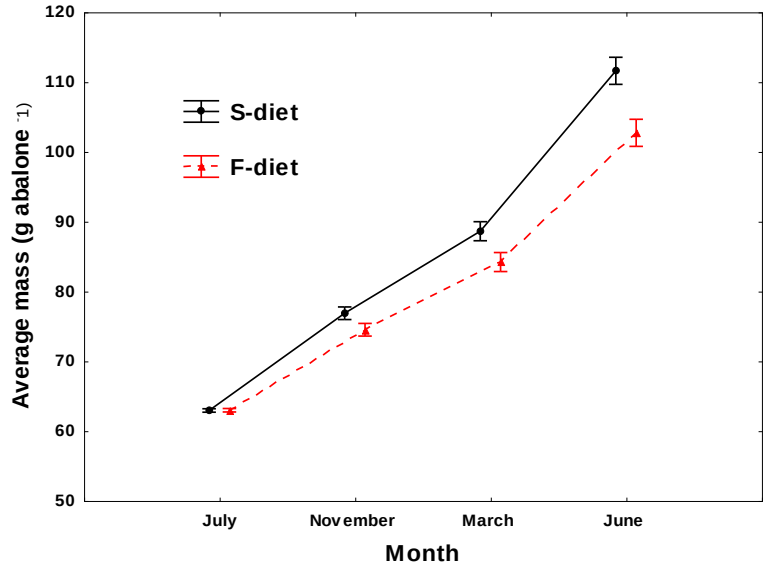


Figure 2.3: The change in average mass (mean ± standard error) of abalone fed a fishmeal and soya diet (S-diet) and fishmeal only diet (F-diet) from July 2012 to June 2013 (RM-ANOVA, $F_{(2, 28)}$ = 8.35, p = 0.002).

The average change in length (mm) was higher in abalone fed the S-diet compared to those fed the F-diet (RM-ANOVA, $F_{(1, 69)}$ = 49.9, p < 0.001, Figure 2.4) and there was a significant interaction between diet and time with average length changing differently for over time (RM-ANOVA; $F_{(11, 759)}$ = 2.26, p = 0.01; Figure 2.4).

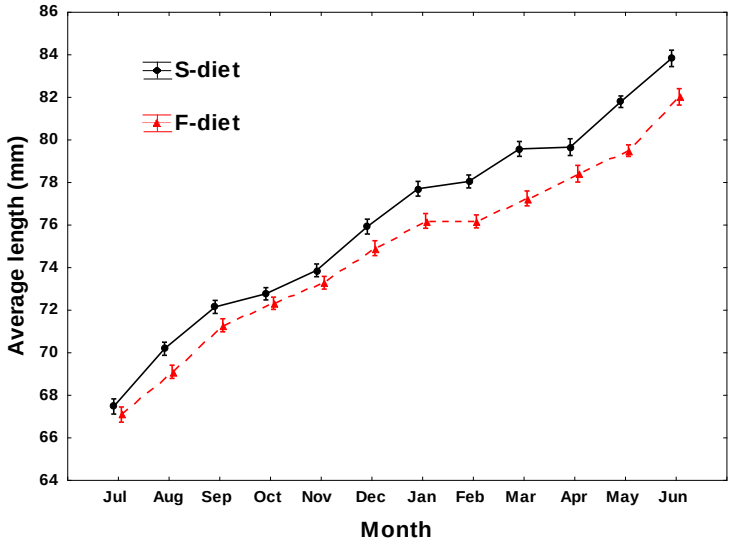


Figure 2.4: The average length (mean ± standard error) of abalone fed a fishmeal and soya diet (S-diet) and fishmeal only diet (F-diet) from July 2012 to June 2013 (RM-ANOVA, $F_{(3, 48)} = 8.34$, $p < 0.001$).

There was no significant interaction between diets for feed conversion ratio (FCR) over time (RM-ANOVA, $F_{(1, 21)} = 0.008$, $p = 0.97$, G-G adjusted, Table 2.2). There was a significant difference in FCR between November and March and between June and March (Tukey's post-hoc analysis, $p < 0.05$). There was no significant difference in FCR between June and November (Tukey's post-hoc; $p > 0.05$). The mean FCR for the S-diet diet was 1.63 ± 0.47 and the F-diet diet averaged 1.71 ± 0.48. There was no significant interaction between diets for change in condition factor over time (RM-ANOVA, $F_{(2, 32)} = 1.67$, $p = 0.2$, Table 2.2). The mean condition factor for the S-diet diet was 1.13 ± 0.03 and the F-diet had a mean of 1.12 ± 0.02.

Table 2.2: The mean (± standard deviation) feed conversion ratio (FCR) and condition factor for each diet over three periods. Significant differences are indicated by different superscripts within each row and column (Tukey's post-hoc test; $p < 0.05$). The S-diet diet consisted of soybean meal and fishmeal as main protein source and F-diet had only fishmeal as the main protein source.

	July 2012 to November 2012	November 2012 to March 2013	March 2013 to June 2013	12 month Average
FCR				
S-diet	1.49 ± 0.16^{a}	2.17 ± 0.44^{b}	1.25 ± 0.14^{a}	1.63 ± 0.47
F-diet	1.55 ± 0.19^{a}	2.23 ± 0.46^{b}	1.33 ± 0.11^{a}	1.7 ± 0.48
Condition factor				
S-diet	1.16 ± 0.02	1.1 ± 0.01	1.13 ± 0.03	1.13 ± 0.03
F-diet	1.14 ± 0.02	1.1 ± 0.02	1.11 ± 0.02	1.11 ± 0.02

Gonad development

Combined sexes (GBI)

Abalone fed the S-diet had a significantly higher GBI ($mm^3\ g^{-1}$) than those fed the F-diet (RM-ANOVA, $F_{(1, 33)} = 16.22$, $p = 0.0003$, Figure 2.5). The mean GBI for the S-diet diet was 27.25 $mm^3\ g^{-1} \pm 15.75$ and the F-diet had a mean GBI of 22.89 $mm^3\ g^{-1} \pm 16.5$. There was no significant interaction in the mean GBI between diets over time showing the trends for both diets changed similarly (RM-ANOVA, $F_{(7, 244)} = 1.21$, $p = 0.29$, G-G adjusted, Figure 2.5).

Separate sexes (GBI)

The GBI was higher in male abalone compared to females (RM-ANOVA, $F_{(1, 33)} = 39.87$, $p < 0.0001$). The mean GBI between each diet for both males and females was significantly different with S-diet-fed abalone showing higher values than those fed F-diet (RM-ANOVA, $F_{(1, 16)} = 6.41$, $p = 0.02$ for males; $F_{(1, 16)} = 9.92$, $p = 0.006$ for females; Figure 2.6a and 2.6b, respectively). There was no significant interaction between diet and time for the GBI in both

sexes (RM-ANOVA, $F_{(11, 176)} = 0.79$, $p = 0.65$ for males; $F_{(8, 92)} = 1.35$, $p = 0.25$, G-G adjusted for females; Figure 2.6a and 2.6b, respectively).

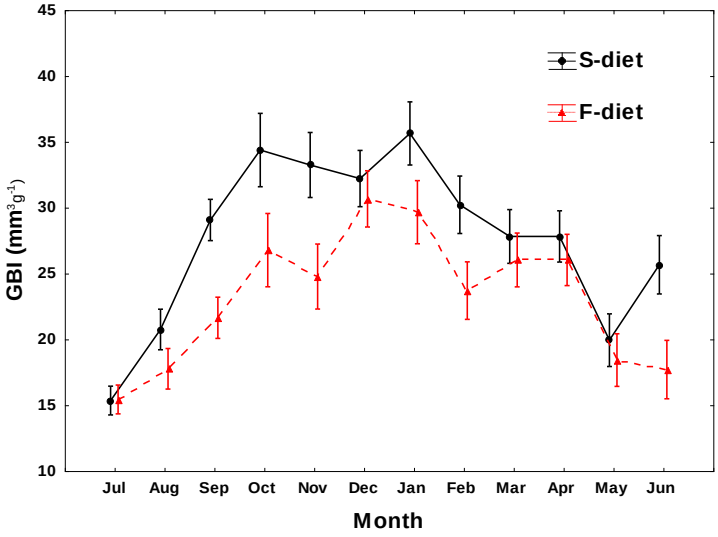


Figure 2.5: The gonad bulk index (GBI) (mean ± standard error) of abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013 (RM-ANOVA, $F_{(7, 244)} = 1.21$, $p = 0.29$, G-G adjusted).

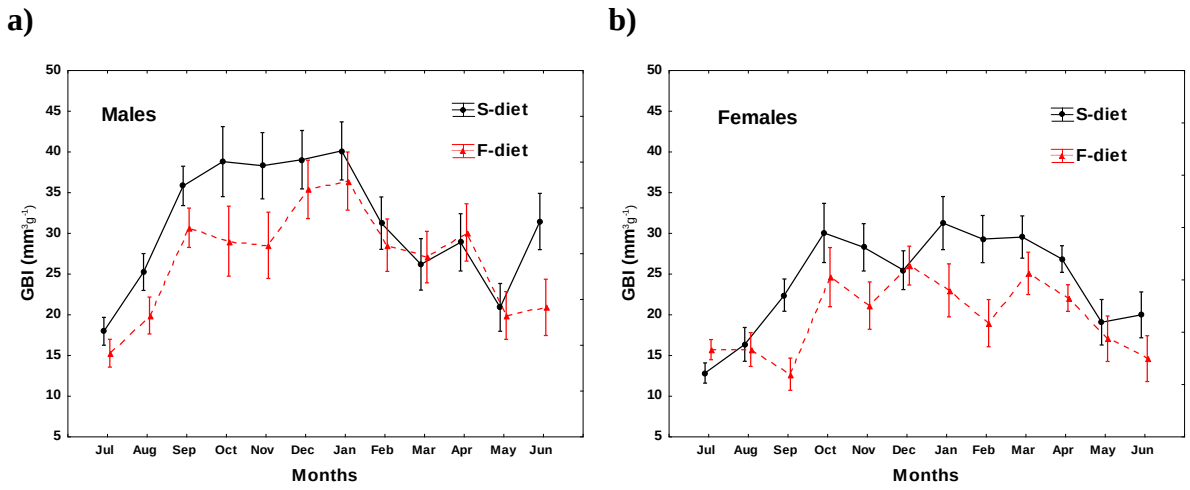


Figure 2.6: The gonad bulk index (GBI) (mean ± standard error) for male (a) and female (b) abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013 (RM-ANOVA, $F_{(11, 176)} = 0.79$, $p = 0.65$ for males; $F_{(8, 92)} = 1.35$, $p = 0.25$, G-G adjusted for females).

Visceral index (%)

Combined sexes

Abalone fed the S-diet had a higher visceral index compared to those fed the F-diet (RM-ANOVA; $F_{(1, 69)} = 68.06$, $p < 0.0001$, Figure 2.7). The mean visceral index was significantly influenced by an interaction between diet and time (RM-ANOVA; $F_{(11, 759)} = 2.53$, $p = 0.004$, Figure 2.7). There was a difference between diets for the months of October, December, February, May and June (Tukey's post-hoc; $p < 0.05$). The visceral index (average ± standard deviation) was 11.19 ± 1.58 % and 10.61 ± 1.53 % for abalone fed the S-diet and F-diet, respectively.

Separate sexes

Male abalone had higher visceral indices (%) than female abalone (RM-ANOVA; $F_{(1, 69)} = 24.35$, $p < 0.0001$) with visceral index changing similarly between sexes over time (RM-ANOVA; $F_{(11, 759)} = 1.65$, $p = 0.08$). The mean visceral index between diets for both males and females was significantly different with S-diet fed abalone showing higher values than those fed F-diet for both sexes (RM-ANOVA, $F_{(1, 34)} = 37.24$, $p < 0.0001$ for males; $F_{(1, 34)} = 31.91$, $p < 0.0001$ for females; Figure 2.8a and 2.8b, respectively). There was no significant interaction between diet and time for the visceral index in both sexes (RM-ANOVA, $F_{(11, 374)} = 1.59$, $p = 0.10$ for males; $F_{(11, 374)} = 1.76$, $p = 0.06$ for females; Figure 2.8a and 2.8b, respectively).

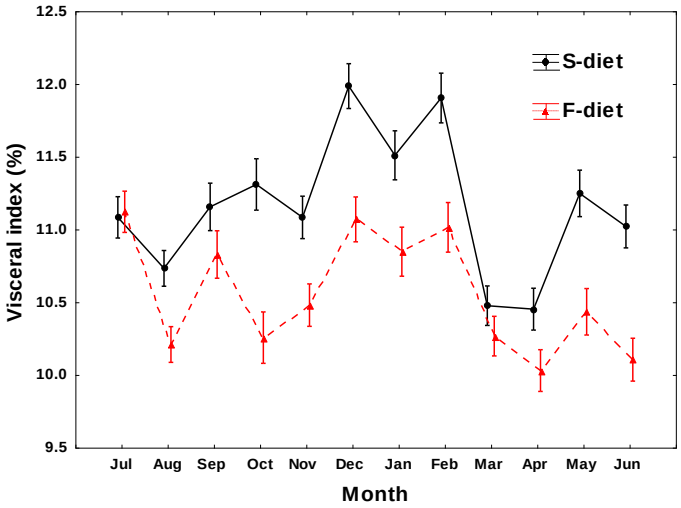


Figure 2.7: The visceral index (mean ± standard error) of abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013 (RM-ANOVA; $F_{(11, 759)} = 2.53$, $p = 0.004$).

a) **b)**

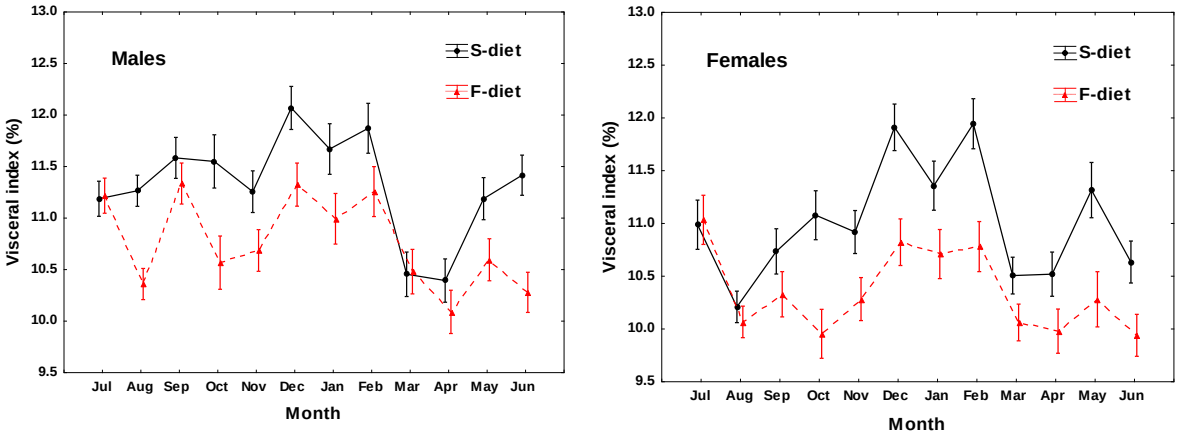


Figure 2.8: The visceral index (mean ± standard error) for male (a) and female (b) abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013 (RM-ANOVA, $F_{(11, 374)} = 1.59$, $p = 0.10$ for males; $F_{(11, 374)} = 1.76$, $p = 0.06$ for females).

Water loss from viscera (%)

Male abalone lost more water from the viscera compared to female abalone (RM-ANOVA; $F_{(1, 33)} = 100$, $p < 0.001$, Figure 2.9). Abalone fed the F-diet diet lost more water from the

viscera than animals fed the S-diet for both sexes (RM-ANOVA, $F_{(1, 16)} = 26.7$, $p < 0.0001$ for males; $F_{(1, 16)} = 38.9$, $p < 0.0001$ for females; Figure 2.9a and 2.9b, respectively). There was no significant interaction between diet and time for the water lost from viscera in both sexes (RM-ANOVA; $F_{(5, 82)} = 0.6$, $p = 0.70$ for males and $F_{(5, 73)} = 1.8$, $p = 0.12$ for females, G-G adjusted for both sexes, Figure 2.9a and 2.9b, respectively). Mean (± standard deviation) percentage water loss from viscera for females was 73.9 ± 2.86 and 75.4 ± 2.9 for S-diet and F-diet diets, respectively. The mean (± standard deviation) percentage water loss from viscera for males was 75.6 ± 2.13 and 76.6 ± 2.13 for S-diet and F-diet diets, respectively. There was a significant difference in percentage water loss from the viscera in females for the months of October, February, March and April (Tukey's post-hoc; $p < 0.05$).

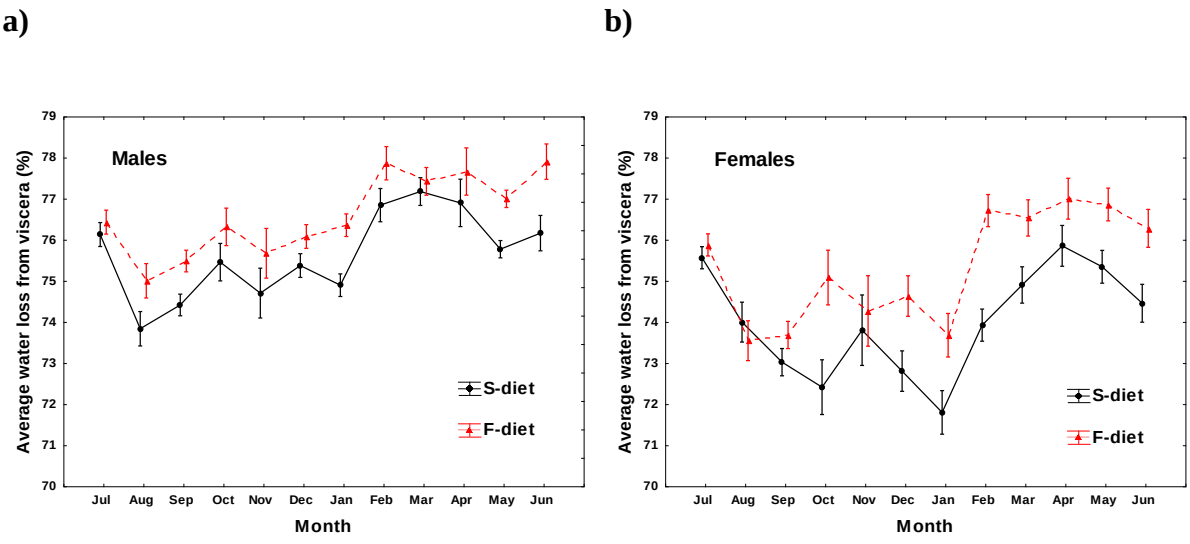


Figure 2.9: The water loss from viscera (mean ± standard error) for male (a) and female (b) abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013 (RM-ANOVA; $F_{(5, 82)} = 0.6$, $p = 0.70$ for males and $F_{(5, 73)} = 1.8$, $p = 0.12$ for females).

Digestive gland (DG) index

The digestive gland index was higher in female abalone compared to male abalone (RM-ANOVA; $F_{(1, 33)} = 15.00$, $p = 0.0004$, Figure 2.10). There was no significant difference in DG index between diets for males (RM-ANOVA; $F_{(1, 16)} = 4.15$, $p = 0.06$, Figure 2.10a). However female abalone fed the F-diet had a higher average DG index than those fed the S-diet (RM-ANOVA; $F_{(1, 16)} = 5.59$, $p = 0.03$, Figure 2.10b). There was no significant interaction between diet and time for the DG index in both sexes, therefore DG index changed similarly over time for both sexes (RM-ANOVA, $F_{(5, 86)} = 0.62$, $p = 0.69$ for males; $F_{(6, 88)} = 1.67$, $p = 0.14$ for females, both sexes G-G adjusted; Figure 2.10a and 2.10b, respectively). The DG index significantly decreased from July 2012 to November 2013 and significantly increased from December 2012 to May 2013 for both diets.

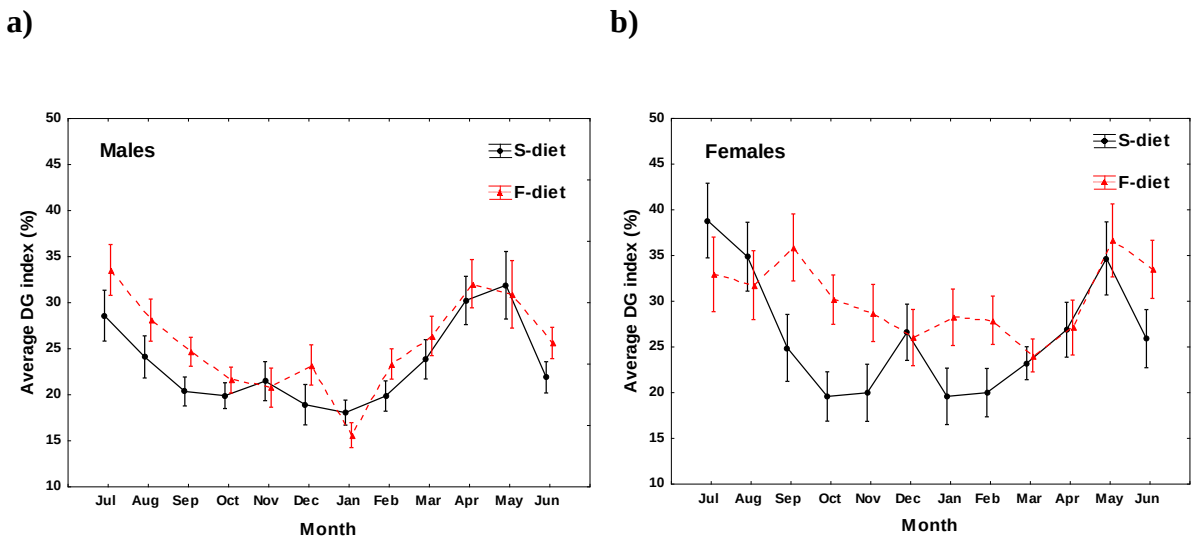


Figure 2.10: The DG index (mean ± standard error) for male (a) and female (b) abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013. RM-ANOVA, $F_{(5, 86)} = 0.62$, $p = 0.69$ for males; $F_{(6, 88)} = 1.67$, $p = 0.14$ for females, both sexes G-G adjusted).

Meat mass index (%)

The meat mass index (%) was similar for both diet and sex over time (RM-ANOVA; $F_{(7, 248)} = 0.80$, $p = 0.60$ for males and $F_{(7, 241)} = 1.7$, $p = 0.11$ for females, G-G adjusted for both sexes, Figure 2.11a and 2.11b, respectively). Meat mass index significantly decreased from September 2012 to February 2013 for both sexes coinciding with the period of high GBI values. The meat mass index (average ± standard deviation) was 61.50 ± 3.01 % and 61.25 ± 2.77 % for abalone fed the S-diet and F-diet, respectively. Soft tissue (meat and viscera) gain over time was significantly higher in abalone fed the S-diet compared to those fed F-diet (RM-ANOVA, $F_{(11, 748)} = 5.03$, $p < 0.0001$) and there was no significant difference between the soft tissue mass for males and females (RM-ANOVA, $F_{(1, 68)} = 0.50$, $p = 0.48$).

a) **b)**

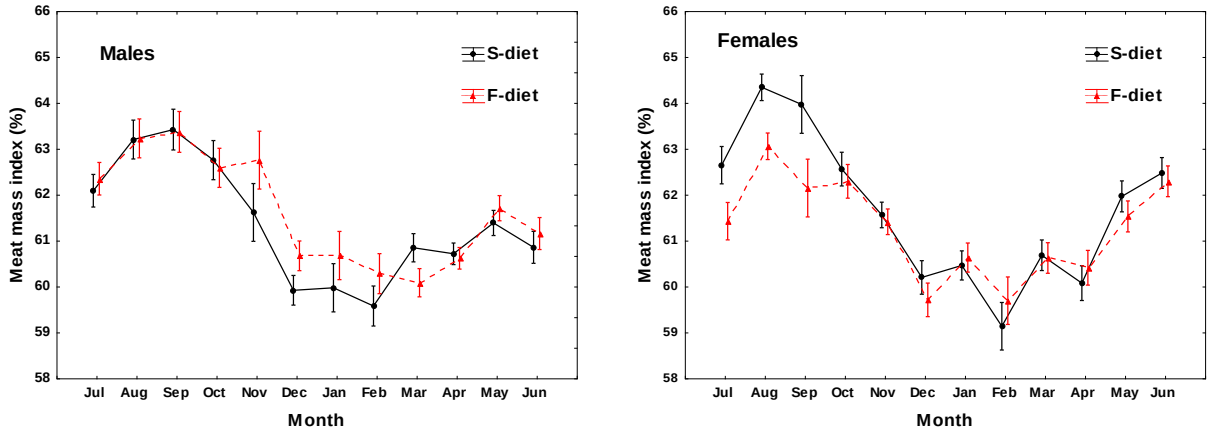


Figure 2.11: The meat mass index (mean ± standard error) for male (a) and female (b) abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013 (RM-ANOVA; $F_{(7, 248)} = 0.80$, $p = 0.60$ for males and $F_{(7, 241)} = 1.7$, $p = 0.11$ for females, G-G adjusted for both sexes).

Water lost from meat (%)

There was no significant difference in the water lost from meat between sexes over 12 months (RM-ANOVA, $F_{(11, 363)} = 1.7$, $p = 0.93$). There was no significant difference in water lost from meat between diets (RM-ANOVA, $F_{(11, 363)} = 0.1$, $p = 0.79$, Figure 2.12). The water loss from meat was not significantly influenced by an interaction between diet and time (RM-ANOVA; $F_{(11, 363)} = 1.7$, $p = 0.08$, Figure 2.12). Water loss from meat (%) significantly increased from September 2012 to April 2013 for both diets coinciding with the period of higher GBI values.

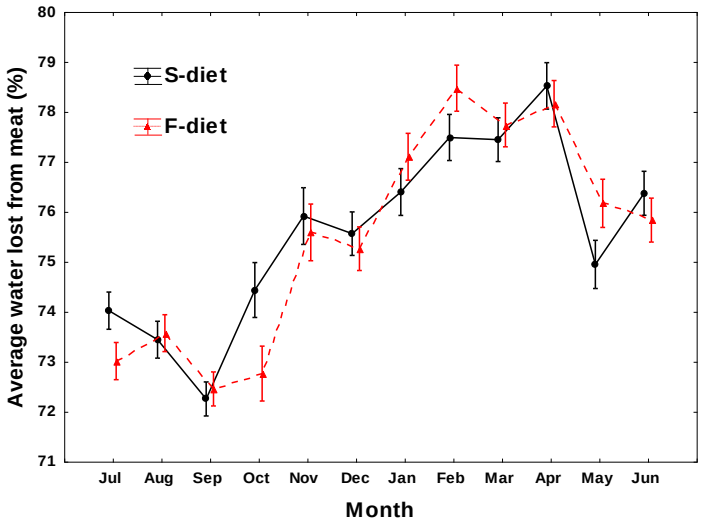


Figure 2.12: The water loss from meat (mean ± standard error) for abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013 (RM-ANOVA; $F_{(11, 363)} = 1.7$, $p = 0.08$).

Phytoestrogens

The S-diet diet had the highest isoflavone content of 64.14 mg / 100 g compared to the F-diet having trace amounts less than 1 mg / 100g (Table 2.3). Genistin and daidzin were the most abundant isoflavones found in soybean meal, which was an ingredient used in the S-diet (Table 2.3). Soybean meal was the only ingredient containing all isoflavones.

Table 2.3: Isoflavone content (mg 100 g^{-1}) found in feed ingredients and experimental diets. The S-diet diet consisted of soybean meal and fishmeal as the main protein source and F-diet had only fishmeal as the protein source. Samples were assayed by Covance Laboratories Inc. Philadelphia, United States of America (AOAC 2000).

	Feed ingredients				**Diets**	
	Fishmeal	**Starch 1**	**Starch 2**	**Soybean meal**	**S-diet**	**F-diet**
Daidzin	< 1	< 1	< 1	139	25	< 1
Glycitin	1.3	1.07	< 1	27.8	5.07	< 1
Genistin	< 1	< 1	< 1	127	30.9	< 1
Daidzein	1.35	< 1	< 1	8.99	1.63	< 1
Glycitein	< 1	< 1	< 1	2.72	< 1	< 1
Genistein	< 1	< 1	< 1	9.21	1.54	< 1
Total as glucosides	**3.51**	**1.07**	**< 1**	**328**	**64.14**	**< 1**

Environmental variables

There was no significant difference between treatments for water temperature, pH, dissolved oxygen (O_2), percentage oxygen saturation, total ammonia-nitrogen (TAN) and free ammonia-nitrogen (FAN) (RM-ANOVA, $p < 0.05$, Table 2.4).

Table 2.4: Mean (± standard deviation), minimum and maximum values for environmental variables tested for each diet from July 2012 to May 2013. Parameters include water temperature, pH, dissolved oxygen (O_2), percentage oxygen saturation, total ammonia-nitrogen (TAN) and free ammonia-nitrogen (FAN). The data were analysed using a RM-ANOVA with the S-diet diet consisting of soybean meal and fishmeal as the main protein source and the F-diet had only fishmeal as the protein source.

	Mean	Minimum	Maximum
S-diet			
Water temperature (°C)	14.68 ± 1.8	11.10	18.70
pH	7.90	6.89	8.80
O_2 (mg L^{-1})	8.18 ± 0.54	7.12	10.13
% Oxygen saturation	98.44 ± 5.21	87.30	119.70
TAN (µg L^{-1})	24.2 ± 12.01	0.21	66.53
FAN (%)	2.24 ± 1.2	0.14	12.51
FAN (µg L^{-1})	0.56 ± 0.42	0.004	2.99
F-diet			
Water temperature (°C)	14.67 ± 1.82	11.10	18.80
pH	7.87	6.92	8.98
O_2 (mg L^{-1})	8.15 ± 0.56	7.14	9.78
% Oxygen saturation	98.27 ± 5.78	88.90	126.00
TAN (µg L^{-1})	24.2 ± 11.6	1.46	55.27
FAN (%)	2.33 ± 1.93	0.15	20.20
FAN (µg L^{-1})	0.55 ± 0.51	0.04	4.74

DISCUSSION

Abalone fed the soya/fishmeal diet grew faster than abalone fed the fishmeal only diet. They averaged 10 g abalone^{-1} more by the end of the experiment. Britz (1996b) found slower growth in *H. midae* using soya oil cake as a sole dietary ingredient compared to a fishmeal-based diet. Shipton and Britz (2001) reported that in terms of growth and feed conversion efficiency, fishmeal and *Spirulina* were the most effective candidates for use as primary protein sources. Feed conversion ratios were similar between diets which suggested that

consumption rates did not differ and that abalone fed the S-diet diet were converting more energy into growth. However, there was a significant increase in FCR-values over the period between November 2012 and March 2013. This FCR increase coincides with the peak GBI-values and low meat mass indices suggesting that abalone were eating more to compensate for increased gonad growth. Since both diets were formulated to be isonitrogenous and isoenergetic, soybean addition was the only difference between diets. Although growth was faster in the S-diet, this was due mostly to the difference between diets in June 2013. Therefore future studies on long-term diet effects on growth should be conducted over a period of at least 12 months. Faster growth rates in abalone fed the S-diet formulated with a combination of plant and animal protein sources corroborate studies documented for other *Haliotis* species (Hahn 1989b, Morrison and Whittington 1991, Nie 1992, Britz 1996b, Tung and Alfaro 2012).

The GBI for animals fed the soya/fishmeal diet was higher than in abalone fed a fishmeal only diet with GBI changing similarly over time for both diets. The GBI was highest between the summer months of October 2012 and January 2013 suggesting that this was the peak spawning period. Abalone are broadcast spawners and the high GBI period confirms that individuals tend to spawn at a certain time of the year (Webber and Giese 1969). Other studies have also shown increased gonad development or vitellogenesis in abalone and other animals fed soya (Pelissero *et al.* 1991, Tung and Alfaro 2012, Wang *et al.* 2012). Male and female gonad investment differed between the times of the year with male abalone investing significantly more mass into gonad tissue than female abalone. Gametogenesis is initiated after each spawning and therefore abalone tend to produce eggs and sperm consistently throughout the year (Wood and Buxton 1996). Females adapt reproductive strategies in response to a surrounding environment and the energy available from food (Podolsky and Strathmann 1996). A large egg size vs. large number of eggs may be an evolutionary trade-

off between two genetic traits having fewer larger and better-provisioned eggs in a batch that may ensure fertilisation on a reef where broodstock density is low or environmental conditions are not favourable (Huchette 2004). A smaller number of eggs will be fertilised less successfully but these eggs will be larger, on the contrary having more smaller eggs is less costly and may be a successful strategy when broodstock density will ensure an optimum or high sperm concentration in the water during spawning peaks (Podolsky and Strathmann, 1996). Considering both diets were isoenergetic and isonitrogenous the energy available for investment into the different strategies of egg size vs. egg number should be similar for both treatments. Males had more gonad tissue than females and the results from the present study contrast with other reproductive studies on abalone that report gonad size in male and female abalone to be similar (Newman 1967, Barkai and Griffiths 1988, Litaay and De Silva 2003, Riddin 2013).

The higher GBI and higher visceral index in soya-fed *H. midae* compared to animals fed no soya may be due to the presence of phytoestrogens in soybean meal. Some authors evaluated the effects phytoestrogens have on gonad development in fish species (El-Sayed *et al.* 2012, Pollack *et al.* 2003). Genistein and daidzein have the strongest estrogenic effects in animals, and studies have shown that soybean meal contains varying levels of genistein (2.6 - 19.4 mg 100 g^{-1}) and daidzein (8.7 - 14.7 mg 100 g^{-1}), depending on the location and soybean variety (Chen and Wei 2008, Eldridge and Kwolek 1983). This falls into the range observed in soybean meal found in this study. Miyahara *et al.* (2003) evaluated the estrogenic activity of phytoestrogens (daidzin, genistin, daidzein and genistein) on fish, amphibians, reptiles and towards human estrogen receptors α (hER-α) and β (hER-β). The estrogenic activity of genistein was one hundred to two thousand times higher than that of daidzein. Miyahara *et al.* (2003) also showed that a diet for carp (containing defatted soybean meal) contained the highest levels of genistein and daidzein while showing the highest estrogenic activity and the

highest relative estrogenic activity for the human estrogen receptors hER-α and hER-β when compared to the other animal diets. These findings suggest that genistein and daidzein are the main contributors to estrogenic activity which demonstrate that there is a possibility of physiological effects occurring during the process of gametogenesis in *H. midae*. Pelissero *et al.* (1991) fed a soybean-based diet to Siberian sturgeon, *Acipenser baeri* and reported that it had a very pronounced effect on plasma vitellogenin levels. The effects of phytoestrogens on abalone gonad development have not been studied, however, these phytoestrogens have a high affinity to bind to estrogen receptors and furthermore inhibit or induce the synthesis and activity of certain enzymes involved in estrogen metabolism (Miyahara *et al.* 2003). A study by Kaushik *et al.* (1995) gives an example of phytoestrogen inhibition, where the plasma vitellogenin levels in rainbow trout, *Oncorhynchus mykiss* fed a diet containing soybean meal were not significantly affected. Thus, phytoestrogens at lower concentrations can work as an antagonistic estrogen, that binds to the estrogen receptors and induce the transcription factors for genes involved in cell growth, proliferation and differentiation (Mueller *et al.* 2004). Phytoestrogens may therefore have the same effects as estrogen or block estrogen's effects as well as alter the sexual development in juvenile stages, which has been observed in *Tilapia* (El-Sayed *et al.* 2012). More work is required to understand the effect phytoestrogens have on the gonad development in *H. midae.* Further studies are required that isolate phytoestrogens into diet formulations and test the effects on gonad development in abalone combined with a study of abalone gonad histology. Furthermore, the dietary ingredient fishmeal, may contain a number of anabolic steroids or other substances that may enhance gonad development in a specific sex which requires the need for future research.

The meat mass index decreased from September 2012 during the period of increased gonad growth (October 2012 to February 2013). The GBI increased from September 2012 to January 2013 and meat mass started to increase after February 2013. There was no significant

difference between diets for meat mass index and therefore it was suggested that this was an event resulting in a meat gain compromise to allow for increased gonadal development. As discussed above the FCR increased significantly during this period for both diets. Thus, abalone may be eating more to balance out the increased gonad growth with meat gain. However, as there was no increase in meat mass index during this time meat growth may decrease during periods of increased gonad growth (Webber 1970). The GBI for both sexes dropped after October 2012 and peaked again in January 2013. The visceral index also dropped after October 2012 and peaked in February 2013. This confirms two peak spawning events that occurred during the trial with lower peaks observed in animals fed the fishmeal only diet compared to soya-fed animals.

There was no significant difference between diets for percentage water lost from viscera and the meat suggesting that these diets did not affect the water retention abilities of these tissues. The moisture content in gonads for wild caught female blacklip abalone, *Haliotis rubra* ranged from 56.7 to 65.8 % (Litaay and De Silva 2003), which falls outside the range observed in the present study. However, studies by Bilbao *et al.* (2012) on the abalone, *Haliotis tuberculata coccinea* revealed that the moisture content in the viscera ranged from 72 to 75 % which is similar to results in present study. More water loss was after the spawning peak in January 2013. Water loss decreased during the spawning season observed from September 2012 to February 2013. Similar water loss percentages in the foot and gonad were also reported by Webber (1970) in black abalone, *Haliotis cracheroidii*. Glycogen molecules in muscle tissue are bound with water and enzymes with one gram of glycogen binding to between two and four grams of water depending on molecule size (Przybylski *et al.* 2006). Glycogen affects the water retention ability in the muscle tissue and as galactogen it acts in the gonad tissue (Webber 1970), suggesting that diets did not affect glycogen levels in the tissues. Instead, water loss changed depending on the time of year. In most molluscs

lipid is the nutritive storage product of eggs, and lower lipid values generally indicate immature gonads (Webber and Giese 1969). In *H. midae* lipid values differed between sexes with ovaries having 22% and testes having 9% (Riddin 2013). The higher lipid values found in the F-diet (Table 2.1) would propose that females fed the F-diet show increased gonad development compared to females fed the S-diet. However this was not supported in the results. Glycogen levels rise when lipid values decrease. Therefore, during peak spawning periods when lipid levels are highest, there was a decrease in water lost from the viscera and meat tissues (Webber 1970). The lack of differences observed for water loss between diets suggest that glycogen levels in the tissues may have been similar. Glycogen levels are strongly affected and increased by feeding high concentrations of digestible carbohydrates as shown in fish species (National Research Council 1993). This has not yet been evaluated in *Haliotis* species, however, it has been suggested that it lowers the yield of meat in cooking due to higher water loss (Fluckiger *et al.* 2011). Glycogen, being the major energy store in the foot muscle is depleted during the metabolically expensive period of gonad growth and stress such as disease and starvation (Webber and Giese 1969, Webber 1970, Fluckiger *et al.* 2011). Considering abalone were fed to satiation and condition factors did not differ between diets, disease and starvation would not affect glycogen levels therefore, gonad growth may have depleted glycogen in the foot muscle.

The digestive gland (DG) index decreased as gonad tissue increased, however it has been reported that the DG remains constant in size throughout the year with no apparent pattern (Najmudeen 2007, Webber 1970). The decrease in digestive gland tissue is owed to the increase in the proportion of gonad tissue. Other studies have reported that the digestive gland will increase in size corresponding to the decrease in the size of the gonad, suggesting that nutritive material may be transported from the digestive gland to the gonad (Litaay and De Silva 2003, Najmudeen 2007). Litaay and De Silva (2003) reported that the digestive

gland acts as a nutrient store and the gonad requires more nutrients for maturation processes which may explain the decrease in DG index as the gonad tissue increased during spawning peaks. The DG is the main organ involved in energy transformations in abalone and its greatest relative size and metabolic activity would be expected to occur in parallel with gametogenesis (Carefoot *et al.* 1998). The digestive gland index was significantly higher in abalone fed no soya indicating a lower proportion of gonad tissue relative to DG size.

Conclusion

Soybean meal as a dietary ingredient in formulated feed promoted gonad development in farmed *H. midae*. Males had more reproductive tissue than females, however, both sexes were equally influenced by the presence of soybean meal in the formulated feed. Growth was also faster in *H. midae* fed a diet with soybean meal and fishmeal as main protein source. Since meat mass indices were similar between diets, it was concluded that growth in body mass, shell length, visceral and gonad mass was affected by diet. Similar condition factors for both diets showed that diets did not compromise the weight/length relationship of abalone suggesting that the proportion of energy invested into various tissues differed because growth was different between diets. There were no significant differences in feed conversion ratio between diets suggesting that the difference in gonad development was not affected by consumption rate. However, increased FCR-values were observed between November 2012 and March 2013 during the period of increased gonad development and decrease in meat mass indices. Phytoestrogens found in the soybean meal may have a significant effect on gonad development. The soybean meal used in this study contained the phytoestrogens genistein and daidzein, which have been known to exemplify the strongest estrogenic effects. Farms need to consider product quality. Live abalone would require less emphasis on reducing gonad development since the gonad forms part of the product, whereas the gonad is

discarded when abalone are canned. Ultimately, this discarded viscera may seem a waste of potential energy if dietary energy was invested into gonad development, and therefore it is important to consider manipulating feeding regimes if soybean meal had an effect on gonad development. For example, knowing the acclimation period for a diet that reduces gonad development would be useful to switch diets during spawning season instead of using the same diet all year. This would require a change in diet at different times of the year corresponding with peak gonad development.

CHAPTER 3

A HISTOLOGICAL STUDY ON THE EFFECT OF DIET ON FEMALE GONAD DEVELOPMENT

INTRODUCTION

Egg quality has important effects on the performance of offspring and abalone produce eggs variable in size and quality to balance the risks between egg size and number in a changeable environment (Huchette 2004). During oogenesis the oocytes require lipids and other nutrient reserves from the food, and by transfer from muscle and digestive reserves to the gonad (Soudant *et al.* 1996). To understand and measure egg quality a histological perspective can be effective. Histological studies on the gonad development for annual reproductive cycles, cytological structures of maturing germ cells and oocyte-morphometrics have been conducted on various abalone species (Newman 1967, Tutschulte and Connell 1981, Martin *et al.* 1983, Hahn 1994, Wood and Buxton 1996, Capinpin *et al.* 1998, Grubert and Ritar 2004, Najmudeen and Victor 2004, Fukazawa 2007, Najmudeen 2008, Bilbao *et al.* 2010, Roux *et al.* 2013). However, histological information on the effect of dietary ingredients on gonad development in mature abalone is limited. Vitellogenesis in marine molluscs is a complex process where yolk formation via nutrients being deposited in the oocytes involves both autosynthetic and heterosynthetic pathways that contribute to an accumulation of more than one type of storage product (Eckelbarger and Young, 1997, Eckelbarger, 2005). Yolk components may be formed in various ways directly or indirectly from Golgi bodies, mitochondria, nucleolar material or independently in the cytoplasm (Eckelbarger and Young 1997). This source of nutrients may be attributed to a dietary effect which has been

demonstrated in Siberian sturgeon, *Acipenser baeri* fed soybean meal resulting in large changes in plasma vitellogenin levels indicating that soybean meal contains compounds that bind to hepatic estradiol receptors (Pelissero *et al.* 1991). Non-steroidal estrogenic substances found in soybean meal are commonly distributed among many animal feeds. With increasing interest in replacing fishmeal with alternative proteins attention must be drawn towards the potential long-term effects that steroidal and non-steroidal "estrogens" have on reproduction and growth (Pelissero *et al.* 1991).

There have been many studies on the classification of oocytes into oogenetic processes and maturity stages, however, approaches to achieve this differed (Apisawetakan *et al.* 1997). Najmudeen and Victor (2004) classified oogenesis of *Haliotis varia* into six stages based on the development of maturation. Sobhon *et al.* (1999) also classified six maturity stages in *Haliotis asinina* including the oogonium stage and five stages of growing oocytes. Martin *et al.* (1983) identified five stages of oogenesis in *Haliotis rufescens*: namely oogonium, presynthetic oocyte, synthetic oocyte, early postsynthetic oocyte and fully developed postsynthetic oocyte. These stages were classified using high resolution transmission electron microscopy (TEM) to study relative abundance of organelles and development of endoplasmic reticulum and Golgi complexes within the cells. Young and De Martini (1970) suggested four stages of female oocyte development in *H. rufescens*. Wood and Buxton (1996) classified four oogenesis stages in *H. midae* based on cell size, structure and orientation within the gonad, but not standard histological characteristics. Roux *et al.* (2013) divided oogenesis into nine stages in *H. midae* based on histological structures. These included oogonia, previtellogenic oocytes divided into stage one and two, vitellogenic stages three to six and late mature stages seven and eight. The majority of these classifications are based on oocyte size alone, however, this does not represent the true maturity criterion of oogenesis because cells are undergoing continuous development (Apisawetakan *et al.* 1997).

Histological features, which show evidence of definite characteristics for distinct developmental stages exemplify more fundamental approaches to classifying maturity stage criteria.

The research aims of this study were to histologically evaluate the effect of the dietary ingredient soybean meal on female gonad development and maturation processes in farmed *H. midae*. The focus of the histological examinations were on the female reproductive cycle as it was easier to observe maturation processes compared to males (Gurney and Mundy 2004). This aim was addressed by quantifying the maturation processes of oogenesis into maturity stages and to examine the relative frequency distribution of these stages in abalone fed a diet with soybean meal and fishmeal as the protein source, and a diet with fishmeal only. This will provide an understanding of the dietary effects on abalone reproduction at a microscopic level which lead to the following hypothesis:

H_{01}: The distribution of maturity stages in oogenesis for both treatments in farmed South African abalone, *Haliotis midae* are similar.

H_{a1}: The distribution of oogenetic maturation stages is affected by dietary composition in farmed South African abalone, *Haliotis midae*.

METHODS AND MATERIALS

The experimental system, diet preparation and experimental abalone were the same as described in methods and materials from Chapter 2. At the beginning of every month three female abalone were randomly selected from each of the two dietary treatments. Length (0.01 mm, vernier callipers) and weight (g abalone^{-1}) measurements, meat mass (g) and visceral mass (g) were obtained using an electronic scale (Kern PLS 4200-2F, serial number:

WIC1200486) and the viscera were stored in Davidson's fixative (20 % formalin, 10 % glycerol, 10 % glacial acetic acid, 30 % absolute ethanol, 30 % seawater). The fixed gonads were sent to the Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa for histological preparation.

Gonads were subjected to standard histological procedures. Fixed samples were dehydrated in a series of dilutions of ethanol (70 % to 100 %) for 180 minutes each, cleared with xylene for 150 minutes, and then embedded in paraffin wax for 120 minutes at 60°C. Three sections according to zones were made from each gonad, i.e., the apical, mid section and basal zones. Sections were cut using a microtome at a thickness of 4-5 micrometres, and incubated at 60°C for 60 minutes in an autoclave. The sections were rinsed in a series of dilutions of ethanol (100, 96 and 70 %), stained with haematoxylin for 10 minutes and rinsed in tap water. They were then differentiated in acid alcohol and blued in running tap water. The sections were then suspended in 70 % alcohol for three minutes and counter-stained with Eosin for three minutes. They were then dehydrated in 96 % and 100 % alcohol for three minutes each and cleared in xylol. Each section was subsequently mounted on a microscope slide (26 mm X 76 mm X 1 mm) with dibutyl phthalate in xylene (DPX) mounting medium, and enclosed with a cover slide.

Each slide was observed under a light microscope (UOP, UB200i compound microscope, serial number: 201101137) at magnifications of 40, 200 and 400 X. A photograph (UOP, Microscope camera DCM900) was taken randomly of each zone and a measuring program, SIGMASCAN® PRO 5 (Systat Software, San Jose, CA, USA) was used to manually count and stage every oocyte within the photograph. Actual lengths and area were calculated using photographs of calibration slides with a one mm scale bar for each magnification. Frequency distributions (%) (Equation 1) of each maturity stage as described in the results section of this

chapter were then calculated to construct stacked bar graphs. The number of oocytes / mm^2 (Equation 2) was calculated from egg counts. Male histological samples were collected, however, these were omitted from this thesis due to difficulty in quantifying characteristics with light microscopy that could be used to compare treatments. The slower developing, more easily detectable characteristic properties of oogenesis were suitable to making comparisons between treatments. Testes sections will be kept for future research such as Transmission Electron Microscopy (TEM).

$$Frequency\ distribution\ (\%) = \frac{O_S}{E_T} \times 100 \quad [1]$$

Distribution (%) of each maturity stage was calculated using Equation 1 where O_S was the total number of oocytes for each maturity stage and E_T was the total number of oocytes counted within the photograph.

$$Number\ of\ oocytes\ per\ mm^2 = \frac{E_T}{A_T} \quad [2]$$

The number of oocytes / mm^2 was calculated using Equation 2 where E_T was the total number of oocytes counted within the photograph and A_T was the total area (mm^2) in the photograph.

Environmental variables

Temperature (°C), pH, dissolved oxygen concentration (mg L^{-1}), percent oxygen concentration, concentration of total ammonia nitrogen (TAN, µg L^{-1}) and free ammonia nitrogen (FAN) were measured as described in the methods and materials from Chapter 2. Environmental variable results were the same as illustrated in the results from Chapter 2.

Statistics

The frequency distributions (%) of maturity stages from abalone fed each diet were analysed using a multivariate analysis of variance (MANOVA) at an error level of 5 % ($p \leq 0.05$). Multivariate tests Wilk's lamda (Lee 1972, Davis 1979), Pillai's trace (Muller 1998) and Hotelling-Lawley's trace (Davis 1970, 1980) were made to construct exact p-values. Wilk's lambda was used to show the amount of variance in the maturity stage distribution accounted for by the independent variable. Pillai's trace test was used to calculate the amount of variance in the maturity stage distribution which is accounted for by the largest separation of the independent variables. The Hotelling-Lawley trace test was used to represent the most significant linear combination of the maturity stage distribution. Frequency distributions were log-transformed (X + 0.5) to achieve equality of variance. The treatment means for the number of oocytes / mm^2 were compared using a repeated measures analysis of variance (RM-ANOVA) at an error level of 5 % ($p \leq 0.05$). The assumptions that were met included homogeneity of variance (Levene 1960) and normal distribution of the residuals (Shapiro and Wilk 1965). Tukey's post-hoc tests were used to compare all possible differences of distributions for treatment means. Correlations between the percentage of maturity stage and oocyte number / mm^2 were tested with Pearson's correlation coefficient. All analyses were performed using Statistica 11 software (Copyright © 1984-2012 StatSoft, Inc.).

RESULTS

The conical appendage included the digestive gland surrounded by the ovary, which could be classified into three zones (basal, midpoint and apical). In the basal zone the digestive gland occupied most of the cross-section, in the midpoint zone the ovarian tissue filled most of the cross-section, and the apical region showed very little digestive gland tissue. The connective tissue extended perpendicularly into the gonads to form the trabeculae, dividing the gonad

into compartments where oocytes were developing around lumens. Two epithelial cell walls enclosed the ovary from the mantle and the digestive gland. Earlier maturity stages were attached to the trabeculae (Figure 3.1B), whereas mature oocytes were separated and suspended in the lumen. The trabeculae decreased in thickness as the female gonad ripened. Microscopic staging of oogenesis were described and identified into nine stages using illustrations from Newman (1967), Wood and Buxton (1996), Sobhon *et al.* (1999) and Roux *et al.* (2013).

Oogonia (Og)

These were round or ovoid shaped cells closely adhering to trabeculae, usually clustered in groups (Figure 3.1A). The cytoplasm was stained dark blue due to the presence of ribosomes. Cell diameter ranged from 10-20 µm and the nucleolus was present, but not as prominent as seen in later stages. Each oogonium was surrounded by flat, squamous shaped follicular cells.

Stage 1 oocyte (Oc_1)

Oc_1 cells were oval scallop-shaped, 20 to 25 µm in diameter (Figure 3.1C). The nucleus was approximately 12 µm in length and displayed signs of densely packed chromatin stained light blue. The cytoplasm was stained a dark blue highlighting its strong basophilic properties. They were generally clustered and closely adhered to the trabeculae. Oc_1 were surrounded by fewer follicular cells than oogonia.

Stage 2 oocyte (Oc_2)

Oc_2 were larger and changed into a more columnar shape ranging from 30 to 40 µm in size (Figure 3.1D). The nucleus was approximately 20 µm with a decondensed chromatin network and a more visible nucleolus. Thus, the nucleolus and nuclear membrane were clearly distinct

because of the clearer nucleoplasm and the presence of mostly euchromatin. The cytoplasm was stained a light blue and is clustered with clear lipid droplets. Stage 2 oocytes were packed less dense around the trabeculae compared to the earlier stages.

Stage 3 oocyte (Oc_3)

These scallop shaped cells were between 45 and 60 µm in diameter with an enlarged nucleus of approximately 25 µm (Figure 3.1E). The nucleus contained transparent nucleoplasm hence making the nucleolus clearly visible. The nucleolus was stained dark blue and the nucleus was stained a lighter blue than stage 2 oocytes. The cytoplasm showed an increasing number of lipid droplets. The follicular cells surrounded the cell and were seen around the trabeculae.

Stage 4 oocyte (Oc_4)

Stage four cells were pear shaped and ranged from 60 to 80 µm in length with a larger nucleus diameter of approximately 40 µm (Figure 3.1F). The nucleus was transparent making the nucleolus clearly visible and enlarged due to complete uncoiling of the chromatin. The cytoplasm showed a number of lipid droplets and a thin jelly coat that began to form on the outside of the cell membrane. Spaces between two cells increased due to the formation of the jelly coat. The base of Oc_4 was attached to the connective tissue of the trabeculae in the ovarian lumen near the digestive gland.

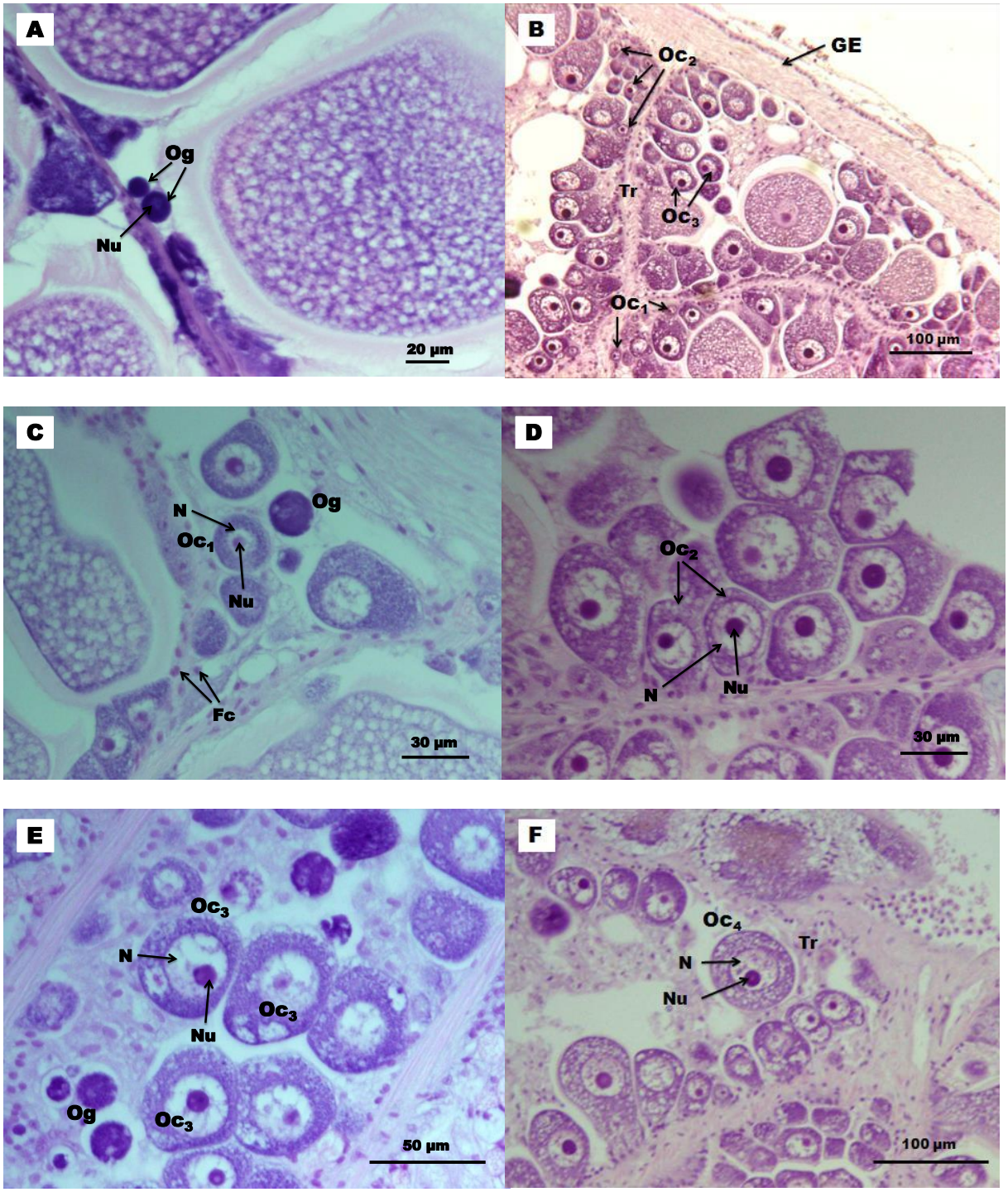


Figure 3.1: Transverse sections of ovarian H. midae tissue stained with haematoxylin-eosin depicting successive stages of oogenesis. (A) Dark blue stained oogonia (Og) (B) early stages (O_{C1}, O_{C2} and O_{C3}) of oogenesis attached to trabeculae (Tr) around edge of gonad epithelium (GE) (C) Stage one oocyte (O_{C1}) with numerous follicular cells in surrounding trabeculae. (D) Stage two oocytes (O_{C2}). (E) Stage three oocytes (O_{C3}). (F) Stage four oocytes (O_{C4}). All stages showed prominent light blue stained nucleus (N) and dark blue stained nucleolus (Nu).

Stage 5 oocyte (Oc_5)

These larger columnar shaped oocytes ranged from 100-150 µm in length (Figure 3.2A). The transparent nucleus was approximately 50 µm in diameter. The nucleolus was a lighter blue colour and ranged between 20 and 25 µm. The chorion attachment to the connective tissue of the trabeculae was clearly visible forming a cytoplasmic stalk. The cytoplasm was stained pink due to the formation of yolk platelets and there was an increasing number of lipid droplets visible in the cytoplasm.

Stage 6 oocyte (Oc_6)

Stage six oocytes were tear-dropped in shape and ranged from 160 to 250 µm in length (Figure 3.2B). The nucleus became more ovoid in shape and approximately 50 µm in diameter. They were attached to the connective tissue of the trabeculae. However they had a thinner cytoplasmic stalk than stage 5 oocytes. This was the last stage before the oocyte completely detached from the trabeculae to be freely suspended in the gonad lumen. They were enveloped by a thick jelly coat which made the oocytes sit loosely within the lumen. Cytoplasm was stained pink due to the yolk platelets and lipid droplets became larger and about 4 µm in size. The nucleus stained light blue and the nucleolus stained a darker blue.

Stage 7 oocyte (Oc_7)

These oocytes were roughly 200 to 250 µm in length and had an oval-shaped nucleus with a diameter of 100 µm, stained light blue (Figure 3.2C). The cytoplasmic stalk was completely separated from the connective tissue of the trabeculae and the oocyte and suspended freely in the gonad lumen. The cytoplasm was stained pink and the oocytes had a thick jelly coat surrounding the cell epithelium.

Stage 8 oocyte (Oc_8)

Stage eight oocytes were the largest, fully mature cells that were released into the water during spawning (Figure 3.2D). Their size ranged between 250 and 300 µm with a large oval nucleus between 100 and 150 µm in diameter. In very gravid females the oocyte changed shape due to the tight compact characteristics in the gonad lumen. Clear lipid droplets occurred in large clusters and the jelly coat became thinner. The cytoplasm still remained pink in colour due to the yolk platelets. All stage 8 oocytes were completely detached from the trabeculae.

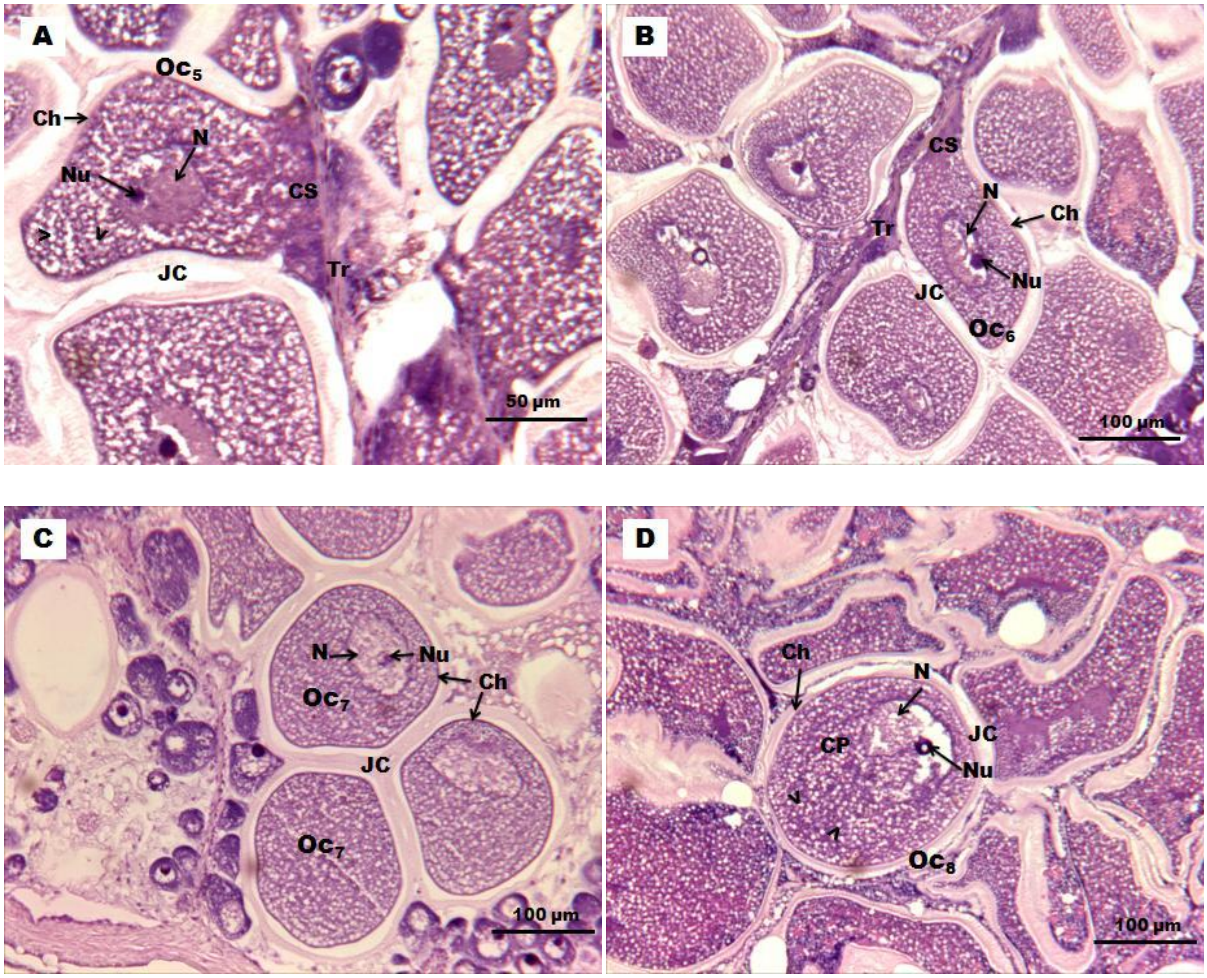


Figure 3.2: Transverse sections of ovarian *H. midae* tissue stained with haematoxylin-eosin depicting successive stages of oogenesis. (A) Stage five oocyte (Oc5) attached to the trabeculae (Tr) by a wide cytoplasmic stalk (CS). A thick layer of jelly coat (JC) surrounds the other surface of the cell membrane. (B) Stage six oocyte (O_{C6}) with a narrow cytoplasmic stalk attached to trabeculae. (C) Stage seven oocytes (O_{C7}) are completely separated from the connective tissue of the trabeculae with thick jelly coat surrounding cell epithelium. (D) Fully mature stage eight oocytes (O_{C8}) separated from trabeculae have a thin jelly coat and full rounded shape. All stages show a prominent nucleus (N), dark blue stained nucleolus (Nu), chorion (Ch) with cytoplasm stained pink due to formation of the eosinophilic yolk platelets (>).

Oocyte number

The number of oocytes / mm^2 remained similar for each zone (RM-ANOVA, $F_{(2, 14)} = 0.73$, $p = 0.49$). There was no significant interaction between time and zone (RM-ANOVA, $F_{(22, 154)} = 0.72$, $p = 0.81$). Oocyte number / mm^2 was not significantly different between diets (RM-ANOVA, $F_{(1, 14)} = 0.18$, $p = 0.18$, Figure 3.3). There was no interaction effect between time

and diet on the oocyte number / mm^2 (RM-ANOVA, $F_{(11, 154)} = 0.78$, $p = 0.66$, Figure 3.3). However, oocyte / mm^2 was significantly different between months (RM-ANOVA, $F_{(11, 154)} = 3.15$, $p < 0.0001$, Figure 3.3). Oocyte number / mm^2 significantly increased from March 2012 to May 2013.

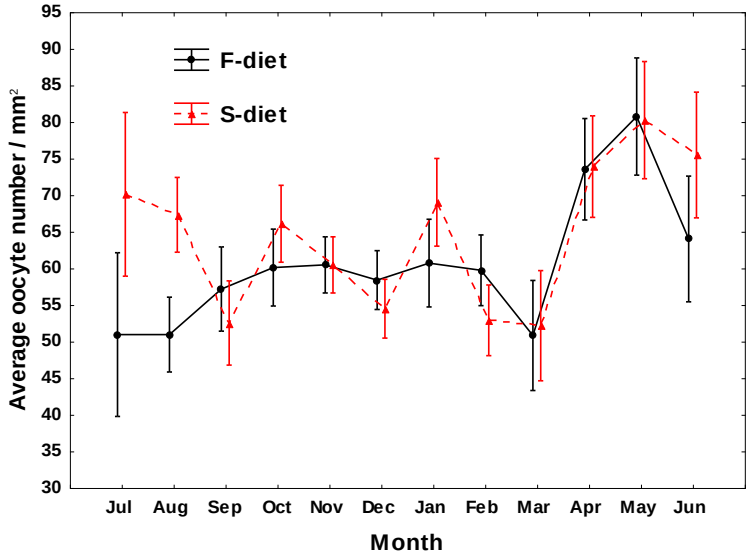


Figure 3.3: The oocyte number / mm^2 (mean ± standard error) of abalone fed a soya and fishmeal (S-diet) diet or a fishmeal only (F-diet) diet from July 2012 to June 2013 (RM-ANOVA, $F_{(11, 154)} = 0.78$, $p = 0.66$).

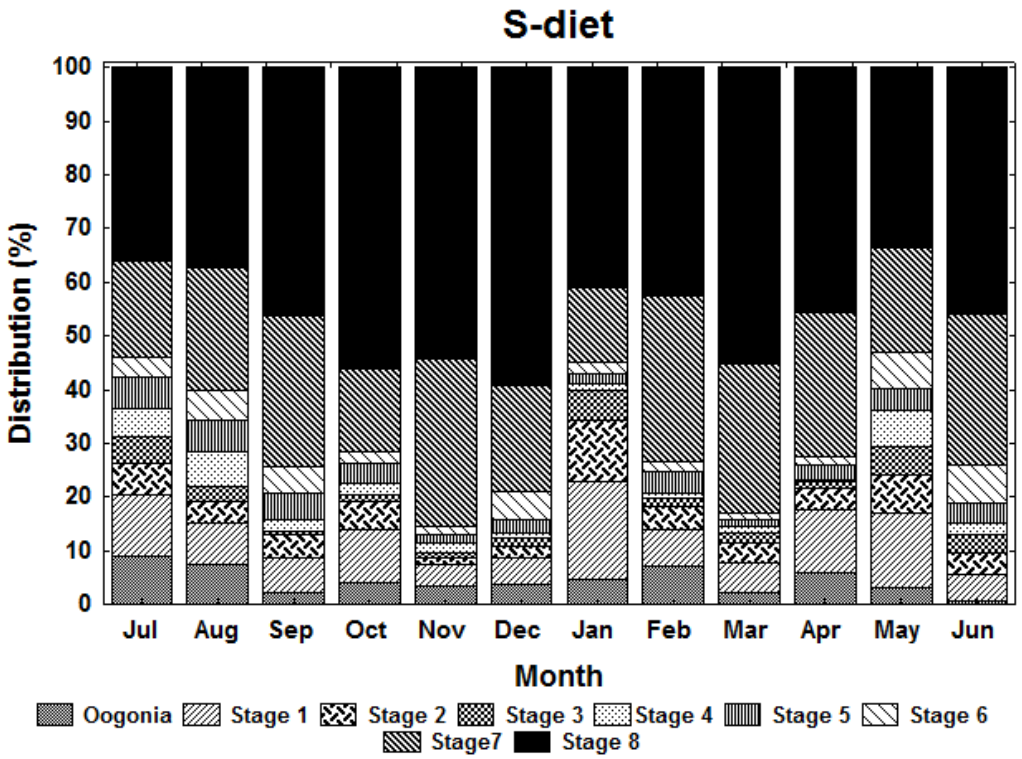
Maturity stage distribution (%)

There was no significant difference in the maturity stage distribution between the three zones (apical, midpoint and base) of the gonad (MANOVA, $F_{(16, 274)} = 0.94$, $p = 0.52$). For this reason the data from all zones were pooled to obtain maturity stage distribution (%) for every gonad. The maturity stage distribution (%) differed between the two diets (MANOVA, $F_{(9, 40)} = 6.52$, $p < 0.0001$, Figure 3.4). The distribution of maturity stages differed between months (Wilk's $\Lambda = 0.008$, $F_{(99, 294)} = 2.96$, $p < 0.001$; Pillai's trace = 2.9, $F_{(99, 432)} = 2.07$, $p < 0.0001$; Hotelling's trace = 12.62, $F_{(99, 344)} = 2.07$, $p < 0.0001$, Figure 3.4). The interaction between the independent variables month and diet had no influence on the distribution of maturity

stages in female abalone gonads (Wilk's $\Lambda = 0.16$, $F_{(99, 294)} = 0.88$, $p = 0.76$; Pillai's trace = 1.55, $F_{(99, 432)} = 0.91$, $p = 0.72$; Hotelling's trace = 2.23, $F_{(99, 344)} = 0.86$, $p = 0.81$).

Gonads from abalone fed the diet containing both soya and fishmeal comprised of oocytes that were predominantly mature in stage eight (Figure 3.4a). The majority of oocytes from abalone fed a fishmeal only diet were at a maturity stage seven (Figure 3.4b). The early maturity stages (O_g to Oc_4) made up 23.25 % and 44.06 % of the oocyte distribution in the gonads for abalone fed the S-diet and F-diet, respectively. Later stages (Oc_5 to Oc_8) made up for 76.7% and 55.9% of oocyte distribution within the gonad for abalone fed the S-diet and F-diet, respectively. During October 2012 abalone from both treatments illustrated very similar maturity stage distributions. After October 2012 the percentage of stage eight remained high in S-diet fed abalone compared to F-diet fed abalone, where mature O_8 oocytes decreased to 4.08 % (Figure 3.4). Stage eight oocyte distribution remained low (2.15 % to 22.2 %) in abalone fed the F-diet after October 2012 to June 2013 (Figure 3.4). All stages from both diets were observed in the gonads every month.

a)



b)

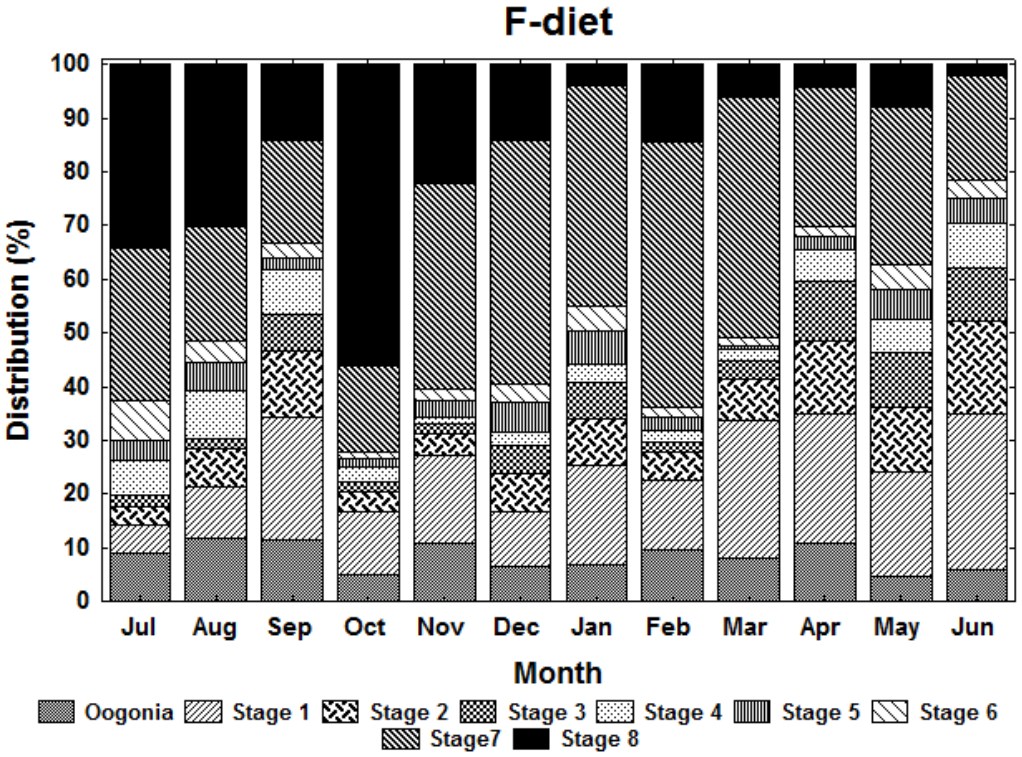


Figure 3.4: Average distribution (%) of maturity stages involved in oogenesis for *H. midae*. Abalone (n = 72) were fed diets with soybean meal and fishmeal (a) and fishmeal only (b) as the protein sources from July 2012 to June 2013.

Abalone fed the soya/fishmeal diet had fewer intercellular spaces in gonads compared to abalone fed the fishmeal only diet (Figure 3.5). Oocytes were densely packed with fewer spaces found in the gonad lumen in S-diet animals (Figure 3.5B). F-diet-fed abalone showed more clusters of oocytes in early maturity stages compared to S-diet fed abalone (Figure 3.5A). The shape of the oocytes for the majority of soya/fishmeal fed abalone were distorted in shape due to them being tightly compacted in a full gonad. There was no correlation between the percentage of maturity stage eight oocytes and oocyte number / mm^2 (Pearson's correlation, $r^2 = -0.33$, $p = 0.052$ for S-diet and $r^2 = -0.11$, $p = 0.51$ for F-diet). Similarly, there was no correlation between oocyte number and the percentage of maturity stage seven oocyte for abalone fed the S-diet (Pearson's correlation, $r^2 = -0.27$, $p = 0.12$). However, as the percentage of stage seven oocyte in abalone fed the F-diet increased oocyte number / mm^2 decreased (Pearson's correlation, $r^2 = -0.42$, $p = 0.01$).

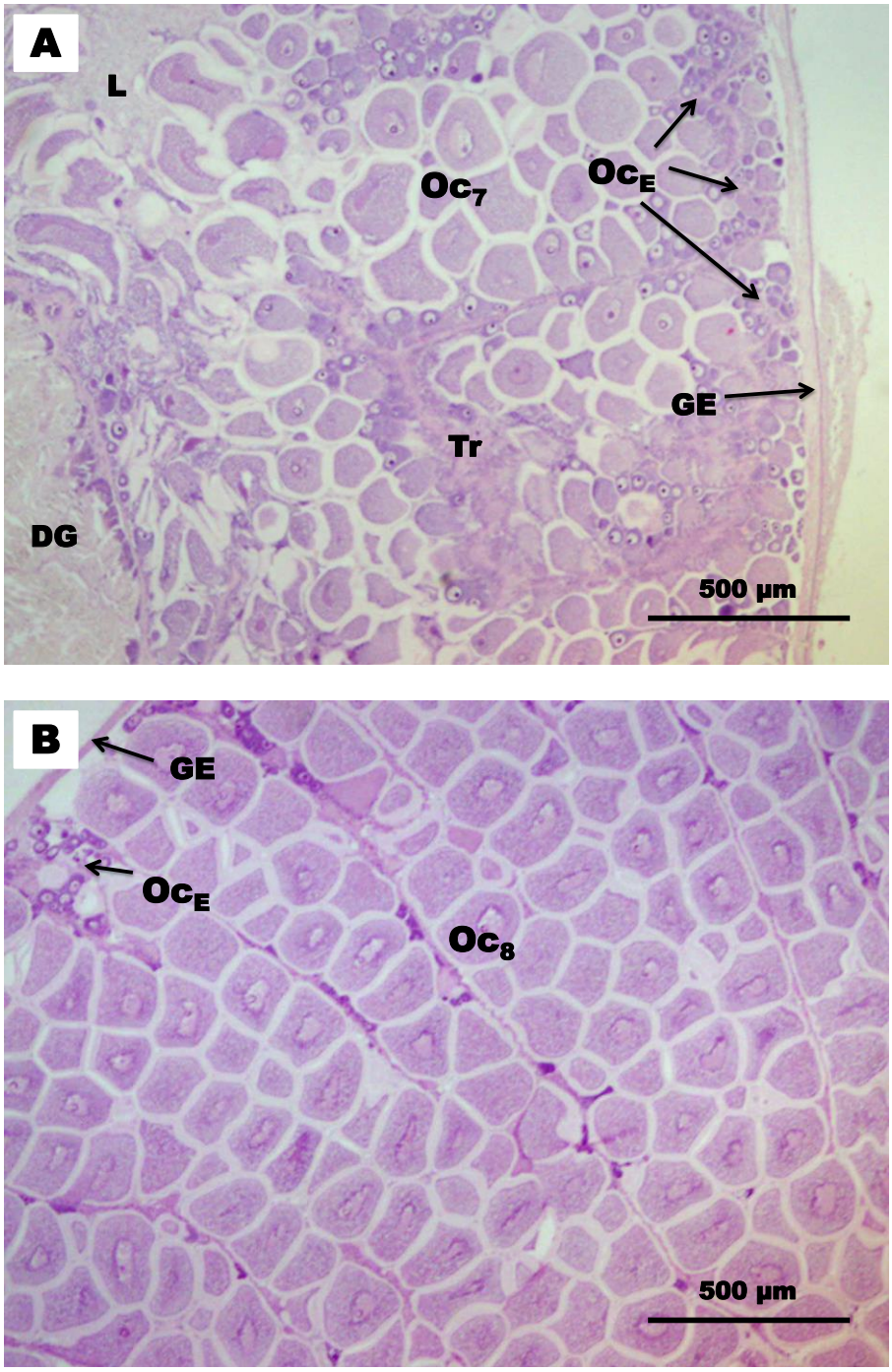


Figure 3.5: Transverse sections of ovarian *H. midae* tissue stained with haematoxylin-eosin depicting oocyte arrangement and development. Ovarian tissue from an abalone (80.09 g) fed the F-diet (A) consists of a gonad lumen (L) amongst oocytes, many clusters of early maturity stages (Oc_E) and a majority of stage seven oocytes (Oc_7). An abalone (82.42 g) fed the S-diet (B) had a fuller gonad with many compacted stage eight oocytes (Oc_8). Trabeculae tissue (Tr) and digestive gland tissue (DG) were also visible. Both abalone were from November 2012.

DISCUSSION

The distribution of maturity stages differed between abalone fed the S-diet and F-diet illustrating changes in relative proportions. Described maturity stages were similar to those reported by Newman (1967), Wood and Buxton (1996), Sobhon *et al.* (1999) and Roux *et al.* (2013) based on cell size and key histological features. These results indicated that soybean meal in artificial abalone diets influenced oogenesis in *H. midae* in a way that was different from fishmeal. The highest amount of mature stage 8 oocytes observed in December 2012 and January 2013 can be compared to the high GBI observed in females from Chapter 2, Figure 2.6b. The GBI was similar for both diets in December 2012. However, in January 2013 it decreased in F-diet-fed abalone and peaked in S-diet-fed abalone (Chapter 2, Figure 2.6b). Histological results corroborate this as the distribution (%) of stage 8 oocytes followed similar trends. Furthermore, abalone from both diets showed a high proportion of stage 8 oocytes in October 2012 which was also a peak observed in the GBI from Chapter 2, Figure 2.6b. All maturity stages in gonads were observed throughout the study period even though the proportion of maturity stages differed from the proportions outside spawning season which could be suggestive for the unsynchronised spawning behaviour that occurs randomly in wild abalone (Litaay and De Silva 2003). Oogenesis in molluscs has been divided into a generative (proliferative) and vegetative (growth) phase (Anderson 1974). The proliferative phase involves oogonial cells that increase in the gonad by mitotic multiplication and the vegetative phase development is due to vitellogenesis and requires uptake and synthesis of nutritive materials. As the diets were isonitrogenous and isoenergetic the only difference between the diets tested here was the ratio of soybean meal to fishmeal protein sources, and this may have affected the vegetative phase and vitellogenesis through nutrient uptake. Farmed *A. baeri* produced large changes in plasma vitellogenin levels when fed a diet comprising of soybean meal, and a diet containing no soybean significantly decreased plasma

vitellogenin levels (Pelissero *et al.* 1991). This provides evidence that soybean meal affected vitellogenesis possibly explaining the difference observed in maturity stage distribution of *H. midae.* This is the first study to show this effect in *H. midae.*

Soya-fed animals had a higher proportion of stage 8 oocytes and a relatively lower proportion of stage 7 oocytes compared to abalone fed a diet with only fishmeal as the protein source. Abalone fed the fishmeal only diet had a relatively higher proportion of oocytes in maturity stage 7. Although GBI was highest in October 2012 and January 2013 for both diets (Chapter 2), there was a difference in the proportion of maturity stage 8 and stage 7 oocytes between diets. The thickness of the jelly coat was the main characteristic that distinguished these two maturity stages apart from each other. Broadcast spawners are often sperm-limited and larger eggs in laboratory and field studies are preferentially fertilised under sperm-limited conditions (Levitan *et al.* 1992). The jelly coat might be an efficient mechanism for enhancing fertilisation success by increasing the overall target size and ultimately increase the sperm-egg collision frequency (Podolsky and Strathmann 1996, Styan 1998). Farley and Levitan (2001) observed gametes of the sea urchin, *Lytechinus variegates* to quantify the difference in the number of collisions between sperm and eggs with, and without jelly coats. Jelly coats were stripped off the eggs using seawater and the collision frequencies among eggs with and without jelly coats and inert plastic beads were compared. Authors also assayed fertilisation to explore fertilisation success of eggs without jelly coats and eggs with jelly coats. There was no difference in the fertilisation efficiency of eggs with or without jelly coats however, jelly coats increased collision rates due to larger target size (Farley and Levitan 2001). The jelly coat material has a lower energy value than the egg material and it has been suggested that selection might favour minimising egg size and maximising jelly coat size in order to maximise parental reproductive success in echinoids (Bolton *et al.* 2000,

Podolsky 2004). The organic density of jelly coat is very low and the large volume of the jelly coat can make up 7 % of the total energy investment into an egg (Bolton *et al.* 2000) Investments in egg and jelly coat material do not provide equal returns and larger eggs not only provide an increased target but also provide an increased investment into caloric egg material (Farley and Levitan 2001). Abalone fed the F-diet had a majority of stage 7 oocytes (thicker jelly coats) which may suggest they make use of the differences in energy investment between egg material and jelly coat to their advantage by increasing target size of the eggs with less caloric jelly coat material. The energy content of the oocyte and the quality of the yolk may be a result of the variation in the quality and quantity of nutrients available to the adult (Bolton *et al.* 2000). Although jelly coats do not contribute energy to embryonic or larval development they require energy costs during the growth of each oocyte (Bolton *et al.* 2000). The amount of energy available (isoenergetic diets) for reproduction, and the number of oocytes / mm^2 was similar between diets, therefore the partitioning of energy to jelly coats should be similar. However this was not the case in this study. Investment into jelly coats may reduce the amount of energy that could be potentially invested into egg material therefore compromising reproductive output and reducing fecundity (Bolton *et al.* 2000). Again, this addresses the concept of an evolutionary trade off between a large egg size vs. large number of eggs. Soya-fed abalone had a relatively higher proportion of stage eight oocytes which may be indicative of a riper stage of maturation.

The many intercellular spaces observed in gonads of abalone fed the F-diet in relation to S-diet fed abalone could be due to oocyte degeneration. Oocyte degeneration and resorption in molluscs is caused by a variety of natural environmental conditions, desiccation, or low levels of nutrition (Dorange and Le Pennec 1989). The breakdown of the oocytes is related to a deficit in energy due to a lack of food (Lubet *et al.* 1987) which may indicate a possible

reason for intercellular spaces observed in fishmeal only-fed abalone. However, there was no significant difference in feed conversion ratio, diets were isoenergetic and abalone were fed to satiation daily. Therefore the 'fullness' characteristic of gonads from soybean fed abalone need to be tested in future histological studies as the presence of phytoestrogens may influence this. The similarity of the functional structure of phytoestrogens and endogenous estrogens may mimic the action of hormones (Roepke *et al.* 2005) and therefore alter reproductive investment into oocytes. The relative percentage of stage 7 oocytes increased as number of oocytes decreased in F-diet fed abalone, suggesting that larger eggs take space differently within the ovarian lumen. Oocyte number / mm^2 remained low during the spawning season (September 2012 to February 2013) therefore a few larger mature oocytes may take up more space than many smaller oocytes. Oocyte number / mm^2 significantly increased after spawning season which may suggest more smaller oocytes within the gonad. The large intercellular space occupied by stage seven oocytes in F-diet-fed abalone may be due to the combined presence of the thick jelly coat around each oocyte. Oocyte number / mm^2 were similar for zones and diets indicating that frequencies of maturity stages was a characteristic that can be used to histologically tell the treatments apart. Oogenesis was similar at the base, midpoint and apex of the gonad as there was no significant difference in the maturity stage distribution between the three zones. However, the digestive gland tissue at the basal zone occupied more area when compared to the apex region of the gonad. Najmudeen (2007) and Webber (1970) reported that the digestive gland remains constant in size throughout the year with no apparent pattern suggesting that the stage of maturity was not affected by the proportions of digestive gland tissue relative to gonad tissue.

Conclusion

The distribution of maturity stages in ovarian tissue was affected by the dietary ingredient, soybean meal in artificial abalone feed. The majority of oocytes within abalone fed a diet with soybean meal as a protein source were fully mature stage 8 oocytes. A possible explanation may be due to the presence of phytoestrogens found in soybean meal. The average number of oocytes / mm^2 did not differ between diets however, it increased after spawning season. The difference between oocytes from each diet may be identified by the difference in jelly coat investment. After the spawning peak in December 2012 F-diet-fed abalone had a smaller proportion of later maturity stages for the remainder of the experiment compared to S-diet-fed abalone. The division of oogenesis into nine stages and distribution (%) of each stage is suggested as a method for comparing the effects of the two diets histologically. Histological methods supported GBI results in Chapter 2 as similar trends were observed. This is the first histological study in *H. midae* using this approach. Future studies on the histological effects of soybean meal on male gonad development will add value to this study. In addition, histological studies on which the effect of selected phytoestrogens on gonad development should be tested in isolation which may be able to support the hypothesis that some compounds in soya are responsible for the effects on gonad development described here.

CHAPTER 4

THE EFFECT OF SEX-SORTING ABALONE ON GROWTH AND GONAD DEVELOPMENT

INTRODUCTION

Sex-sorting has been successfully applied in livestock husbandry and in aquaculture as exemplified by the intensive production of *Tilapia* species (Beardmore 2001, Dan and Little 2000, Chakraborty *et al.* 2011). Differences between males and females in growth rate, nutritional requirements, behavioural patterns and characteristics of the marketed product dictate the need to establish management systems adjusted to the sex of the animal (Sagi 1986). A monosex population is non-breeding and therefore energy for somatic growth should be diverted to gonadal growth instead of reproduction, however, there are no published studies on mollusc species. Numerous other studies have been carried out on other aquatic species. Graf *et al.* (2010) conducted trials on the effects of sex separation on somatic growth and lifespan in killifish, *Nothobranchius furzeri*. Female reproductive effort and offspring investment in *N. furzeri* was significantly reduced after separation suggesting that the trade-off between reproduction and somatic growth depended on the presence of males. Female killifish on their own grew significantly faster and larger than females in a mixed-sex population (Graf *et al.* 2010). In Australia, freshwater crayfish, *Cherax albidus* were sex sorted by hand before they are stocked into ponds and crayfish in male-only ponds grew on average 53 % faster than those in mixed-sex ponds (Lawrence 2004). Dan and Little (2000) compared the performance between monosex and mixed-sex Nile tilapia, *Oreochromis niloticus* and showed that fish in monosex groups grew 10% faster than fish in mixed-sex

groups. There have been no sex-sorting studies carried out on *Haliotis midae* under farming conditions or any other abalone or mollusc species. Therefore, it is important to explain sex-specific changes to growth and reproduction.

Pheromones play a significant role in coordinating reproductive activity in marine invertebrates (Cummins *et al.* 2005, Nhan *et al.* 2010). Chemical communication has been shown to have a range of functions in the reproductive system from gametogenesis to spawning (Nhan *et al.* 2010). Hamel and Mercier (1999) observed that chemicals in the secreted mucous from the sea cucumber, *Cucumaria frondosa* helped maintain gametogenic synchrony between individuals and initiate gametogenesis. Studies have been done on the presence of gametes that induce spawning in the opposite sex in haliotids (Murayama 1935, Morse *et al.* 1977, Counihan *et al.* 2001, Nhan *et al.* 2010, Kuanpradit *et al.* 2012). Counihan *et al.* (2001) demonstrated that the frequency and synchronicity of female ejaculations from captured *Haliotis asinina* from Heron Reef, Australia was not affected by the presence of males. However, the presence of *H. asinina* females appeared to affect male spawning behaviour resulting in more frequent sperm release (Counihan *et al.* 2001). Females generally release their gametes after males, and therefore, it may seem that females release a factor prior to egg release that affects male spawning behaviour (Counihan *et al.* 2001). Sex pheromones released by recently-ovulated female fish contain prostaglandins (PGFs) which are endocrine mediators of ovulation and female spawning behaviour (Stacey and Sorensen 2009, Lim and Sorensen 2011). Lim and Sorensen (2011) showed that common carp, *Cyprinus carpio*, released a PGF-based sex pheromone that is potent and species specific. The response to this released PGF requires the olfaction detection threshold to be maintained in other individuals making PGFs reliable indicators of sexual activity in males (Stacey and Sorensen 2009).

Nhan *et al.* (2010) illustrated the first direct evidence that *H. asinina* uses sexual attraction pheromones with significantly more mature females being attracted to mature males indicating that only males are releasing the attractants. Chemical cues may be derived from mucous glands in combination with the reproductive tract or from one or more mucous-secreting glandular sites associated with the pedal muscle, hypobranchial and buccal tissue (Kuanpradit *et al.* 2012). Physicochemical properties of this mucous may help to keep hold of signalling molecules and preventing dilution in surrounding seawater as mucous-associated proteins (MAPs) are water soluble (Kuanpradit *et al.* 2012). The MAPs can be synthesised and released into the water column by secretory cells and induce a chemo-stimulatory response in conspecific abalone. Thus, abalone spawning behaviour may be modified by chemicals released by both sexes, however, no information is known on the effects this may have on gonad development. Separating abalone into monosex and mixed-sex populations may affect reproductive behaviour, somatic growth and gonad development.

The aim of this study was to examine the effects of sex-separation on growth and gonad development in farmed *H. midae*. This aim was addressed by separating abalone into monosex and mixed-sex groups and comparing growth, gonad bulk indices (GBI) and visceral and meat indices between treatments. The following hypotheses were tested:

H_{01}: There is no difference in growth and gonad development between abalone in monosex and mixed-sex treatments.

H_{a1}: Growth and gonad development is similar between treatments.

METHODS AND MATERIALS

Experimental system

The experiments were conducted at HIK Abalone (Pty) Ltd. farm in Hermanus, Western Cape (34º26'04.35"S; 19º13'12.51"E) from July 2012 to June 2013. The serial-use tank system described by Naylor *et al.* (2011a, 2011b) was used (Figure 4.1). The serial-use system consisted of 21 tanks (0.9 m X 0.6 m X 0.6 m) with each tank having its own water inflow (Naylor *et al.* 2011a, 2011b). Each tank held one 'oyster mesh' basket that contained seven vertical acrylonitrile butadiene styrene plastic plates with a surface area of 3.2 m^2, and a horizontal feeder plate that was submerged 15 cm under the water. The incoming seawater was filtered by a microscreen drum filter (100 µm) in the HIK Abalone Farm (Pty) Ltd. main header tank. Water was supplied from this header tank where it was gravity-fed through polyvinyl chloride (PVC) piping (40 mm outer diameter) to the 21 experimental tanks (Naylor *et al.* 2011a, 2011b). All tanks were aerated through 20 mm PVC airlines that were suspended 50 mm off the tank bottom. Each tank was fixed with a drain and a 40-mm PVC upstand pipe at the opposite end of the incoming water for drainage (Naylor *et al.* 2011a, 2011b). Flow rate was maintained at one tank volume exchange per hour (300 L h^{-1}). Tanks were cleaned every two weeks. Eighteen tanks were used with the addition of a row of three tanks without animals to allow for baskets with abalone to be moved when tanks were cleaned.

Figure 4.1: Experimental system comprising of 21 tanks, each with its own water inflow.

Experimental abalone and acclimation

Haliotis midae spawned by several females in November 2009 on HIK Abalone Farm (Pty) Ltd. were kept aside. These abalone were fed a formulated abalone feed (34.7 % protein, 2.4 % lipid, 57.3 % carbohydrate, 1.6 % fibre, 5.6 % ash; Abfeed® S34, Marifeed (Pty) Ltd, Hermanus, South Africa) prior to the start of the acclimation period. Mature abalone (70 – 80 g abalone^{-1}) were used to allow for accurate sex sorting and fed the S-diet (Chapter 2) for one month before the start of the experiment. After acclimation the abalone were stocked into each basket at a density of 18 % of available surface area with 139.6 ± 0.69 abalone basket^{-1}. This stocking density was maintained for 12 months with abalone in each tank being size-graded every four months.

Experimental design

Abalone were randomly selected and sorted into three treatments. These were males (M), females (F) and equal numbers of males and females (MF) (Table 4.1). Each treatment was replicated six times and allocated to the 18 tanks using a randomised block design. Animals

were fed to apparent satiation once a day at 16h00 every day for 12 months. To minimise feed waste the feed was given to each basket that had less than 15 pellets on the feeder plate from a cup that contained 66.6 ± 3.2 g of pellets (Britz *et al.* 1997). Each tank was allocated a one-litre bucket of S-diet from which the remaining food was weighed before the bucket was refilled to calculate the feed conversion ratio (FCR) (Equation 1, Chapter 2). The FCR was calculated at the end of each four-month period after farm-specific size-grading procedures during which each basket was weighed. Every four months the condition factor (CF) was calculated (Equation 10, Chapter two), where weight is the mass of the animal (g abalone^{-1}) and shell length is in mm (Britz 1996a). Thirty animals from each basket were randomly sampled every four months during size-grading, weighed (0.01 g) using an electronic scale (Kern PLS 4200-2F, serial number: WIC1200486) and measured (0.01 mm) using vernier callipers, respectively.

Table 4.1: Abalone sex-separated into three treatments. There were six replicates per treatment.

Treatment	Description
M	Male *H. midae* only
F	Female *H. midae* only
MF	Equal numbers of male and female *H. midae*

At the beginning of every month four abalone were removed from each tank with equal numbers of males and females removed from MF treatment. These animals were placed into labelled cotton bags and taken to the processing plant (SPP Canning (Pty) Ltd.). Abalone were not fed two days prior to the sampling day. Dry weight, gonad bulk index analysis, and visceral and meat mass indices were obtained using the experimental procedures and equations described in Chapter 2 (Equations 2 to 9).

Environmental variables

Temperature (°C), pH, dissolved oxygen concentration (mg L^{-1}, O_2), percent oxygen concentration, concentration of total ammonia nitrogen (TAN, µg L^{-1}) and free ammonia nitrogen (FAN) were measured using the methods and materials described in Chapter 2.

Statistical analysis

The treatment means for each of the dependent variables were compared using repeated measures analysis of variance (RM-ANOVA) at an error level of 5 % ($p \leq 0.05$). Data for males from the MF treatment were tested against the M treatment and data for females from the MF treatment were tested against the F treatment, except for growth and FCR data that were tested for M, F and MF treatments. The assumptions that were met included homogeneity of variance (Levene 1960) and normal distribution of residuals (Shapiro and Wilk 1965). If sphericity was significant, p-values and degrees of freedom were corrected using the Greenhouse-Geisser (G-G) method (Greenhouse and Geisser 1959). Tukey's post-hoc HSD test was used to compare all treatment means. All analyses were performed using Statistica 11 software (Copyright © 1984-2012 StatSoft, Inc.).

RESULTS

Growth

Average mass at the start of the experiment was 74.74 ± 0.30 g abalone^{-1}, and by the end of the trial, mass had increased to 131.49 ± 14.76 g abalone^{-1}. Change in average mass did not differ between M, F and MF treatments over the 12-month growth period (RM-ANOVA, $F_{(6, 45)} = 2.28$, $p = 0.052$, Figure 4.2).

Similarly, the change in shell length (mm) did not differ between treatments over time (RM-ANOVA, $F_{(6, 45)} = 2.10$, $p = 0.06$, Figure 4.3). At the start of the experiment the length (mean ± standard deviation) was 72.13 ± 0.31 mm and it increased to 87.72 ± 3.49 mm.

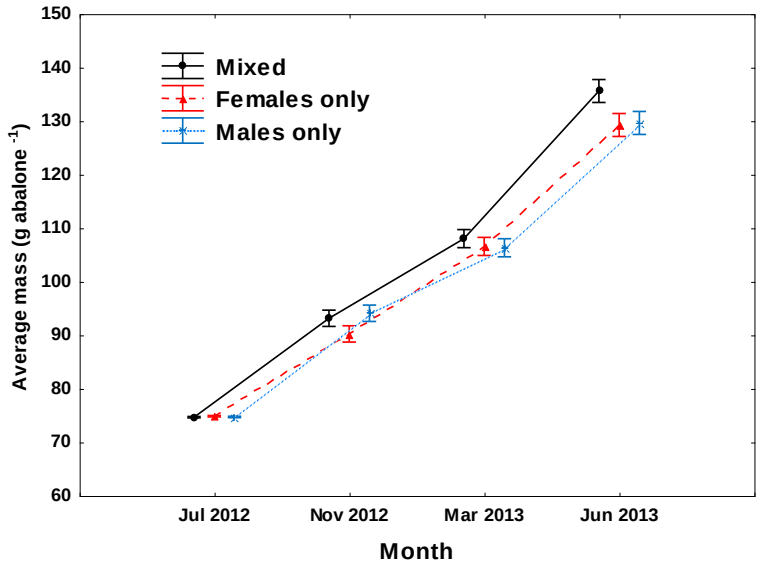


Figure 4.2: Average mass (mean ± standard error) of abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed-sex group (MF) with equal numbers of males and females. Mean weights were recorded from each tank every four months during standard farm size-grading procedures (RM-ANOVA, $F_{(6, 45)} = 2.28$, $p = 0.052$).

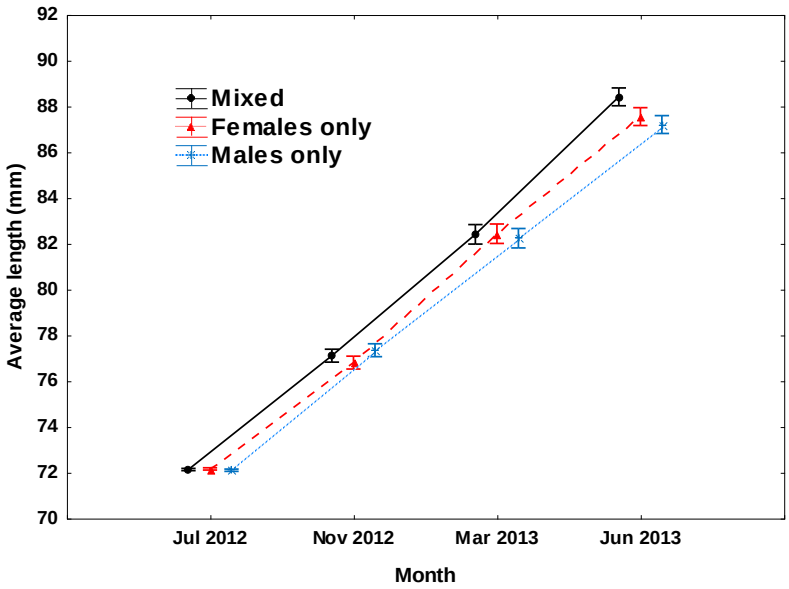


Figure 4.3: Average length (mm) (mean ± standard error) of abalone assigned to three sex groups namely males only (M), females only (F) and a mixed group (MF) with equal numbers of males and females. Mean length values were recorded from each tank every four months during standard farm size-grading procedures (RM-ANOVA, $F_{(6, 45)} = 2.10$, $p = 0.06$).

For average FCR–values, there was a significant interaction between treatment and time (RM-ANOVA, $F_{(2, 18)} = 3.65$, $p = 0.04$, G-G adjusted, Table 4.2), however, there was no significant difference in average FCR between M, F and MF treatments (RM-ANOVA, $F_{(2, 15)} = 3.30$, $p = 0.07$, Table 4.2). Average FCR increased significantly from November 2012 to March 2013 in all treatments. The M treatment had a significantly higher FCR between November 2012 and March 2013 compared to MF and F treatments (Tukey's post-hoc; $p < 0.05$, Table 4.2). The FCR-values for the period between November and March 2013 were significantly higher than the periods from July to November 2012 and March to June 2013 (Tukey's post-hoc; $p < 0.05$, Table 4.2). Average condition factor was similar for all treatments throughout the experiment (RM-ANOVA, $F_{(4, 30)} = 0.18$, $p = 0.95$, Table 4.2).

Table 4.2: The mean (± standard deviation) feed conversion ratio (FCR) and condition factor for abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and females. Significant differences are indicated by different superscripts across rows and columns (RM-ANOVA, $F_{(2, 18)} = 3.65$, $p = 0.04$, G-G adjusted and $F_{(4, 30)} = 0.18$, $p = 0.95$ for FCR and condition factor, respectively).

	July to November 2012	**November 2012 to March 2013**	**March to June 2013**	**Average**
FCR				
Mixed (MF)	1.43 ± 0.38^{a}	2.91 ± 0.41^{c}	1.14 ± 0.11^{a}	1.83 ± 0.20
Females only (F)	1.66 ± 0.30^{ab}	2.68 ± 0.33^{bc}	1.16 ± 0.08^{a}	1.83 ± 0.11
Males only (M)	1.31 ± 0.14^{a}	4.03 ± 1.49^{d}	1.20 ± 0.13^{a}	2.18 ± 0.42
Condition factor				
Mixed (MF)	1.18 ± 0.04	1.12 ± 0.03	1.14 ± 0.02	1.14 ± 0.03
Females only (F)	1.16 ± 0.03	1.11 ± 0.04	1.13 ± 0.01	1.13 ± 0.02
Males only (M)	1.18 ± 0.02	1.11 ± 0.04	1.14 ± 0.04	1.14 ± 0.03

Gonad development

Males from the MF treatment were tested against the M treatment and females from the MF treatment were tested against the F treatment over time. There was no interaction for GBI ($mm^3\ g^{-1}$) between treatment and time, with abalone from the M and F treatments having similar values compared to the same respective sex in the MF treatment (RM-ANOVA, $F_{(11, 110)} = 0.99$, $p = 0.45$ for males and $F_{(11, 110)} = 1.20$, $p = 0.30$ for females, Figure 4.4a and 4.4b respectively, Table 4.3).

Within the MF treatment, male abalone showed higher GBI values than females (RM-ANOVA, $F_{(1, 10)} = 9.69$, $p = 0.01$, Table 4.3). There was no interaction between sex and time in the MF treatment with GBI in both sexes changing equally over time (RM-ANOVA, $F_{(11, 110)} = 1.11$, $p = 0.36$, Table 4.3). GBI (mean ± standard deviation) was highest during November 2012 for both sexes with males (52.88 ± 18.99 $mm^3\ g^{-1}$) having higher GBI values than females (45.88 ± 15.79 $mm^3\ g^{-1}$) (Figure 4.4). The lowest GBI-values of 23.49 ± 7.67 $mm^3\ g^{-1}$ and 12.06 ± 9.26 $mm^3\ g^{-1}$ occurred in April 2013 for males and females, respectively (Figure 4.4).

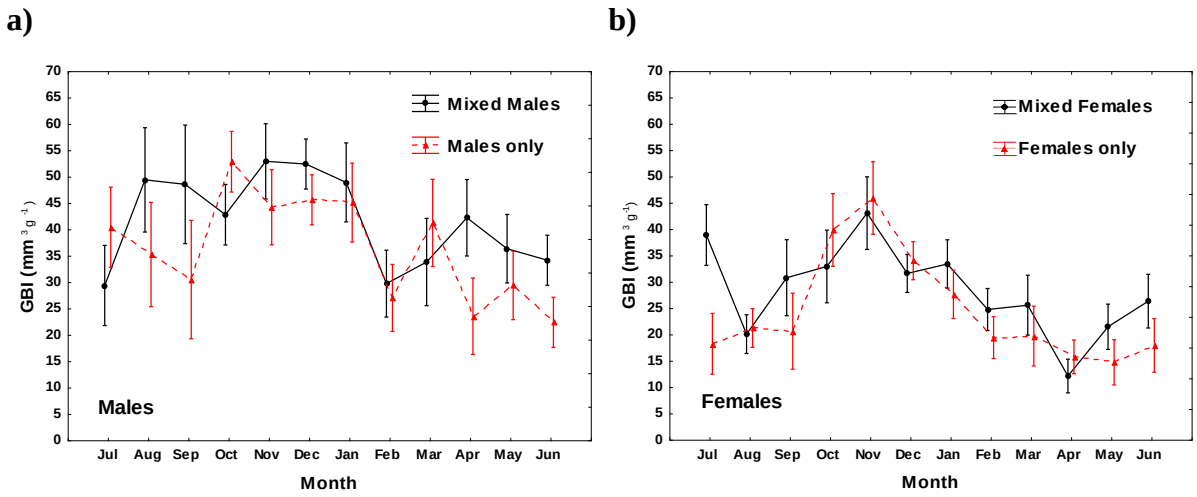


Figure 4.4: Average (± standard error) gonad bulk index (mm^3 g^{-1}) of abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and females. Males from treatment MF were compared with values of males from treatment M (graph a) and values for females from the treatment F were compared with females from treatment MF (graph b) from July 2012 to June 2013 (RM-ANOVA, $F_{(11,110)} = 0.99$, $p = 0.45$ for males and $F_{(11,110)} = 1.20$, $p = 0.30$ for females).

Visceral index (%)

There was no interaction for visceral index (%) between treatment and time, with abalone from the M and F treatments having similar values compared to the same respective sex in the MF treatment (RM-ANOVA, $F_{(11, 242)} = 0.71$, $p = 0.73$ for males and $F_{(11, 242)} = 0.9$, $p = 0.54$ for females, Figure 4.5a and 4.5b, respectively, Table 4.3).

Within the MF treatment, male abalone had a higher visceral index (%) than females (RM-ANOVA, $F_{(1, 12)} = 12.10$, $p = 0.002$, Table 4.3) and no interaction between sex and time was observed (RM-ANOVA, $F_{(11, 242)} = 0.54$, $p = 0.87$, Table 4.3). The visceral index (%) decreased from December 2012 for all treatments as this was the highest visceral index recorded for both sexes (12.77 ± 1.43 % for males and 12.62 ± 1.64 % for females) (Figure 4.5). The visceral index (%) increased from August 2012 to December 2012 and from March

2013 to June 2013 for both sexes. The visceral index (%) increased in males from MF treatment and decreased for abalone in M treatment only in April 2012 (Figure 4.5).

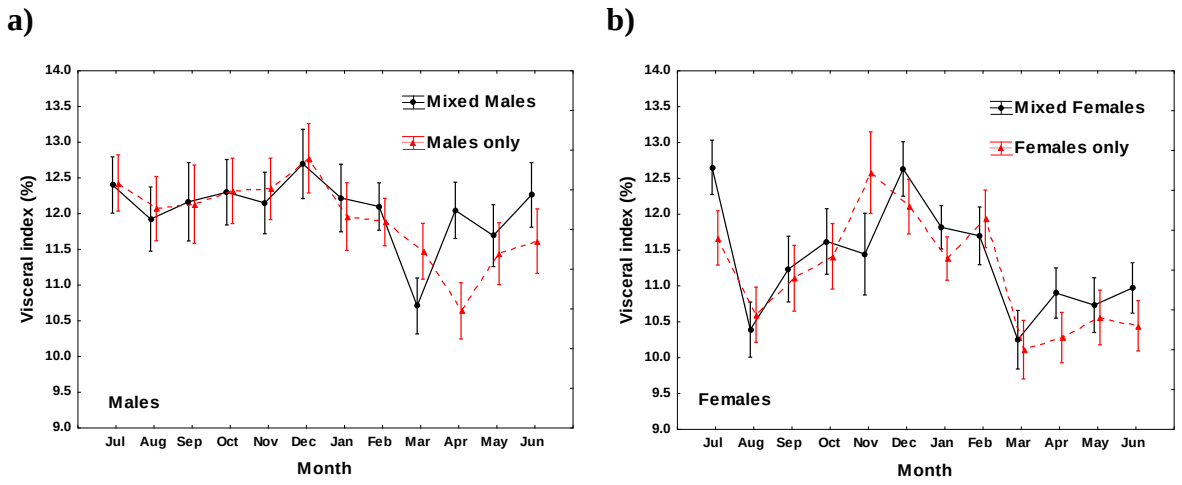


Figure 4.5: Average (± standard error) visceral index of abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and females. Males from treatment MF were compared with values of males from treatment M (graph a) and values for females from the treatment F were compared with females from treatment MF (graph b) from July 2012 to June 2013 (RM-ANOVA, $F_{(11, 242)} = 0.71$, $p = 0.73$ for males and $F_{(11, 242)} = 0.9$, $p = 0.54$ for females).

Water loss from viscera (%)

There was no interaction for water loss from viscera (%) between treatment and time, with abalone from the M and F treatments having similar values compared to the same respective sex in the MF treatment (RM-ANOVA, $F_{(11, 110)} = 1.22$, $p = 0.29$ for males and $F_{(11, 110)} = 1.06$, $p = 0.40$ for females, Figure 4.6a and 4.6b respectively, Table 4.3).

Within the MF treatment, males lost more water from the viscera (%) compared to females (RM-ANOVA, $F_{(1, 10)} = 5.7$, $p = 0.04$, Table 4.3) and there was no significant interaction between sex and time (RM-ANOVA, $F_{(11, 110)} = 1.8$, $p = 0.07$, Table 4.3). There was a

significant increase in water loss from the viscera (%) between the months of January and February 2013 for females from MF and F treatments (Figure 4.6b).

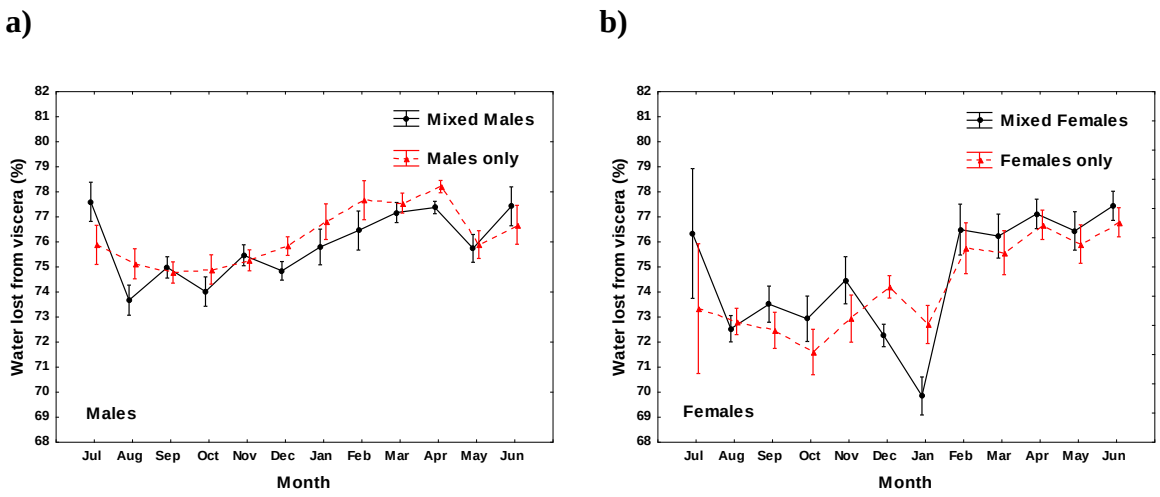


Figure 4.6: Average (± standard error) water loss from viscera (%) of abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and females. Males from treatment MF were compared with values of males from treatment M (graph a) and values for females from the treatment F were compared with females from treatment MF (graph b) from July 2012 to June 2013 (RM-ANOVA, $F_{(11, 110)} = 1.22$, $p = 0.29$ for males and $F_{(11, 110)} = 1.06$, $p = 0.40$ for females).

Digestive gland (DG) index

There was no interaction for DG index (%) between treatment and time, with abalone from the M and F treatments having similar values compared to the same respective sex in the MF treatment (RM-ANOVA, $F_{(11, 110)} = 0.90$, $p = 0.55$ for males, and $F_{(11, 110)} = 0.37$, $p = 0.96$ for females, Figure 4.7a and 4.7b respectively, Table 4.3).

Within the MF treatment, there was no significant difference in DG index (%) between sexes (RM-ANOVA, $F_{(1, 10)} = 2.65$, $p = 0.14$, Table 4.3).

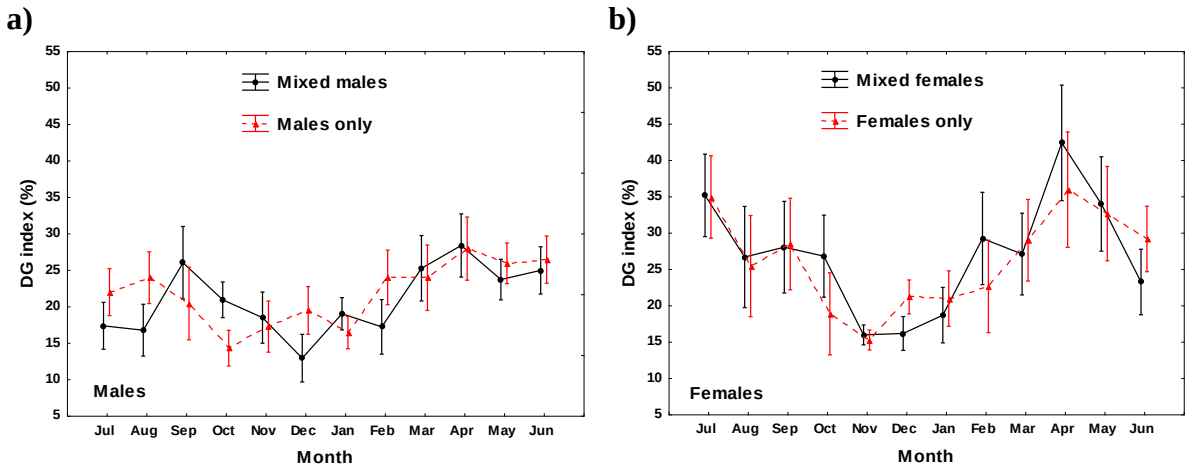


Figure 4.7: Average (± standard error) DG index of abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and females. Males from treatment MF were compared with values of males from treatment M (graph a) and values for females from the treatment F were compared with females from treatment MF (graph b) from July 2012 to June 2013 (RM-ANOVA, $F_{(11, 110)} = 0.90$, $p = 0.55$ for males and $F_{(11, 110)} = 0.37$, $p = 0.96$ for females).

Meat mass index (%)

There was no interaction for meat mass index (%) between treatment and time, with abalone from the M and F treatments having similar values compared to the same respective sex in the MF treatment (RM-ANOVA, $F_{(11, 242)} = 0.69$, $p = 0.74$ for males and $F_{(11, 242)} = 0.76$, $p = 0.68$ for females, Figure 4.8a and 4.8b respectively, Table 4.3).

Within the MF treatment, there was no significant difference in meat mass index (%) between sexes (RM-ANOVA, $F_{(11, 22)} = 3.57$, $p = 0.07$, Table 4.3). The meat mass index for both sexes decreased from August 2012 to December 2013 and increased from December 2012 to the end of the trial (Figure 4.8).

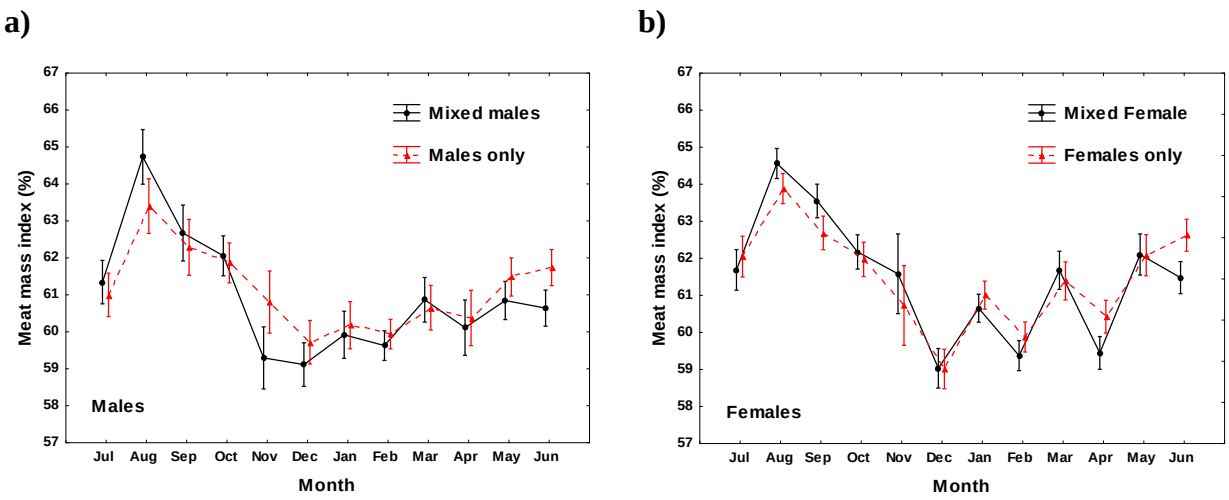


Figure 4.8: Average (± standard error) meat mass index of abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and females. Males from treatment MF were compared with values of males from treatment M (graph a) and values for females from the treatment F were compared with females from treatment MF (graph b) from July 2012 to June 2013 (RM-ANOVA, $F_{(11,242)} = 0.7$, $p = 0.74$ for males and $F_{(11, 242)} = 0.8$, $p = 0.68$ for females).

Water loss from meat (%)

There was no interaction for water loss from meat (%) between treatment and time, with abalone from the M and F treatments having similar values compared to the same respective sex in the MF treatment (RM-ANOVA, $F_{(11, 110)} = 0.64$, $p = 0.79$ for males, Figure 4.9a and $F_{(11, 110)} = 1.56$, $p = 0.12$ for females, Figure 4.9a and 4.9b respectively, Table 4.3).

Within the MF treatment, water loss from meat (%) was the same for both sexes (RM-ANOVA, $F_{(11, 10)} = 1.53$, $p = 0.24$, Table 4.3). Water loss from meat (%) increased from September 2012 to March 2013 for all treatments.

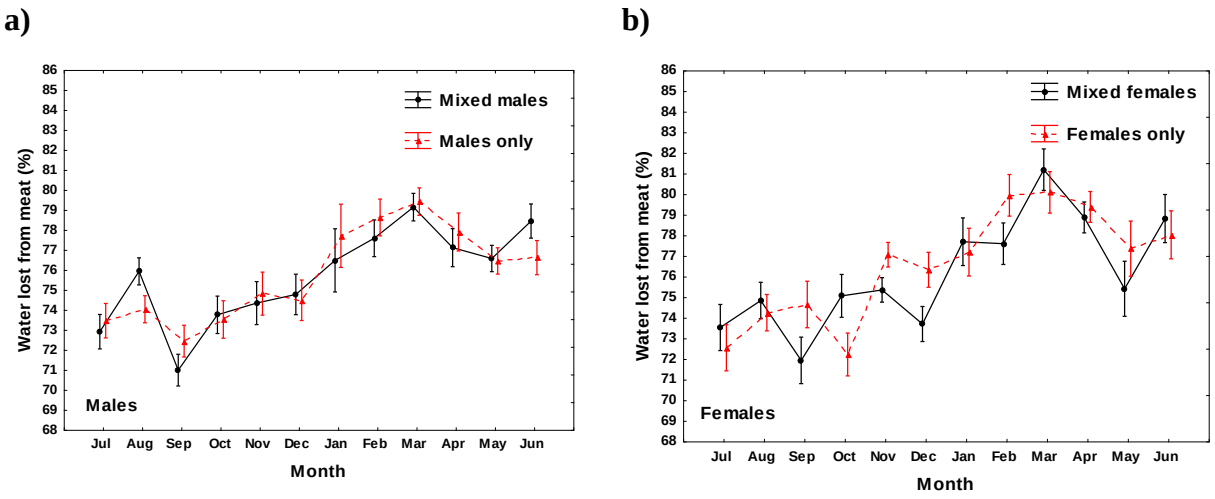


Figure 4.9: Average (± standard error) water loss from meat of abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and females. Males from treatment MF were compared with values of males from treatment M (graph a) and values for females from the treatment F were compared with females from treatment MF (graph b) from July 2012 to June 2013 (RM-ANOVA, $F_{(11, 110)} = 0.6$, $p = 0.79$ for males and $F_{(11, 110)} = 1.6$, $p = 0.12$ for females).

Table 4.3: Repeated measures ANOVA results for abalone assigned to three treatments, i.e., males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and females, tested against all of the dependent variables. Males from the MF treatment were tested against the M treatment and females from the MF treatment were tested against the F treatment. Males were compared with females in the MF treatment. Significant differences are indicated by the p-value superscript * ($p < 0.05$).

	GBI		**Visceral index**		**Water lost from viscera (%)**		**DG index (%)**		**Meat mass index (%)**		**Water lost from meat (%)**	
	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value
Males (M and MF)												
Effect												
Treatment	1.78	0.21	0.58	0.45	3.79	0.08	0.32	0.59	0.60	0.45	0.11	0.75
Repeated measures												
Time (months)	2.31	**0.01***	2.19	**0.02***	7.72	**0.00***	2.37	**0.01***	8.52	**0.00***	11.42	**0.00***
Time and treatment	1.00	0.45	0.71	0.73	1.22	0.29	0.90	0.55	0.69	0.74	0.64	0.79
Females (F and MF)												
Effect												
Treatment	1.42	0.26	0.92	0.35	1.27	0.29	0.04	0.85	0.02	0.89	1.08	0.32
Repeated measures												
Time (months)	5.93	**0.00***	6.72	**0.00***	7.04	**0.00***	3.63	**0.00***	14.95	**0.00***	12.13	**0.00***
Time and treatment	1.20	0.30	0.90	0.54	1.06	0.40	0.37	0.96	0.76	0.68	1.56	0.12
Mixed (MF)												
Effect												
Sex	9.69	**0.01***	12.10	**0.00***	5.72	**0.04***	2.65	0.14	3.57	0.07	1.53	0.24
Repeated measures												
Time (months)	1.70	0.08	2.89	**0.00***	4.43	**0.00***	2.47	**0.01***	11.77	**0.00***	9.62	**0.00***
Time and treatment	1.11	0.36	0.54	0.87	1.76	0.07	0.88	0.56	0.76	0.68	0.49	0.91

Environmental variables

There was no significant difference among treatments for water temperature, pH, dissolved oxygen (O_2), percentage oxygen saturation, total ammonia-nitrogen (TAN) and free ammonia-nitrogen (FAN) (RM-ANOVA, $p > 0.05$, Table 4.4).

Table 4.4: Mean (± standard deviation), minimum and maximum values for environmental variables tested for abalone assigned to three treatments males only (M), females only (F) and a mixed sex treatment (MF) with equal numbers of males and female from July 2012 to May 2013. Variables included temperature (°C), pH, dissolved oxygen concentration (mg L^{-1}, O_2), percent oxygen concentration, concentration of total ammonia nitrogen (TAN, µg L^{-1}) and free ammonia nitrogen (FAN). There were no differences in water quality among treatments analysed using a repeated measures ANOVA ($p > 0.05$).

Treatment	Mean	Minimum	Maximum
Mixed (MF)			
Water temperature (°C)	15.09 ± 1.83	11.10	19.80
pH	7.84	7.12	8.91
O_2 (mg L^{-1})	8.11 ± 0.61	6.75	10.13
% Oxygen saturation	97.40 ± 5.59	82.40	126.00
TAN (mg L^{-1})	24.91 ± 11.80	6.47	55.27
FAN (%)	2.01 ± 1.22	0.28	12.26
FAN (µg L^{-1})	0.55 ± 0.64	0.06	6.47
Males only (M)			
Water temperature (°C)	15.11 ± 1.84	11.20	19.90
pH	7.85	7.10	8.47
O_2 (mg L^{-1})	7.92 ± 0.49	6.55	9.10
% Oxygen saturation	98.03 ± 3.80	86.20	111.00
TAN (mg L^{-1})	25.22 ± 11.09	5.22	56.52
FAN (%)	2.03 ± 0.99	0.28	5.97
FAN (µg L^{-1})	0.51 ± 0.36	0.05	2.25
Females only (F)			
Water temperature (°C)	15.09 1.81	11.30	19.90
pH	7.87	7.11	8.76
O_2 (mg L^{-1})	8.03 ± 0.50	6.80	9.50
% Oxygen saturation	97.78 ± 4.11	84.00	110.00
TAN (mg L^{-1})	25.33 ± 11.04	0.21	69.03
FAN (%)	2.17 ± 1.24	0.27	11.38
FAN (µg L^{-1})	0.53 ± 0.39	0.01	2.16

DISCUSSION

The growth in body mass of *H. midae* in monosex tanks (treatments M and F) did not differ from that of abalone stocked in mixed-sex (treatment MF) tanks. This was not the case in studies comparing monosex and mixed-sex populations of fish and invertebrates. Chakraborty *et al.* (2011), for example, reported faster growth in male monosex Nile tilapia, *Oreochromis niloticus,* which has been related to the lack of energy expenditure on courtship by males. Abalone do not display such courtship behaviour (Clark *et al.* 2007), which may suggest that there was no energy requirement for these behaviours and therefore this may have contributed to the lack of difference in growth between mixed-sex and monosex groups. These results provide evidence that growth in *H. midae* appears to be independent of behavioural interactions between sexes. Grow-out comparisons between mixed-sex and monosex culture of *Cherax albidus* showed that the male monosex population grew faster than the female and mixed-sex populations, respectively (Sagi *et al.* 1986, Lawrence *et al.* 2000). Studies on redclaw crayfish, *Cherax quadricarinatus* also demonstrated that males from monosex ponds grew faster and attained a larger size at harvest than females from monosex and mixed-sex ponds, respectively (Curtis and Jones, 1995, Rodgers *et al.* 2006). Male channel catfish, *Ictalurus punctatus* grew significantly faster than females, whether in mixed-sex or monosex culture (Goudie *et al.* 1994). These authors also reported significantly higher FCR-values in monosex males than in mixed and monosex treatments. Similarly, in the present study FCR-values were significantly higher in treatment M than in treatments F and MF from November 2012 to March 2013, which is during presumed spawning season. The FCR-values were higher for all treatments during November 2012 to March 2013 compared to other periods. In November and December the GBI and visceral index peaked and gradually decreased until February and March 2013. These results were similar to those observed in Chapter 2 with male abalone having larger GBI-values than females. There was

no significant difference in meat mass index (%) between sexes, with no significant change between November 2012 and March 2013 (Figure 4.8) and therefore allocation of energy into gonad development (GBI) was higher in males. This may also explain the higher FCR-values observed in the M treatment because more energy may be converted into gonad mass. Furthermore, the FCR-values were higher between November 2012 and March 2013 suggesting a possible spawning event that may have occurred because there was a decrease in GBI and visceral index (%) (Figure 4.4 and 4.5). A higher gonad development and lower growth in meat mass resulted in higher FCR-values which may be due to gonad mass that was lost as gametes during this spawning period. Thus, it is suggested that the energy invested into gonad development was independent of whether abalone were kept in monosex or mixed-sexed populations except for the spawning season (November 2012 to March 2013).

Pheromones have been associated with the control of some reproductive behaviours in molluscs with a water-borne peptide pheromone being an attractant that is released during ovulation to attract other individuals so as to form mating aggregations (Susswein and Nagle 2004). If pheromones were secreted more frequently or in larger quantities in mixed-sex populations, then investment into gonad development and frequency of spawning may have been influenced more than in monosex treatments. This was, however, not the case in monosex *H. midae* treatments, suggesting that pheromones or other attractants were not used to communicate with the opposite sex because there were no significant differences between treatments. Cummins *et al.* (2005) illustrated that different species of the marine mollusc, *Aplysia spp.* attracted each other with pheromones, the effect of which was independent of sex and species. Pheromones given off by *H. midae* should be tested in future studies to examine if these chemicals are sex and species-specific. On the contrary, pheromones may be used by each sex to influence the same sex which should also be conducted in future studies.

Results from this study provide a possible explanation that pheromones were not responsible for influencing gonad development in sex-separated abalone. Counihan *et al.* (2001) suggested that the spawning behaviour observed in abalone was a result of a higher density of abalone rather than the opposite sex specifically. A higher density of abalone may stimulate abalone to release gametes more frequently, suggesting that they delay releasing gametes until more abalone surround them. Stocking densities were maintained the same for all treatments resulting in a lack of differences observed in gonad development. This may have been due to the fact that density stimulated spawning behaviour instead of a sex-specific pheromone response. Higher densities might trigger competitive behaviour within male monosex groups, thus affecting gonad development. Testes extracts from marine polychaete worms have heterospecific activity as they initiated a response in another sexually mature conspecific (Watson *et al.* 2003). However, using data for growth and gonad development did not provide evidence of this in *H. midae*. There was no significant difference in gonad development between monosex and mixed-sex groups over time as there was no interaction between time and treatment. The majority of marine invertebrates release pheromones at the same time as they release gametes (Hardege *et al.* 1996). This provides a possible reason for simultaneous gonad development that changed in the same way over time for all treatments and both sexes.

Male abalone lost more water than females from viscera, but water loss was not dependent on whether abalone were sorted into monosex groups or mixed-sex groups. Water content in viscera remained high in males for most of the year, however, in females in treatment MF it significantly decreased from November 2012 to January 2013. It should be tested whether this may be due to an increase in the number of oocytes, thus possibly lowering the water content of the gonad. Zhou *et al.* (2012) found a water content of 76 ± 6 % in the viscera of Pacific abalone, *Haliotis discus hannai*, and this falls within the range (60.7 to 87.45 %)

found in the present study. The water lost from meat was also similar in all treatments. Meat mass decreased during periods of increased gonad growth (Webber 1970). Trends in meat growth in the present study were similar to that observed in Chapter 2, thus suggesting that this is an event resulting in a meat gain compromise to allow for increased gonad development.

Conclusion

Meat and gonad growth in *H. midae* were not affected by sex-sorting. No differences were observed between monosex and mixed-sex groups for GBI, visceral and meat mass indices and body mass growth. Higher FCR-values were observed during the months of November 2012 to March 2013 compared to the rest of the trial. These high FCR-values coincide with the period of increased gonad development. Applying sex-separation techniques to a commercial abalone farm would not be recommended if the primary objective was to mitigate the loss in growth caused by increased gonad development. Sex-sorting of abalone has to be viable since the labour involved in sexing abalone is not practical in the production of a monosex crop. For example, an inexperienced labourer was able to sex-sort approximately seven abalone per minute compared to an experienced person who sorted 10 to 15 abalone per minute. This is a labour-intensive approach with no benefits to production.

CHAPTER 5

GENERAL DISCUSSION

The main focus of this study was to address the issue of reduced growth rate due to increased gonad development in abalone during the spawning season. The research produced data on the effects of diet and sex-sorting on growth, body composition and gonad development during a 12-month experiment. Experiments were conducted on a farm so that results reflect commercial conditions in order to arrive at conclusions that are applicable in the abalone industry. Over two thousand kilograms of abalone were reared for the treatments in Chapter 2. Obtaining data involved weighing, measuring and processing over 1600 kg of abalone.

Effect of the dietary ingredient, soybean meal on growth and gonad development

Abalone fed a formulated diet containing soybean meal and fishmeal as the major protein sources grew faster and showed enhanced gonad development than abalone fed a fishmeal-based diet with no soybean meal (Chapter 2). Although abalone are herbivorous, a combination of dietary plant and animal protein sources appears to be necessary to attain high growth rates (Bautista-Teruel *et al.* 2003). Fishmeal digestibility is lower in *H. midae* than plant proteins but it is high and balanced in amino acids, which is not true of plant proteins (Sales and Britz 2003). The isonitrogenous and isoenergetic attributes of the S-diet and F-diet make soybean meal addition the only difference between diets. Using abalone in the size range of 50 to 70 g abalone^{-1} insured that abalone were sexually mature at the start of the experiments. Immature abalone may have used metabolic energy for somatic growth as there are no gonads to maintain. Gonad growth was seasonal as illustrated by two peaks in soya-fed

animals, which were higher than in abalone fed no soya. Seasonal fluctuations in gonad development were the same for abalone fed both diets, but abalone fed a diet with soybean meal showed higher gonad bulk indices. The increasing and decreasing trends in GBI (Chapter 2 and 4) imply that gonad development in abalone fluctuated with a response to possible unknown environmental cues despite the fact that abalone fed the S-diet had faster gonad growth. Environmental seasonal changes may vary from year to year and therefore it cannot be assumed that results observed from this study will be the same in following years due to the highly variable seasonal changes in South Africa. Irrespective of environmental cues that trigger periods of higher gonad development, it was still evident that a combination of soybean and fishmeal initiates better growth and gonad development than just fishmeal as a protein source. The objectives of this study could be addressed as the dietary ingredient responsible for gonad development was identified as soybean meal. The objective to describe and validate the drop in growth can be explained by the meat mass index that decreased from September 2012 during the period of increased gonad growth (October 2012 to February 2013). The GBI increased from September 2012 to January 2013 and meat mass increased after February 2013. There was no significant difference between diets for meat mass index and therefore it was suggested that this was a period of meat gain compromise to allow for increased gonad development. A common observation in all chapters was the fact that FCR-values were highest during November 2012 to March 2013 which coincided with peak gonad development in this species. This increased FCR may be due to abalone eating more to compensate for increased gonad growth coupled with the significant drops in gonad mass after January 2013 when gametes were possibly released over spawning season.

Phytoestrogens

One of the objectives of this study required the need to explain which dietary ingredient was responsible for gonad development. Soybean meal enhanced gonad development and a

possible reason may be further explained by antinutrients such as phytoestrogens (daidzin, genistin, daidzein, genistein, coumestrol and equol) found in this ingredient. The discussion in Chapter 2 suggested that the phytoestrogens present in soybean products may alter reproductive physiology in abalone through an effect on gonad development. They either bind directly to estrogen receptors or convert into compounds that have estrogenic effects such as equol (Francis *et al.* 2001). Genistein and daidzein have the strongest estrogenic effects in animals (Miyahara *et al.* 2003) with plasma levels significantly affected in *A. baeri* fed a soybean-based diet. Future studies should be aimed at lowering phytoestrogens in diets without compromising the current growth observed in the grow-out *H. midae* stock. Alternatively, future studies can be aimed at isolating phytoestrogens or enhancing soybean isoflavone content in diets fed to abalone broodstock. The higher gonad development observed in S-diet-fed abalone may be as a result of phytoestrogens thus, it may be beneficial to enhance broodstock gonad quality and growth.

Gonad bulk index

The gonad bulk index (GBI) values cannot be used to calculate gonad tissue mass in abalone as the size and the shape of the digestive gland within the section was not consistent. However, the area-based calculations can give a good estimate of size and shape. The gonadosomatic index (GSI) method has been used in many studies on various abalone and other invertebrates (Webber and Giese 1969, Dorange and Le Pennec 1989, Brewin *et al.* 2000, Najmudeen and Victor 2004, Najmudeen 2007, Tung and Alfaro 2011). However, the GSI based on the mass of the gonads as a percentage to animal mass may bias results if variations in moisture contribute error to the analysis (Litaay and De Silva 2003). Furthermore, using mass only does not fully represent abalone gonad investment as the gonad is incorporated into the viscera (gut and gonad). Since there was a significant difference between diets for the water content in viscera (Chapter 2) the variations can contribute to an

error in mass-based GSI analyses. If the mass of the gonads of an animal relative to its total mass had been used, it may have been biased due to the difference in water loss from viscera between diets. It was therefore important to use GSI calculations based on gonad area, and this approach should also be used in future research. Using area-based GBIs in the present study was a valuable method of measuring gonad development considering the difficult nature of studying abalone gonads as the gonad tissue surrounds the digestive gland tissue. Thus, the experimental approach of combining gonad bulk indices and changes in histology (descriptive), spawning events and gonad development was used as a novel and better method to explain the effect of diet on growth and gonad development.

Benefits of combining gonad bulk indices with histology

It is important that data are collected using multiple techniques instead of just one to understand reproductive development (Hahn 1989a). Young and De Martini (1970) used one technique (descriptive histology) to investigate the spawning periodicity and identified two periods in red abalone, *Haliotis rufescens.* Mature gametes were observed throughout the year and conclusions suggested that there was no defined spawning period. Quayle (1971) used only histological descriptions for a reproductive study on northern abalone, *Haliotis kamtschathana* in British Columbia and also found mature gametes throughout the year, concluding that spawning period could not be defined. Sainsbury (1982) used primarily gonad bulk indices to monitor the breeding seasons of the black-footed abalone, *Haliotis iris* over four years and failed to identify a spawning event during two of these years. Some spawning events may have occurred and may have been observed if histological data had been used. Various authors combined gonad bulk index and histology on abalone species to identify spawning seasons. Wood and Buxton (1996) showed that *H. midae* were iteroparous, asynchronous spawners identifying two spawning seasons using histological examination and gonad bulk indices. Litaay and De Silva (2003) used the same combination of techniques and

histological examinations and showed that there ripe gonads throughout the whole study period even though prevalence was low outside spawning season. However, highest GSI confirmed spawning season. Shepherd and Laws (1974) identified spawning seasons of five *Haliotis* species using a combination of such techniques. Wilson and Schiel (1995) successfully identified the spawning seasons in *Haliotis iris* and *Haliotis australis* using GBI and histology data. These authors found an apparent seasonal change using GBI data while average oocyte area and frequency histograms showed spawning peaks coinciding with high GBI.

Combining gonad bulk index analysis and histology may help in comparing the effects of diet on reproduction. For example, the maturity stage distribution showed that abalone fed the S-diet developed a higher percentage of mature stage 8 oocytes compared to F-diet-fed abalone (Chapter 3). Two peaks in GBI were observed for the summer months October 2012 and December/January 2013 with abalone fed soya showing higher GBI-values (Chapter 2, Figure 2.6b). Histology revealed the mature stage 8 was the most abundant maturity stage during October 2012 for abalone from each diet. Although maturity stage 8 for F-diet abalone decreased during the second spawning peak they still had a majority of stage seven oocytes. Lower GBI values observed in F-diet-fed abalone can be confirmed from histological data suggesting that abalone had a majority of maturity stage seven oocytes, one stage below S-diet-fed abalone. The more intercellular spaces between oocytes observed in gonads from histological data of F-diet fed abalone (Chapter 3, Figure 3.4) may also contribute to the explanation of lower GBI values observed. Fewer oocytes may contribute to the lower bulk in gonads for F-diet fed abalone and this should be tested in future studies. The addition of histological techniques helped reveal the effect of diet on gonad development thus, achieving one of the objectives for this study.

Economic considerations

The profitability of the S-diet and F-diet fed to abalone on a 150 ton commercial farm was modelled in Table 5.1. These models were based on the assumptions that abalone stock, average abalone size, pumping costs to provide water for a flow-through system, other expenses, export costs and price per kg of live abalone were independent of diet. Thus, the effect of diet on profits can be estimated. Model calculations and descriptions are presented in Table 5.2. There was a profit difference of 86 % between the two diets with the S-diet earning higher profits. Abalone fed the S-diet had faster growth rates than those fed the F-diet (Chapter 2) therefore, the total monthly production and the amount of feed required was higher. The visceral mass (gut and gonad) as a percentage of whole abalone mass was higher in S-diet fed animals (Chapter 2) and this was alternatively represented by the lower "FCR-values for viscera" for the S-diet. Higher "FCR-values for viscera" suggest that more feed was required to produce more viscera illustrating that the low viscera FCR for the F-diet was less effective at producing visceral mass. The percentage of production that produces viscera was lower on the hypothetical Farm B. However, if we assume that abalone on both farms had equal monthly weight gains, Farm B would produce 5.2 % less viscera than Farm A. There was no significant difference in meat gain between the two diets (Chapter 2) explaining the similarity of the "FCR-values for meat" between Farm A and Farm B. The profitability of Farm B was highly dependent on the US dollar / Rand exchange rate and if the exchange rate dropped below R9.76/$ Farm B would be at a loss compared to Farm A which will still maintain a profit over R700 000 at R9.76/$. The higher feed cost/kg of F-diet were due to higher market prices of fishmeal coupled with the higher ratio of fishmeal in the diet compared to the S-diet. A high inclusion of fishmeal questions the environmental sustainability of this diet and the potential of fishmeal as an abalone feed component. Producing abalone using less fishmeal creates a low forage fish efficiency ratio which has

been proposed by the WWF to reduce fishing pressure on wild forage fish stocks (WWF 2011). In conclusion abalone farmers would be advised to consider using the S-diet as this was more effective than the F-diet despite the higher gonad development.

Table 5.1: Modelled scenarios of abalone farm profitability as a function of diet used. Farm A represents abalone stock that were fed the S-diet and Farm B represents abalone stock fed the F-diet. Both farms were hypothetically equal in size and capacity.

	Farm A	**Farm B**
Abalone stock (tons)	150	150
Average abalone mass (g)	60	60
Total number of abalone on farm	2500000	2500000
Total water pumping cost per month	430 000	430 000
Diet (*Chapter 2*)	**S-diet**	**F-diet**
FCR	1.63	1.7
FCR (Meat)	2.41	2.73
FCR (Viscera)	**14.1**	**19.5**
Feed required (kg):		
Total	18215.25	15555.00
Meat	16563.03	15299.94
Viscera	17631.80	18930.89
Feed price (R/ kg)	R 20.80	R 22.89
Feed cost:		
Feed cost for meat production	R 344 510.95	R 350 215.71
Feed cost for viscera production	**R 366 741.51**	**R 433 328.13**
Total feed cost	R 378 877.2	R 356 054.0
Other expenses	R 1 800 000.00	R 1 800 000.00
Export costs	R 715 000.00	R 715 000.00
Total costs	**R 3 323 877.20**	**R 3 301 053.95**
Monthly weight gain (g) (*Chapter 2*)	4.47	3.66
Total production per month (kg):	11175	9150
Total meat production	6872.63	5604.38
Total viscera production	**1250.48**	**970.82**
Price per live kg ($)	37.00	37.00
R/ $ exchange rate	10.12	10.12
Price per kg (Rands)	374.44	374.44
Turnover:	R 4 184 367.00	R 3 426 126.00
Profit	**R 860 489.80**	**R 125 072.05**
% profit relative to Farm A		**-85.47%**

Table 5.2: Model parameter descriptions and calculations used in Table 5.1.

Model parameter	**Description**
Abalone stock (tons)	Tonnage of abalone standing on farm
Average abalone mass	Average mass in grams of a single abalone in stock
Total number of abalone on farm	Used to calculate total production = ((Abalone stock * 1000)* 1000) / Average abalone size
Total water pumping cost per month	Value based on electricity required to pump water supplying a 150 ton commercial flow-through abalone farm in 2013.
Diet (*Chapter 2*)	S-diet (soybean meal and fishmeal as protein source) F-diet (fishmeal only as protein source)
FCR	Feed conversion ratio's obtained from average for each diet in *Chapter 2.* Unit dry food fed per unit wet weight gain
FCR (Meat)	Calculated from total meat biomass gained in *Chapter 2.* Unit dry food fed per unit wet weight gain
FCR (Viscera)	Calculated from total viscera biomass gain in *Chapter 2.* Unit dry food fed per unit viscera weight gain
Feed required (kg): Total	=FCR * Total production per month
Meat	=FCR (Meat) * Total meat production per month
Viscera	=FCR (Viscera) * Total viscera production per month
Feed price (R/ kg)	Current price for each experimental diet quoted from Marifeed (Pty) Ltd. pers. comm (2013). Calculated from ingredient ratios for each diet and current soybean meal and fishmeal market prices.
Feed cost for meat production	=Feed required (Meat) * Feed price
Feed cost for viscera production	=Feed required (Viscera) * Feed price
Total feed cost	=Feed required (Total) * Feed price
Other expenses	Obtained from a 150 ton commercial abalone farm (Naylor pers. comm 2013). Other expenses include salaries and wages, overhead costs, marketing and other running costs
Export costs	Obtained from 150 ton commercial abalone farm related to farm abalone stock (tons)
Total costs	=Total water pumping cost + Total feed cost + Other expenses + Export costs
Monthly weight gain (Chapter *2*)	Obtained from biomass gain data used in calculating FCR in *Chapter 2* for each diet. =Average total monthly biomass gained / Average total number of abalone
Total production per month (kg):	=(Monthly weight gain * Total number of abalone on farm) / 1000
Total meat production	Average percentage of meat to whole mass obtained from *Chapter 2*: S-diet = 61.5% F-diet = 61.25% =Total meat production * Average percentage of meat to whole mass
Total viscera production	Average percentage of viscera to whole mass obtained from *Chapter 2*: S-diet = 11.19% F-diet = 10.61% =Total meat production * Average percentage of meat to whole mass
Price per kg ($)	$ 37 kg^{-1}. Average price of abalone product (50 - 100g) sold from a commercial abalone farm
R/ $ exchange rate	$ 1 = R10.12 (average for October 2013)
Price per kg (Rands)	=Price per kg * exchange rate
Turnover:	=Total production per month (kg) * Price per kg (Rands)
Profit	=Turnover - Total costs

Sex-sorting

Although testing the effect of diet on gonad development was the main focus of this study, the possibility of a pheromonal effect from the opposite sex may have also occurred. Chapter 4 illustrated that growth and gonad development was not affected by the presence or absence of the opposite sex. Pheromones given off by each sex play an important role in reproductive behaviour in fish species (Stacey and Sorensen 2009). Gonads developed and changed equally for males and females in the mixed-sex group and monosex group suggesting that a pheromone effect on gonad development was independent of sex-separation. Within the mixed-sex group, males had a faster rate of gonad development than females, which was similar to results presented in Chapter 2. To address the concern about reducing gonad development over spawning season energy investment must favour meat growth rather than gonad development. Results from Chapter 4 suggest that the reaction to pheromones by opposite sexes could be immeasurable if these pheromones seem universal and non sex-specific. On the contrary pheromones may be less concentrated due to the high flow rates in the experimental tanks despite the fact that abalone are broadcast spawners and evolved methods of sexual attraction in a marine environment (Nhan *et al.* 2010). Other possible hormonal cues pumped in from the sea may be unavoidable explaining no difference in growth or gonad development observed between monosex and mixed sex groups. These assumptions require the need for future research to be done in controlled laboratory experiments to examine the specific effect these pheromones may have on growth and gonad development for each sex. In requisite of addressing the study objective, these results confirm that sex-sorting abalone into monosex and mixed-sex treatments had no effect on growth and gonad development.

Application to management and future research

The marketable product on an abalone farm determines the need for specific production or management strategies. Farmers need to consider the most important and beneficial approach to maximise the marketable product. For example, a canned product, such as meat only, would require as little gonad as possible because money invested into growth would seem a waste if abalone switch energy investment into the discarded visceral portion. On the other hand, a live product would draw attention away from gonad mass if a less expensive diet was used on the basis that it promotes faster overall and gonad growth. Abalone fed a diet including soybean meal had significantly faster growth which may outweigh the advantages of using an alternative diet that lowers gonad growth. Farms may also consider dividing stock into canned-product or live-product sections and grow abalone accordingly to whether meat or whole growth is priority depending on diet. The FCR-values increased during the spawning period of increased gonad development proposing the need for future research on developing diets for abalone during this period. Research into replacing protein sources adds value to any commercial aquaculture business (Britz 1996a 1996b, Britz and Hecht 1997, Tung and Alfaro 2012). Gonad development in abalone fed soybean meal was higher than F-diet-fed abalone during spawning peaks (Chapter 2) suggesting that it may be practical to modify formulated diets during this time and remove such ingredients without disadvantage to the nutritional importance of the feed. To achieve a benefit from this would require to first determine whether phytoestrogens are the main cause of the observed changes in histology, and second to conduct studies to test how long it takes for abalone to respond to a dietary change with a change in gonad development. This study has provided the foundations and the motivation for such experiments, which are currently being conducted. Future research aimed towards finding alternative proteins without phytoestrogens may be beneficial to reduce gonad development in farmed abalone without reducing growth. This may also propose aims

at modifying abalone broodstock diets with higher levels of soybean meal to promote faster gonad development and higher gamete quality. In addition to this, the costs of formulated feeds for broodstock diets will be reduced because fishmeal is replaced by less expensive soybean meal.

Conclusion

This study highlighted the effects of the dietary ingredient, soybean meal in formulated feeds on the gonad development in farmed *H. midae*. Growth was faster and gonad development was higher in *H. midae* fed the soybean meal diet compared to *H. midae* fed the diet with no soybean meal. Histology revealed higher maturity stages in oocytes from soybean fed abalone than abalone fed no soybean meal. A difference in the distribution of the maturity stages in the gonad was also affected by diet. Future studies should test whether abalone growth in soya-fed animals will be less predictable due to occasional spawning events that lead to a loss in body mass. Phytoestrogens have been hypothesised as the primary reason for the difference in growth and gonad development. However, future studies should be done under controlled conditions to test the effect of selected phytoestrogens on gonad development. Sex-sorting abalone into monosex and mixed-sex populations had no influence on growth and gonad development. Future studies should establish the management implications of changing diet formulations according to spawning season and marketable products.

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APPENDIX

Ammonium chloride standard curves showing least-square linear regression coefficients used for the calculation of total ammonia nitrogen (TAN) in Chapter 2, Chapter 3 and Chapter 4.