A holistic approach in understanding the effects of dietary protein sources on the growth and reproductive development of farmed abalone, *Haliotis midae*

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ABSTRACT

The combination of fishmeal and soya in the formulated feed of farmed South African abalone, *Haliotis midae*, not only improved abalone growth, but also the sustainability of the compound diets by reducing the reliance on fishmeal. However, the presence of soya produced larger gonads compared to those of abalone fed kelp or single-source protein diets. There is an increasing drive to control sexual maturation and reduce undesirable spawning events in farmed abalone. However, the reasons for the reported effects of soya inclusion on the reproductive development of farmed *H. midae* remain unresolved.

The aim of this research was to use a combination of techniques to contribute to the understanding of the relationship between growth, reproductive and nutritional physiology of farmed *H. midae* fed diets of varying protein sources. These techniques included stable isotope analysis (SIA), fatty acid analysis, gonad histological assessments and haemolymph sex-steroid hormone analysis. The objectives were to examine the allocation of protein and nutritional components to somatic and reproductive tissues. This study also included the first attempt to investigate the role that sex steroid hormones play during gametogenesis. Abalone (40 – 50 g abalone^{-1}) were fed one of four diets: either a single-source protein-based diet, i.e., (1) fishmeal-only (FM) or (2) soya-only (S), or a combination diet of (3) fishmeal-soya (FM S) or (4) fishmeal-sunflower meal (FM SM). Sampling occurred in 45-day intervals over one year.

Dietary protein source had an effect on the growth of abalone, with enhancements in growth linked to the combination of fishmeal and a plant-protein source. The fishmeal ingredient was the most utilised protein source throughout the temporal changes in protein allocation into somatic and reproductive tissue, followed by soya and then sunflower meal. The mean whole-

body mass of abalone was significantly influenced by an interaction between time and dietary treatment, with average whole-body mass changing differently over time between treatments (RM-ANOVA, $F_{(24, 96)} = 2.13$, $p = 0.005$). Overall, abalone that were fed FM S had higher mean whole-body mass values than abalone that were fed the single-protein based diets, while abalone that were fed FM SM were similar to animals from all dietary treatments (RM-ANOVA, $F_{(3, 12)} = 5.75$, $p = 0.01$).

Male abalone had significantly higher gonad bulk index (GBI) values compared to females (RM-ANOVA, $F_{(1, 24)} = 49.03$, $p < 0.001$) and this was independent of dietary treatment. Within each sex, female abalone fed the FM S diet (15.92 ± 1.88 $mm^3\ g^{-1}$) had significantly higher GBI values than abalone fed S (9.76 ± 1.08 $mm^3\ g^{-1}$), while abalone fed FM (11.96 ± 1.71 $mm^3\ g^{-1}$) and FM SM (11.90 ± 0.80 $mm^3\ g^{-1}$) were similar to abalone from all other dietary treatments (Tukey's HSD, $p < 0.05$). Male abalone fed the FM S (21.59 ± 2.10 $mm^3\ g^{-1}$) and FM SM diet (19.30 ± 2.63 $mm^3\ g^{-1}$) had similar GBI values and they were significantly higher than in abalone fed the S diet (14.74 ± 1.27 $mm^3\ g^{-1}$), while abalone fed FM S had significantly higher GBI values than abalone that consumed the FM diet (15.08 ±1.63$mm^3\ g^{-1}$) (Tukey's HSD, $p < 0.05$).

Although sunflower meal was poorly utilised, it produced similar overall growth to abalone that were fed the FM S diets. Yet, feed conversion ratio values were significantly lower for abalone fed FM S (1.30 ± 0.13) compared to those in the other three treatments, with ratios ranging from 1.65 – 1.72 over the one-year. The fatty acid compositions of the somatic and gonadal tissues were similar between treatments (PERMANOVA, $p = 0.21$), while fatty acid composition was influenced by sampling day, tissue type and abalone sex (PERMANOVA, $p < 0.05$). The essential fatty acids (EFAs) eicosadienoic acid and α-linolenic acid were present

in abalone tissue, but they were not detected in the diets, suggesting the important role that an alternate food source (e.g. farmed abalone also had access to diatoms) may have played and the ability that *H. midae* may have in converting long-chain polyunsaturated fatty acids from C18 precursors. A change in the abundance of EFAs in the gonad tissue during highest and lowest GBI values suggested that arachidonic, eicosapentaenoic, γ-linolenic and linoleic acid were important in females, while eicosapentaenoic, eicosadienoic and α-linolenic acid were important for male reproductive development.

Dietary protein sources had an effect on the frequency distribution of maturity stages, where females that were fed FM S produced more ripe gonads and more males that were fed FM SM contained testes that showed signs of ripeness over the one-year study. Although dietary protein influenced the sex steroid concentrations in females and males, exhibiting fluctuations throughout the one-year period, no distinct pattern linked to gametogenesis were observed.

The results from this study illustrate: (1) the importance of conducting laboratory studies when implementing SIA and mixing models in aquaculture nutrition; (2) conducting nutritional studies on mature, grow-out abalone; and (3) assessing the importance of naturally occurring diatoms in their diet and their contribution to growth and reproduction. The novel contribution of this research towards abalone nutritional physiology, the implications of these findings to industry as well as potential considerations for future studies were addressed.

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LIST OF ABBREVIATIONS

ALA	α-linolenic acid
ANCOVA	Analysis of covariance
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ARA	Arachidonic acid
A_T	Total area in the photograph
DG	Digestive gland
DHA	Docosahexanoic acid
DPA	Docosapentaenoic acid
EDA	Eicosadienoic acid
EFA	Essential fatty acid
EGV	Effective gonad volume
EPA	Eicosapentaenoic acid
E_T	Total number of oocytes in the photograph
FA	Fatty acid
FAMEs	Fatty acid methyl esters
FCR	Feed conversion ratio
FM	FM-only based diet
FM S	Fishmeal and soya-based diet
FM SM	Fishmeal and sunflower meal-based diet
γ-LA	Gamma-linolenic acid
GBI	Gonad bulk index
G_{ep}	Gonad epithelium

GSI	Gonadosomatic index
HUFA	Highly unsaturated fatty acid
LA	Linoleic acid
L_{ca}	Length of the conical appendage
LC-PUFA	Long chain polyunsaturated fatty acid
L_f	Final shell length
L_t	Initial shell length
M_f	Final wet whole0body mass
M_m	Wet meat mass
M_t	Initial wet whole-body mass
MUFA	Monounsaturated fatty acids
nd	Not detected
NMDS	Non-metric multidimensional scaling analysis
n-3	Omega-3
n-6	Omega-6
OA	Oleic acid
O_g	Oogonia
PAL	Palmitic acid
PERMANOVA	Permutational multivariate analysis of variance
PUFA	Polyunsaturated fatty acid
RM-ANOVA	Repeated measures analysis of variance
S	Soya-only based diet
SA	Stearic acid
SD	Standard deviation
SE	Standard error

SFA	Saturated fatty acid
SIAR	Stable isotope analysis in R
SIMPER	Similarity percentage analysis
St	Stage
V_m	Wet visceral mass
W_m	Wet whole-body mass
W_s	Shucked mass

CHAPTER 1

GENERAL INTRODUCTION

1.1 Problem statement

Farmed abalone, *Haliotis midae*, that are a fed a compound formulated feed that includes soya, *Glycine max*, as the sole protein source or as a partial substitute for fishmeal, produced larger gonads compared to those of abalone fed kelp or diets that include only fishmeal as the main protein source (Tung and Alfaro, 2012; Ayres, 2013; Riddin, 2013; Wu *et al.*, 2019). Soya also influenced reproduction in female abalone by producing more mature oocytes throughout the year relative to abalone that were fed non-soya diets (Ayres, 2013), as well as by exhibiting a dose-dependent effect on the proportion of mature oocytes (Wu *et al.*, 2019). In an industry where the somatic tissue forms the principal monetary value of the marketed product since the gonad is often discarded when abalone is dried or canned (Tao *et al.*, 2016), this increase in investment into reproductive tissue growth is not favourable. Moreover, the addition of soya in the diet can lead to unpredictable spawning events of farmed abalone, which leads to a sudden loss of biomass in the tanks and the resultant difficulties for the estimation of feeding rates and growth. However, the inclusion of soya not only improves overall abalone growth rates, but also improves the sustainability of the compound diets by reducing the reliance on fishmeal (Guzmán and Viana, 1998; Shipton and Britz, 2001a; Dlaza *et al.*, 2008; Ayres, 2013; Wu *et al.*, 2019).

It was hypthesised that the phytoestrogens found in soya were potentially responsible for the increased reproductive development (Ayres, 2013; Riddin, 2013), however a study that included crystalline isoflavones in a fishmeal-only based diet could not verify the effects seen in abalone fed fishmeal with increased rates of soya inclusion (Wu *et al*., 2019). Therefore, the reasons for the effects of soya inclusion on the reproductive activity in both males and females as well as its influence on the distribution of oocyte stages in farmed *H. midae* remain unresolved.

1.2 Literature review

1.2.1 Haliotids

Abalone are marine gastropods, located along the coastlines of temperate to tropical regions where they dwell in the tidal to subtidal zones (Mau and Jha, 2018). The genus *Haliotis* consists of approximately 100 species, 56 which are extant and of the five species endemic to southern Africa, *H. midae* is the largest and only species of commercial importance (Venter *et al*., 2016). It has a coastal distribution that spans the warm waters of Port St Johns, Eastern Cape to the colder waters of St Helena Bay in the Western Cape (Hecht, 1994; Venter *et al*., 2016).

Abalone are herbivorous, with distinct ontogenetic shifts from diatoms to microalgae and macroalgae (Mau and Jha, 2018). They are sedentary animals and move small distances at night to feed when the supply of macroalgae is restricted (Morash and Alter, 2016; Venter *et al*., 2016). The anatomy of abalone consists of three main components: the shell, foot muscle and visceral mass that comprises of the reproductive, digestive, respiratory and circulatory organs (Morash and Alter, 2016). The shells of abalone are used for jewellery and the inlaying of furniture (Grindley *et al*., 1998), while the viscera are typically discarded or processed into low-value feedstuff (Tao *et al*., 2016). The foot muscle of the abalone is processed for human consumption and it is a prized delicacy worldwide, but predominantly in Asian countries with China being the largest consumer of abalone (Cook, 2014; Morash and Alter, 2016; Tao *et al*., 2016).

1.2.2 Haliotis midae industry in South Africa

The abalone fishery in South Africa began in 1949, but farming only began in 1981 as a result of the successful capturing and spawning of wild-caught abalone (Sales and Britz, 2001a). Commercial farming facilities typically have a hatchery, nursery area and on-growing facilities. The hatchery holds the broodstock and the larvae during their settlement and early post-settlement development during which time they are fed diatoms (Iba, 2008). They are then weaned from the diatoms on to either macro-algae or a pelleted feed at the juvenile stage (Kawamura *et al*., 1995; Venter *et al*., 2016). After weaning, the spat is moved to the grow-out facility, where they are reared on macroalgae or formulated feed until the abalone reach market size (Venter *et al*., 2016). The size at which abalone are harvested varies from approximately 80 g abalone^{-1} (cocktail size) to larger abalone of up to 200 g abalone^{-1}, depending on consumer preferences. Under conditions on farms with a consistent food supply, some animals have been observed to reach full sexual maturity at two years , measuring approximately 48 mm in length (Roux *et al*., 2013), compared to wild caught abalone which can take between 3 – 7 years to reach full sexual maturity (Newman, 1967; Wood and Buxton, 1996), and may lead to unexpected spawning and resultant weight loss due to the high gonadal index of abalone (Riddin, 2013).

South Africa is the largest producer of abalone outside of Asia with 12 farms currently in operation (Cook, 2016; Roux *et al*., 2016). Products sold include canned, dried, frozen and live abalone, with production increasing from 1023 to 1400 t between 2010 and 2015, respectively (Cook, 2016). Low meat recovery relative to gonad size upon harvest is a problem for abalone farmers since only the foot adductor muscle forms part of the product, unless animals are sold live (Jumah *et al*., 2016). The gonad encompasses the bulk of the visceral mass (Newman, 1967), with the viscera comprising up to approximately 12 % of the whole-body mass (Ayres, 2013; Wu *et al*., 2019). Thus, investment into reproductive tissue is only beneficial for broodstock animals as well as animals used for live export, as the gonads form part of the whole-body weight. However, farmers may need to compensate for weight loss as a result of spawning that can occur during transport by adding extra abalone during packing.

1.2.3 Formulated feed, growth and reproduction

In order to support the economic feasibility, convenience, efficiency and sustainability of the abalone aquaculture industry, a compound feed was formulated to maintain or improve the growth rate (Fleming *et al*., 1996; Sales and Britz, 2001a; Sales and Janssens, 2004; Iba, 2008). Research has been conducted to determine inclusion rates of the various components of the compound feed for *H. midae*. Examples are studies on protein requirement and quality (Britz, 1996a; Sales *et al*., 2003), the importance of dietary lipids (Green *et al*., 2011a), ingredient digestibility (Sales and Britz, 2001b; Shipton and Britz, 2001b; Sales and Britz, 2002a; Shipton and Britz, 2002; Sales *et al*., 2003), the relevance of the ratio of protein to energy (Britz and Hecht, 1997; Green *et al*., 2011b; Riddin, 2013), the suitability of ingredients (Britz, 1996b; Shipton and Britz, 2001a), ingredient utilization (Kemp, 2018) and amino acid requirements (Lloyd, 2016). Many of these studies were conducted on *H. midae* that had not yet reached sexual maturity, therefore the effects of formulated feed on reproductive development in abalone were either not incorporated or remained unquantified until abalone farmers started reporting spawning events and changes in reproductive development of abalone.

A number of protein sources were tested on the growth of *H. midae*, including different types of fishmeal, soybean meal, spirulina, casein, torula yeast, sunflower meal, cotton seed meal, abalone viscera silage and brewery waste (Britz, 1996b; Shipton and Britz, 2001b). These authors found that fishmeal, spirulina and sunflower meal resulted in the best growth and feed efficiency when used as primary protein source, while differences in the response of juveniles and young adults indicated size-specific nutritional requirements in farmed *H. mI* (Shipton and Britz, 2001a). These authors also determined that fishmeal could be replaced by 30 and 50 % of sunflower meal and soybean meal, respectively without significantly affecting growth (Shipton and Britz, 2001a). The abalone industry in South Africa relies on a commercially available diet comprised of fishmeal and soya for the protein component, as it produces favourable growth, reduces the reliance on fishmeal and seaweeds, particularly in areas where the preferred species is not readily available (Dlaza *et al*., 2008). However, it has been shown that the presence of soya also resulted in larger gonads in sexually mature farmed abalone (40 – 70 g abalone^{-1}) compared to those fed kelp or a fishmeal-only based diet (Ayres, 2013; Riddin, 2013; Wu *et al*., 2019). The production of gametes is energy-expensive (Ault, 1985) and there has been an effort to control sexual maturation and undesirable spawning (Botwright *et al*., 2014; Morash and Alter, 2016**).** *Haliotis midae* are slow growing animals, taking approximately four years to reach market size (Troell *et al*., 2006) and considering one of the highest recurring cost in the culturing of abalone is the feed cost (Sales and Janssens, 2004), adjustments in the diet that promote growth, particularly somatic growth are beneficial, while focusing on protein as it is the most expensive component that is essential for soft tissue growth (Fleming *et al*., 1996).

Soybean meal is one of the main plant protein sources in animal diets worldwide, due to its good essential amino acid profile, continuous supply and lower cost compared to other plant protein sources (El-Sayed *et al*., 2012). However, soya also contains antinutritional compounds, including phytoestrogens, phytic acid and protease inhibitors (Burrells *et al*., 1999; Francis *et al*., 2001) that may influence growth and/or reproductive development. On the other hand, sunflower meal has been found to be a suitable alternative to soya in prawns and fish (Sanz *et al*., 1994; Hossain and Chakraborty, 2017) with a major benefit in that it does not contain as many antinutritional factors as those found in soya.

Feeding an animal a diet and measuring its growth output gives us an indication of the diet as a whole on the growth and not how the individual components are influencing particular tissues, such as the somatic and reproductive organs and how this may change over time. The utilization of individual ingredients is made possible through the use of stable isotope analysis and mass-balance mixing models. This approach has been increasingly used in aquaculture nutrition studies to determine the contributions of alternate feed ingredients to tissue growth of animals (Gamboa-Delgado and Le Vay, 2009; Martínez-Rocha *et al.*, 2013; Gamboa-Delgado, 2014; Gamboa-Delgao *et al*., 2016; Cyrus *et al.*, 2019), but has not been studied in abalone within the context of understanding the allocation of dietary ingredients to somatic and reproductive tissues.

The protein sources used in formulated feeds are comprised of variable important and essential nutrients required for growth and reproduction, such as essential fatty acids (EFAs), which are typically mobilised to the gonads and considered important components for reproduction (Bautista-Teruel *et al*., 2001). There has been increasing evidence suggesting that fatty acids are important in gametogenesis in abalone (Webber, 1970; Uki *et al*., 1986; Uki and Watanabe, 1992; Bautista-Teruel *et al*., 2001; Nelson *et al*., 2002), particularly n-3 and n-6 polyunsaturated fatty acids (Floreto *et al*.,1996; Mai *et al*., 1996; Nelson *et al*., 2002; Hu *et al*., 2009; Bautista-Teruel *et al*., 2011; Mulvaney *et al*., 2015). The fatty acid composition of abalone tissue is dependent on the diet consumed (Nelson *et al*., 2002), but no studies have been conducted on how the different protein sources used in the formulated diet influence the tissue composition and subsequently growth and reproduction on the South African species, *H. midae*. Understanding the allocation of particular EFAs to the development of reproductive organs will shed light on how the availability and allocations of EFAs in the protein ingredients incorporated in H. midae formulated feeds are linked to somatic and reproductive development.

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One of the most accurate methods of measuring reproductive development in abalone is with gonad histological assessments (Litaay and De Silva, 2003). However, for abalone, gametogenesis is an ongoing process and gonads often contain oocytes and sperm cells at several developmental stages at any given point in time, therefore the stage of development of the gonad is typically based on the prevalence of cell types (Capinpin *et al.,* 1998). It is common to study gonad histology in only female abalone as females are easier to monitor compared to males (Gurny and Mundy, 2004), thus histological assessments in males should also be considered in future studies.

Another method of measuring the reproductive cycle is the measurement of sex steroid hormones, particularly 17β-oestradiol and testosterone (Zebrzanska *et al.*, 2015), however, this method has not yet been assessed in abalone. By monitoring gonad histology in conjunction with sex steroid hormone levels, one may be able to demonstrate the role that sex steroids play in gametogenesis in abalone and test whether their levels are influenced by diet

1.3 Aims and objectives

While a plentiful amount of research has served to establish and sustain the commercial abalone industries worldwide, “we have surprisingly, little understanding of the unique nutritional physiology of these organisms” (Kemp, 2018).

The aim of this study was to contribute to the standard approach of feeding abalone specific compound diets and measuring their growth and reproductive outputs, by also studying the allocation of the nutrients provided by different dietary protein ingredients to somatic and reproductive tissue growth, particularly in mature, reproducing animals. The study contributes to the existing body of literature on diet formulation for this species by using the most accurate methods of assessing reproductive development simultaneously to elucidate the potential link between sex steroid hormones and gametogenesis in farmed *H. midae* fed a range of diets. Thus, the research approach aiming to better understand abalone growth, nutrient allocation and reproductive development by using a wide range of methods that have not been previously tested simultaneously in any study on this species.

The aim of the study was met by carrying out the following objectives:

1) monitor the growth and reproductive development of abalone fed single-protein based diets (fishmeal-only and soya-only) and combination diets (fishmeal-soya and fishmeal-sunflower meal) over one-year;
2) determine the potential temporal changes in the relative contribution of fishmeal, soya and sunflower meal to somatic and reproductive tissue growth with the use of stable isotope analysis;
3) determine the differences in the allocation of essential fatty acids to the somatic and reproductive tissue from fishmeal, soya and sunflower meal and its link to reproductive investments; and
4) use gonad histological assessments of female and male abalone to accurately stage reproductive development in abalone fed fishmeal, soya and sunflower meal while also studying the importance of 17ß-oestradiol and testosterone for gametogenesis.

CHAPTER 2

USING STABLE ISOTOPE ANALYSIS AS A TOOL IN UNDERSTANDING THE EFFECTS OF DIETARY PROTEIN SOURCES ON GROWTH AND REPRODUCTION IN FARMED *Haliotis midae*

2.1 INTRODUCTION

Protein is one of the main components in the formulated feeds used for culturing abalone and is considered to be the most influential on growth (Lee, 2004). The South African abalone industry has successfully relied on a superior, commercially available formulated feed, Abfeed® S34 (produced by Marifeed (Pty) Ltd, Hermanus), which comprises of fishmeal and soya (*Glycine max*) as the primary protein sources. The use of soya only reduces the reliance on fishmeal, but the combination of these two ingredients had been shown to promote growth of *H. midae* (Guzmán and Viana, 1998; Dlaza *et al.*, 2008; Ayres, 2013; Wu *et al.*, 2019).

Although the combination of fishmeal and soya has resulted in improved growth in farmed *Haliotis midae*, the presence of soya has resulted in increased investment in reproductive tissue at both the macroscopic and microscopic level and it was hypothesised that the phytoestrogens present in soya were responsible for this phenomenon (Ayres, 2013; Riddin; 2013). However, the inclusion of crystalline isoflavones in fishmeal-only based diets failed to verify the occurrence of larger gonads, and hence in importance of phytoestrogens, compared to diets with increased soya inclusion (Wu *et al.*, 2019). Replacing soya with an alternate plant protein source that does not contain isoflavones, but has similar nutritional value to soya may be useful in eliminating the possibility that these isoflavones play a role in increased reproductive tissue growth.

Sunflower (*Helianthus annuus*) meal has been found to be a suitable alternative to soybean meal in a range of cultured animals, including poultry (de Morais Oliviera *et al*., 2016), fish (Sanz *et al*., 1994), prawns (Hossain and Chakraborty, 2017) and swine (Carellos *et al*., 2005). Shipton and Britz (2001b) reported that 30 % of the fishmeal component could be replaced with sunflower meal without significantly reducing growth in *H. midae*. Furthermore, the digestibility of soybean and sunflower meal have both been reported to be very high at 85 and 99 %, respectively in *H. midae* (Sales and Britz, 2002b). One of the major benefits of using sunflower meal is that it does not contain antinutritional factors as those found in soybean meal, but it has a much lower lysine content compared to soybean meal (Florou-Paneri *et al*., 2014), which can be corrected for with a balanced protein diet (Sokarovski *et al*., 1990) or by the addition of lysine. Since lysine has been suggested and shown to be the first limiting amino acid in *H. midae* (Shipton and Britz, 2002; Lloyd, 2016), the addition of lysine would be necessary.

This study will not only contribute to the knowledge on abalone physiology, particularly on larger, sexually mature abalone, but feeding animals formulated feeds comprised of variable protein sources and measuring growth and reproductive outputs will be insightful. However, understanding the utilisation of the individual ingredients would be particularly valuable, especially in an industry where the cost of feed is considered to be the highest recurrent cost (Sales and Janssens, 2004). Furthermore, gamete production is energy-expensive (Ault, 1985) and understanding which ingredients are more utilised in the gonads during periods of peak reproduction will give us a better understanding of the usage of the respective ingredients.

Stable isotope analysis has been used in aquaculture to assess the nutritional contributions of alternative feed ingredients to tissue growth (Gamboa-Delgado and Le Vay, 2009; Martínez-Rocha *et al.*, 2013; Gamboa-Delgado, 2014; Gamboa-Delgado *et al.*, 2016). The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ stable isotopes, denoted by delta, occur naturally and can be used with no environmental contamination (Schroeder, 1983; Gamboa-Delgado, 2014; Gamboa-Delgado *et al.*, 2016). Plant protein-based diets have lower (more negative) values for $\delta^{13}C$, while fishmeal-based diets are less positive for $\delta^{15}N$ than plant protein-based diets (Moreno-Rojas *et al.*, 2008). Different plants have different major pathways for carbon fixation during photosynthesis. For example, soya and sunflowers are C3 plants and have a higher $^{13}C/^{12}C$ ratio than C4 plants, such as corn (Cruz *et al.*, 2004). The $\delta^{13}C$ values of tissues are influenced by lipid content, which varies within or among tissues and can significantly influence $\delta^{13}C$ values and this can be mitigated by chemical extraction (Mintenbeck *et al.*, 2008; Ryan *et al.*, 2012). Both $\delta^{13}C$ and $\delta^{15}N$ have been used to explore the transference and deposition of dietary protein sources in aquaculture (Gamboa-Delgado *et al.*, 2013, 2016).

Mass-balance mixing models are used to determine the relative contribution of dietary ingredients to tissue growth and by using dietary ingredients of isotopically distinct values; these models represent an advantage in estimating turnover rate and nutrient incorporation as the output resolution of the model is improved (Cruz *et al.*, 2004; Gamboa-Delgado and Le Vay, 2009; Gamboa-Delgado *et al.*, 2011). However, the use of mixing models requires certain assumptions and conditions. These consider that the animal is in isotopic equilibrium with its diet and that the isotopic discrimination factors are known (Gamboa-Delgado, 2014). Isotopic equilibrium of an organism's tissue, with its diet, is evaluated through time series sampling and is reached when ratios in the tissues reach a steady state (Le Vay and Gamboa-Delgado, 2011). The discrimination factor is defined as the difference in isotopic values between an animal and its diet once it has reached isotopic equilibrium and may be dependent on a number of factors, such as diet quality, animal species and feed intake and is linked to growth rate (Gamboa-Delgado and Le Vay, 2009; Bloomfield *et al*., 2011). Time taken to reach isotopic equilibrium, also termed turnover rate, varies among species, tissue type and is dependent on sexual maturation (Bloomfield *et al*., 2011). For example, Atlantic salmon (*Salmo salar*) with a starting weight of 48 g fish^{-1} required a 300 % increase in mass to reach complete muscle tissue turnover, which took eight months. Nile tilapia (*Oreochromis niloticus*) fingerlings with a starting weight of about 3.5 g fish^{-1} had to double their weight, which took two months (Zuanon *et al*., 2007). Fast growing fish exhibit faster turnover rates and a shorter half-life of carbon and nitrogen in the tissue than slow growing animals (Li *et al*., 2013). Tissue selection for stable isotope analysis is important as isotopic routing occurs, which is the selective metabolism and incorporation of different dietary elements, instead of being evenly mixed and directed to all tissues (Gamboa-Delgado, 2014). It is therefore possible to trace a specific dietary element, such as nitrogen or carbon to a specific tissue-reservoir (Gamboa-Delgado, 2014), for example muscle or gonad tissue.

Stable isotope analyses in abalone have mainly been focused on ecological studies, evaluating dietary preference and ontogenetic changes in feeding habits of wild abalone (Won *et al*., 2007; Guest *et al*., 2008; Won *et al*., 2010a, 2010b; Vega-García *et al*., 2015). One study has evaluated the applicability of the use of stable isotope analysis by estimating the time taken to reach isotopic equilibrium as well as the $\delta^{13}C$ and $\delta^{15}N$ discrimination factors in juvenile *Haliotis discus hannai* (Won *et al*., 2008). Only one study has implemented stable isotope analysis and mixing models in assessing the contributions of dietary ingredients in formulated feed on the tissue of *H. midae* (Kemp, 2018). However, the reproductive development of the animals was not investigated (Kemp, 2018).

The aim of this study was to determine the utilisation of dietary protein sources in mature abalone, with the use of stable isotope analysis. The relative contribution of the two widely used protein sources, fishmeal and soya, as well as an alternate plant protein source (sunflower meal) to abalone muscle and gonad tissue growth was explored over one-year, encompassing a full reproductive cycle. This was achieved by carrying out the following objectives:

1) quantify the growth and reproductive tissue development of abalone fed single-protein based diets (fishmeal-only based and soya-only based) and combination diets (fishmeal-soya and fishmeal-sunflower meal) over 12 months;
2) determine the time taken for $\delta^{13}C$ and $\delta^{15}N$ in the muscle and gonad tissue to reach isotopic equilibrium with its respective diets;
3) determine the $\delta^{13}C$ and $\delta^{15}N$ discrimination factors for muscle and gonad tissue of abalone fed the respective diets; and
4) quantify the changes in the relative contribution of fishmeal, soya and sunflower meal to the somatic and gonadal tissue of abalone fed combination diets over 12 months.

2.2 MATERIALS AND METHODS

Experimental system

The study was conducted at Whale Rock Abalone Farm (Pty) Ltd, Hermanus (34°26’04.35”S; 19°13’12.51”E), South Africa over 12 months (24 July 2017 to 16 July 2018). Animals were kept in 2800 L canvas tanks (4.3 x 0.85 x 0.67 m), supported by wooden frames and each tank contained six oyster mesh baskets (76 x 55 x 59 cm; Riddin, 2013). Each basket contained seven vertical acrylonitrile butadiene styrene plastic plates to increase the surface area for attachment of abalone and a horizontal floating top plate. Aeration was provided to each tank through two 20-mm polyvinyl chloride tubing with 1 mm holes, which ran perpendicular along the bottom length of the tank (Riddin, 2013). Sea water was pumped from the sea into a header tank where the water was filtered through micro screen drum filters (90 µm; Riddin, 2013; Wu *et al*., 2019), which was then gravity-fed into the tanks with a flow rate provided to achieve 1.5 exchanges per hour (Riddin, 2013). Once a week, tanks were cleaned as baskets were moved into cleaned tanks (Ayres, 2013; Riddin, 2013).

Experimental animals and acclimation

Hatchery-reared abalone, *Haliotis midae* (40 – 50 g abalone^{-1}, 26 – 28 months old, n = 17 352, spawned from several females from three different cohorts were used in the study. Three batches of abalone were included, which were spawned two months apart, with one-month interval in between. Due to the difficulty in obtaining large quantities of abalone for the farm-scale trial, abalone from different cohorts and batches (time of spawning) had to be used. Abalone from each spawning period and cohort were equally distributed amongst the dietary treatments. The abalone were raised in a commercial abalone hatchery (Romansbaai Farm, Gansbaai, South Africa) and were subsequently subjected to farm management procedures before the study commenced. Three weeks prior to the study, abalone were anaesthetised by bubbling carbon dioxide through the water in the holding tanks for 15 min, ensuring the pH was maintained at 4.6 – 4.9 as per industry protocol. Abalone were placed in mesh bags that were kept in crates and were transported to Whale Rock Abalone Farm (Pty) Ltd, Hermanus. Each basket was stocked at 17 % surface area with a biomass of approximately 8.1 kg basket^{-1} for this size class of abalone (Riddin, 2013). Abalone were fed a commercial abalone feed (Abfeed® S34, Marifeed (Pty) Ltd, Hermanus) prior to the study.

Dietary treatments

Animals were given one of four dietary treatments, formulated to contain 30 % crude protein, 6 % lipid and 15.77 – 17.43 MJ kg^{-1} energy (Table 2.2.1). Each treatment included four replicate tanks, containing six baskets per tank. Two diets contained either fishmeal (FM) or soya-oil cake (S) as the main protein source. Soya-oil cake will be referred to as soya/soybean meal. A third diet contained a combination of both fishmeal and soya and the fourth diet comprised of fishmeal and dehulled sunflower meal (SM) as the main protein source. Abalone were fed once daily, between 15h00 and 16h00 and sufficient feed was provided to ensure that they reached apparent satiation (Nel *et al.*, 2017a). Each replicate tank was assigned a bucket with feed that was re-filled as required. Feed weights were recorded and used to determine the feed conversion ratio at six-month intervals (FCR; Equation 1; Britz, 1996b):

$$FCR = \frac{Dry\ feed\ consumed\ (g)}{Wet\ mass\ gained\ (g)} \qquad (1)$$

Table 2.2.1: Formulation of dietary treatments, including a fishmeal-only based diet (FM), combination of fishmeal and soya (FM S), fishmeal and sunflower meal (FM SM) and soya-only based diet (S) and the standard protein, lysine, lipid and energy content to which the treatments were formulated.

	Treatment (main protein source)			
	FM	FM S	FM SM	S
Fishmeal	38.84	27.55	29.45	0.00
Soya meal	0.00	18.00	0.00	61.92
Sunflower meal	0.00	0.00	18.00	0.00
Starch	59.06	51.70	49.26	33.74
Lysi-PEARL™	0.93	0.83	1.46	0.56
Vitamin mixture	0.11	0.11	0.11	0.11
Vegetable oil	1.06	1.82	1.72	3.68
Total	**100.00**	**100.00**	**100.00**	**100.00**
Protein (%)	30.00	30.00	30.00	30.00
Lysine (% of protein)	6.90	6.90	6.90	6.90
Lipids (%)	6.00	6.00	6.00	6.00
Energy (MJ kg^{-1})	17.43	16.94	17.00	15.77

Each diet was analysed for proximate composition, including crude protein, lipid, moisture and ash (Ayres, 2013) and these data are presented in the results in Table 2.3.1.

Sampling protocol

In order to reduce the amount of handling and unwanted spawning due to sampling stress, all abalone were gut purged for 48 h prior to sampling, which occurred over two days. On the first day of sampling, two males and two females were randomly collected by mechanical removal with a spatula from each replicate tank at the beginning of the study (n = 16 per treatment) and again on the first Monday closest to a 45 d interval for one year (Figure 2.2.1). The same animals were used for stable isotope analysis (n = 8 per treatment i.e. one of the two samples was randomly selected) and hormone assays (n = 16 per replicate, Chapter 4). At day 133 and

315, the muscle tissue, gonad and digestive gland of one male and one female per replicate tank was used for fatty acid analysis (n = 8 per treatment, Chapter 3).

Abalone were kept in labelled mesh bags that were carried in a crate and immediately transported to SPP Canning (Pty) Ltd, Hermanus and stored in a chilling room at approximately 10 °C. The mesh bags were removed at random from the chilling room for sampling, where each abalone was placed in an individually marked 350 ml plastic tub. Haemolymph samples were taken from each abalone for steroid hormones assays based on the methods by Hooper *et al*. 2014 (Chapter 4), after which the animals were shucked and the visceral mass was separated from the foot muscle and all body parts were placed into the plastic tub. Whole body mass, which included haemolymph that was present in the tub, meat mass and visceral mass were measured to the nearest 0.01 g using an electronic balance (Kern PLS 4200-2F, serial number: WIC1200486 and Kern PLS 3100-2F serial number: WIC1100813) and shell length was measured to the nearest 0.01 mm using Vernier callipers.

On the second day of sampling, two males and two females were randomly selected from each replicate tank for histological assessment (n = 16 per treatment; Chapter 4), and another four males and four females per replicate tank were used for gonad bulk index (GBI) calculations (n = 32 per treatment). Morphometric data, including whole body mass, meat mass and visceral mass were collected. Gonads used for histological assessment were placed in 40 ml plastic bottles that were filled with 10 % buffered formalin, while gonads used for GBI were placed in 40 ml plastic bottles filled with Davidsons fixative (10 % glycerol, 10 % acetic acid, 20 % formalin, 30 % ethanol and 30 % sea water). Morphometric data from days one and two were pooled.

Baskets were size graded every six months according to farm protocol to maintain the stocking density (Figure 2.2.1). The biomass of each basket was used to calculate FCR at the two size-grading events (Figure 2.2.1).

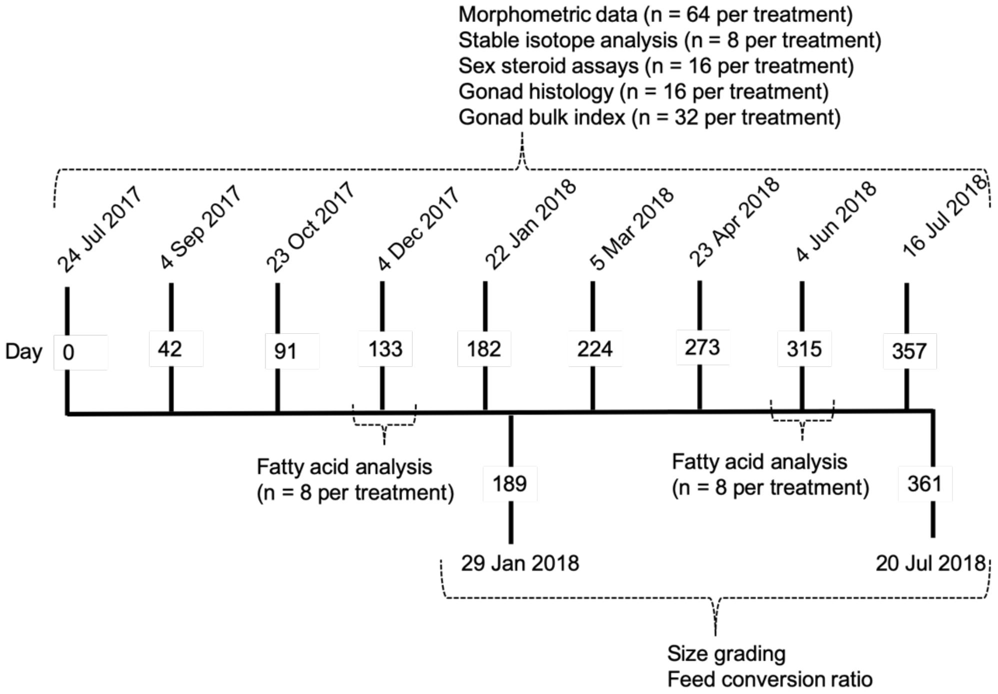


Figure 2.2.1: Timeline of sampling days for and six-month size grading intervals for *Haliotis midae*. Data collected included morphometric data and samples were analysed for stable isotopes, steroid hormones, gonad histology and gonad bulk index. The number of animals represent those that were taken at each sampling event over the one year.

Growth

Wet whole mass and shell length measurements from all abalone samples in each treatment were used to calculate the mean monthly weight gain (g abalone^{-1} month^{-1}) and length gain (mm abalone^{-1} month^{-1}) after 12 months (Equation 2 and 3, respectively), where the initial wet whole-body mass (M_t) was subtracted from the final wet whole-body mass (M_f), divided by the duration of the study (365 days) and multiplied by 30 days to obtain the mean monthly weight gain. Mean monthly length gain was obtained by subtracting the initial shell length (Lt) from the final shell length (Lf), divided by the durations of the study (365 days) and multiplied by 30 days.

$$Weight\ gain\ (g\ abalone^{-1}\ month^{-1}) = \left(\frac{M_f - M_t}{365}\right) \times 30 \qquad (2)$$

$$Length\ gain\ (mm\ abalone^{-1}\ month^{-1}) = \left(\frac{L_f - L_t}{365}\right) \times 30 \qquad (3)$$

Meat mass index, which is the meat mass as a percentage of whole mass was determined at each sampling period (Equation 4), where Mm is the wet meat mass (g abalone^{-1}) and Wm is the wet whole-body mass (g abalone^{-1}). In addition, visceral mass as a percentage of whole-body mass was determined (Equation 5), where Vm is the visceral wet mass (g abalone^{-1}) and Wm is the wet whole mass (g abalone^{-1}). The meat visceral ratio, which is the wet meat mass, M_m (g abalone^{-1}) divided by the wet visceral mass, Vm (g abalone^{-1}) was also determined (Equation 6).

$$Meat\ index\ (\%) = \left(\frac{M_m}{W_m}\right) \times 100 \qquad (4)$$

$$Visceral\ index\ (\%) = \left(\frac{V_m}{W_m}\right) \times 100 \qquad (5)$$

$$Meat\ visceral\ ratio = \frac{M_m}{V_m} \qquad (6)$$

Gonad bulk index (GBI)

Gonad bulk index was determined using the methods described by Tutschulte and Connell (1981). The weighed viscera that were used for GBI were kept in Davidson's fixative for a minimum of one week to ensure complete hardening. Each gonad was removed and placed on a scaled grid. Each grid was labelled and a photo was taken (Sony Cyber-shot DSC-T9, Japan) 400 mm above the grid (Wu *et al.*, 2019). The length of the conical appendage (mm) was determined using ImageJ 1.51n (National Institute of Health, USA; Figure 2.2.2a). The viscera was sectioned through the middle of the conical appendage and linear measurements were taken of the digestive gland (mm) and the gonad (mm) tissue (Figure 2.2.2b) to calculate the effective gonad volume (EGV; Equation 7), where L_{ca} is the length of the conical appendage, x and y is the linear dimensions of the digestive gland, and a and b is the linear dimensions of the gonad. The GBI was then determined by dividing the EGV by shucked mass (W_S) (Equation 8; Tutschulte and Connell, 1981). The linear dimensions of the digestive gland and gonad were used to calculate the digestive gland (DG) index (%) (Equation 9), which is the area of the digestive gland relative to the gonad area as a percentage (Tutschulte and Connell, 1981).

$$EGV\ (mm^3) = \frac{L_{ca}\pi}{96}\left[8(x+y)^2\ \frac{(a+b+x+y)^3}{x+y}\right] \tag{7}$$

$$GBI\ (mm^3\ g^{-1}) = \frac{EGV}{W_S} \tag{8}$$

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

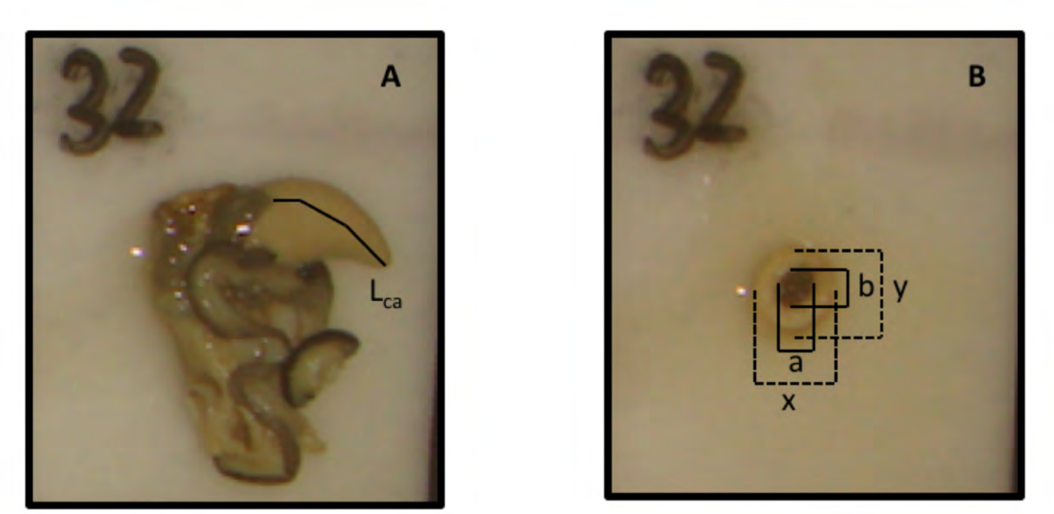


Figure 2.2.2: (A) Photograph demonstrating the measurement of the conical appendage (L_{ca}) in a male *Haliotis midae*; and (B) photograph demonstrating the cross section through the midpoint of the conical appendage. Linear dimensions of the digestive gland (a and b) and gonad (x and y) are illustrated. This method was applied when measuring female gonads.

Stable isotope tissue sample collection

After the visceral mass was weighed, a cross-section was taken through the conical appendage. Tissue samples of approximately 1.00 g of the digestive gland and gonad were rinsed with distilled water and drip-dried for about five seconds. The tissue samples were placed in 2 ml boil-proof Eppendorf vials and samples were dried in an oven at 45 °C until a constant weight was reached. The samples were then processed for lipid extraction.

Lipid extraction

Lipid extraction was conducted using a modified method of Bligh and Dyer (1959). A pestle and mortar were used to crush the dried meat and gonad and digestive gland samples into a fine powder. A solution of 70 % ethanol was used to clean the pestle and mortar in between samples. Samples were covered with 1.5 ml of a 2:1 ethanol: chloroform mix and then shaken for 10 s using a vortex mixer (VM-300, Gemmy Industrial Corp). The samples were then left to stand overnight at room temperature. The ethanol: chloroform solution was removed the following day and if the solution appeared golden/yellow then the samples were washed with fresh ethanol: chloroform solution until the solution became clear. The samples were then dried again at 45 °C for 72 h. The Eppendorf vials were stored at -20 °C until further analysis.

Stable isotope analysis

Aliquots of approximately 0.6 to 0.7 mg were weighed into tin capsules and isotopic analysis was done on a Flash EA 1112 Series coupled to a Delta V Plus stable light isotope ratio mass spectrometer via a ConFlo IV system (Thermo Fischer, Bremen, Germany), housed at the Stable Isotope Laboratory, Mammal Research Institute, University of Pretoria.

A laboratory running standard (Merck Gel: $\delta^{13}C$ = -20.57 ‰, $\delta^{15}N$ = 6.8 ‰, C % = 43.83, N % = 14.64) and blank sample was run after every 12 unknown samples. All results were referenced to Vienna Pee-Dee Belemnite for carbon isotope values, and to air for nitrogen isotope values. Results are expressed in delta notation using a per thousand unit scale using the Equation 10, where X= ^{15}N or ^{13}C and R represents ^{15}N or ^{13}C, respectively (Bligh and Dryer, 1959).

$$\delta X(‰) = \left[\frac{R_{sample-}\, R_{standard}}{R_{standard^{-1}}}\right] \times 1000 \qquad (10)$$

Turnover rates

The one-compartment, first-order kinetics model was used to evaluate the isotopic incorporation rates for abalone meat and gonad tissue by fitting a non-linear model:

$$\delta X_t = \delta X_\infty - (\delta X_\infty - \delta X_0)e^{-t/\tau} \tag{11}$$

where δX_t was the $\delta^{13}C/\delta^{15}N$ value for a tissue at time t, δX_∞ was the $\delta^{13}C/\delta^{15}N$ value for a tissue after the diet switch, δX_0 was the $\delta^{13}C/\delta^{15}N$ value before the diet switch and τ was the estimated turnover rate.

The tissue-diet discrimination factor (Δ^{13} C/Δ^{15} N) is a term used to describe the difference in isotopic values between the consuming organism and its diet after it has reached isotopic equilibrium. The consuming organism is this case would be either the foot muscle tissue or visceral tissue sample.

Nitrogen and carbon contribution from protein sources

The proportions of nutrient contribution were estimated using a two source, two isotope mixing model that used a Bayesian analytical framework and this was conducted using stable isotope analysis in R (SIAR). The discrimination factors from animals that were fed the FM-only and S-only based diets were integrated into the mixing models.

Water quality

Water quality variables including temperature (^{o}C) and dissolved oxygen (mg L^{-1}) were measured once a week using an electronic meter (Handy Polaris, OxyGuard). The pH values were also obtained once-weekly using an electric meter (Handy pH, OxyGuard). Mean pH values were obtained by log-transforming the data, averaging the logged data, and using the anti-log of the average value.

Statistics

Data from the two sampling days were pooled and males and females from each replicate tank were averaged separately prior to analysis. If abalone sex did not significantly affect the growth parameter analysed, then data collected from males and females were pooled. A multi-factorial analysis of variance (ANOVA) was used to determine the differences in mean monthly weight and length gain between abalone sex and dietary treatments. A repeated measures analysis of variance (RM-ANOVA) was used for all growth parameters, feed conversion ratios (FCR) as well as water quality parameters. Tukey's HSD post-hoc test (Tukey, 1960) was used to determine where significant differences occurred between treatments at $p < 0.05$. Linear regression analysis was used to determine the relationship between gonad bulk indices and digestive gland indices for each sex within each dietary treatment and the relationships were also assessed visually to determine the linearity, thus results were presented in a table (Table 2.3.2). Data were tested for homogeneity of variance (Levene, 1960) and for normal distribution of residuals (Shapiro and Wilk, 1965).

Data are presented as means ± standard error unless noted otherwise. All growth, FCR and water quality parameters were conducted using Statistica® 13 software package. The package stable isotope analysis in R (SIAR) was used to analyse isotope data and this was conducted in R.

2.3 RESULTS

Diet proximate analysis

Protein content of dietary treatments ranged between 26.18 – 30.91 % with FM SM containing the lowest and S containing the highest protein content (Table 2.3.1). Moisture content ranged from 10.48 – 11.73 %, lipid ranged from 3.01 – 3.57 % and ash content ranged between 3.21 – 5.42 % (Table 2.3.1).

Table 2.3.1: Proximate analysis of dietary treatments, including a fishmeal-only based diet (FM), combination of fishmeal and soya (FM S), fishmeal and sunflower meal (FM SM) and a soya-only based diet (S).

Treatment	Protein (%)	Moisture (%)	Lipid (%)	Ash (%)
FM	27.72	11.73	3.37	5.42
FM S	28.61	11.41	3.01	4.89
FM SM	26.18	10.48	3.57	5.12
S	30.91	10.96	3.43	3.21

Weight and length gain

Female abalone had a significantly higher overall mean monthly weight gain (3.51 ± 0.08 g abalone month^{-1}) compared to males (3.19 ± 0.19 g abalone month^{-1}) and this was independent of dietary treatment (ANOVA, $F_{(1, 24)} = 8.04$, $p = 0.01$). Diet had no influence on mean monthly weight gain in females (ANOVA, $F_{(3, 12)} = 1.82$, $p = 0.20$), while diet significantly affected weight gain in male abalone (ANOVA, $F_{(3, 12)} = 5.27$, $p = 0.02$), with males that were fed FM (2.84 ± 0.10 g abalone month^{-1}) having significantly lower weight gain per month compared to those that were fed the FM S diet (3.72 ± 0.12 g abalone month^{-1}; Tukey's HSD, $p < 0.05$; Figure 2.3.1a).

Similarly, there was a significant effect of abalone sex on the mean monthly length gain of abalone (ANOVA, $F_{(1, 24)} = 7.69$, $p = 0.01$), where females (1.59 ± 0.04 mm abalone month^{-1}) had a higher mean monthly length gain compared to males (1.47 ± 0.06 mm abalone month^{-1}), independent of dietary treatment. Dietary treatment had no significant effect on the mean monthly length gain of females (ANOVA, $F_{(3, 12)} = 1.81$, $p = 0.20$) and males (ANOVA, $F_{(3, 12)} = 2.87$, $p = 0.08$; Figure 2.3.1b).

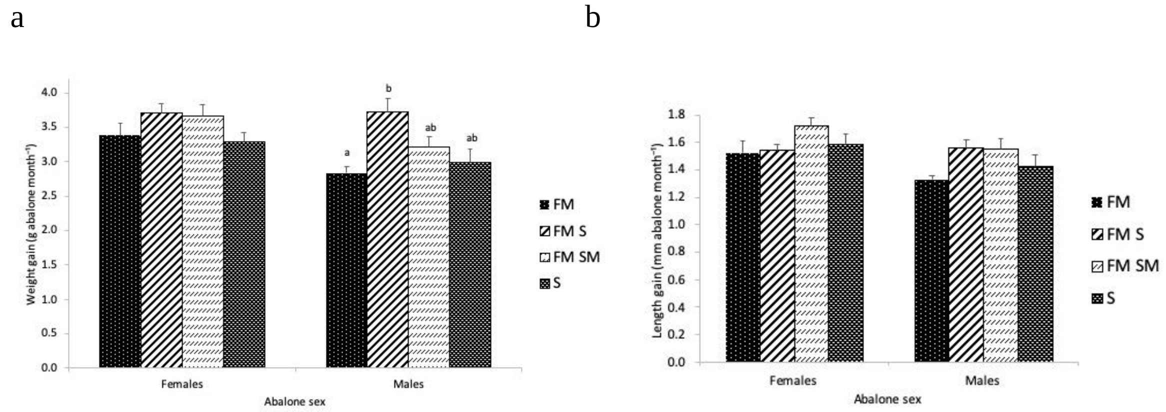


Figure 2.3.1: (a) Mean (± SE) monthly weight gain (g abalone month^{-1}); and (b) length gain (mm abalone month^{-1}**)** of *Haliotis midae* fed diets that included fishmeal (FM), soya (S), fishmeal and soya (FM S), and fishmeal and sunflower meal (FM SM) as the main protein sources (ANOVA, $p < 0.05$). Significant differences are indicated by different superscripts.

Whole-body mass and shell length

The mean whole-body mass of abalone was significantly influenced by an interaction between time and dietary treatment, with average whole-body mass changing differently over time between treatments (RM-ANOVA, $F_{(24, 96)} = 2.13$, $p = 0.005$; Figure 2.3.2a). Overall, abalone that were fed FM S had higher mean whole-body mass values than abalone that were fed the single-protein based diets, while abalone that were fed FM SM were similar to animals from all dietary treatments (RM-ANOVA, $F_{(3, 12)} = 5.75$, $p = 0.01$). This pattern could be seen throughout the study (Figure 2.3.2a). In addition, there was a significant interaction between time and abalone sex (RM-ANOVA, $F_{(8,\,192)}$ = 1.99, p = 0.04; Figure 2.3.2b), where the mean whole-body mass of females and males changed differently over time, independently of diet, with females performing better than males at the end of the study (Figure 2.3.2b).

The mean shell length of abalone was significantly influenced by an interaction between time and dietary treatment (RM-ANOVA, $F_{(24,\,96)}$ = 2.22, p = 0.003; Figure 2.3.2c). In addition, there was a significant interaction between time and abalone sex, with the mean shell length of female and male abalone changing differently over time, independently of diet, where females performed better than males during the middle and end of the study (RM-ANOVA, $F_{(8,\,192)}$ = 2.31, p = 0.02; Figure 2.3.2d).

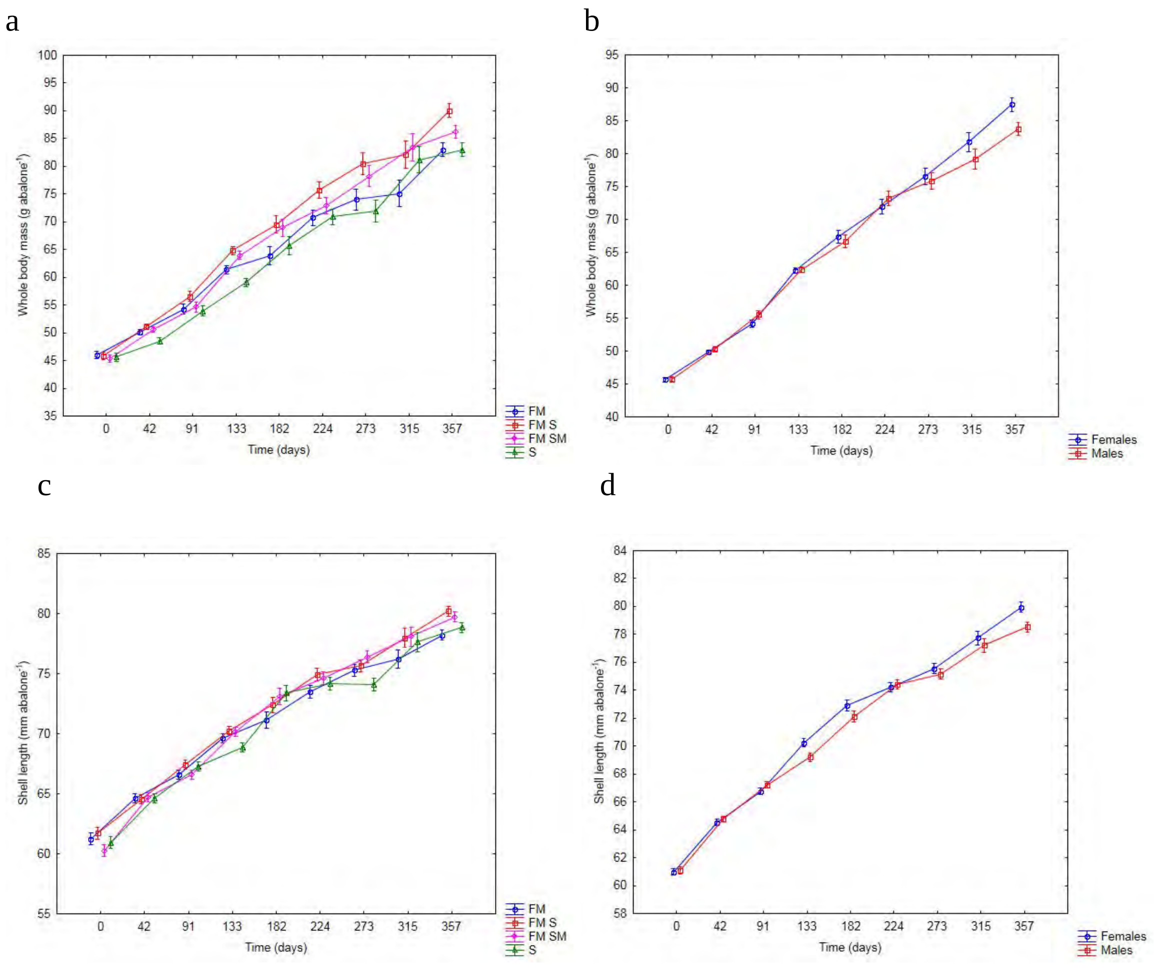


Figure 2.3.2: Mean (± SE) whole body mass (g abalone^{-1}) and shell length (mm abalone^{-1}) of *Haliotis midae* that were fed diets that included fishmeal (FM), fishmeal and soya (FM S), soya (S) and fishmeal and sunflower meal (FM SM) as the main protein sources (RM-ANOVA, $p < 0.05$).

Meat mass index

The average meat index was influenced by a significant interaction between time and dietary treatment, since it varied for different treatments at different times during the experiment (RM-ANOVA, $F_{(24, 96)} = 1.74$, $p = 0.03$; Figure 2.3.3). Overall, abalone that were fed FM SM had higher meat index values than abalone fed the S-only based diet, while abalone fed FM and FM S were similar to those in all other treatments (RM-ANOVA, $F_{(3, 12)} = 6.85$, $p = 0.006$; Figure 2.3.3). This relationship could be seen throughout the study (Figure 2.3.3).

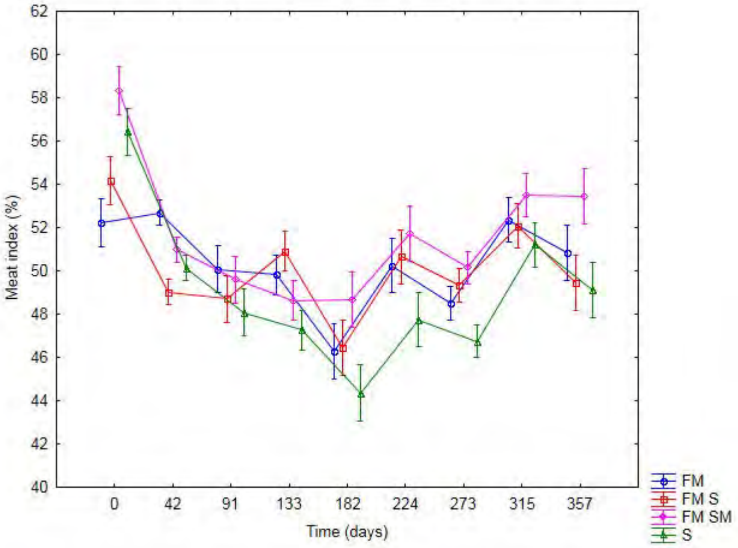


Figure 2.3.3: Mean (± SE) meat index (%) of *Haliotis midae* that were fed diets that included fishmeal (FM), fishmeal and soya (FM S), soya (S) and fishmeal and sunflower meal (FM SM) as the main protein sources (RM-ANOVA, $p = 0.03$).

Visceral index

There was no significant interaction between time and treatment in visceral index values, with values from abalone from all treatments changing similarly over time (RM-ANOVA, $F_{(24, 96)} = 0.50$, $p = 0.97$; Figure 2.3.4). There was a significant effect of diet on the overall visceral index values (RM-ANOVA, $F_{(3, 12)} = 8.53$, $p = 0.003$), where abalone that were fed FM S (10.78 ± 0.25 %) and S (10.59 ± 0.26 %) had significantly higher visceral indices than those that were fed FM (10.16 ± 0.25), while abalone that were fed FM SM (10.48 ± 0.23 %) were similar to the other three treatments (Tukey’s HSD, $p < 0.05$). This pattern was observed throughout the study (Figure 2.3.4).

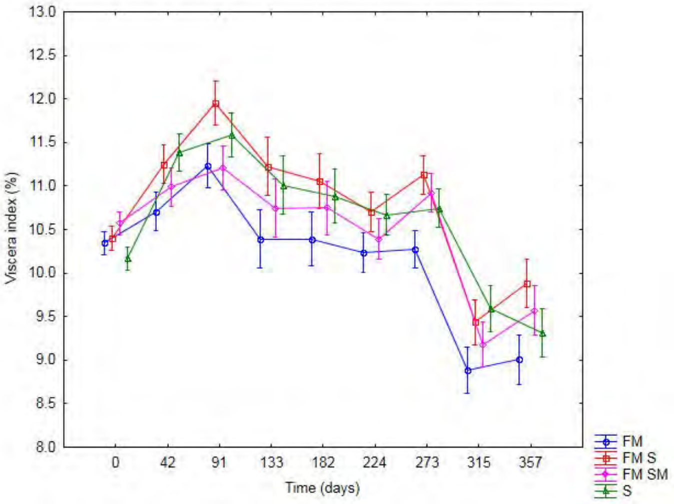


Figure 2.3.4: Mean (± SE) visceral index (%) of *Haliotis midae* fed diets that included fishmeal (FM), soya (S), fishmeal and soya (FM S) and fishmeal and sunflower meal (FM SM) as the main protein sources (RM-ANOVA, $p = 0.97$).

Meat visceral ratio

There was no significant interaction between time and treatment for the meat viscera ratios with ratios of abalone from all treatments showing similar trends over time (RM-ANOVA, $F_{(24, 96)} = 0.81$, $p = 0.71$; Figure 2.3.5). There was a significant effect of treatment on the overall meat visceral ratios (RM-ANOVA, $F_{(3, 12)} = 8.82$, $p = 0.002$). Here, abalone fed FM (4.98 ± 0.16) and FM SM (4.93 ± 0.17) had similar meat visceral ratio values that were significantly higher compared to abalone fed FM S (4.65 ± 0.16) and S diets (4.63 ± 0.17), and this pattern can be seen throughout the study (Tukey’s HSD, $p < 0.05$; Figure 2.3.5)

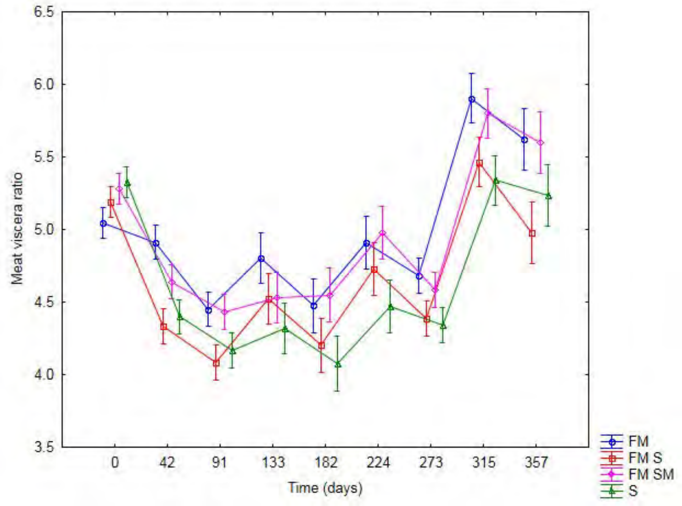


Figure 2.3.5: Mean (± SE) meat visceral ratio of *Haliotis midae* fed diets that included fishmeal (FM), fishmeal and soya (FM S), soya (S) and fishmeal and sunflower meal (FM SM) as the main protein source (RM-ANOVA, p = 0.71).

Gonad bulk index (GBI)

Male abalone had significantly higher GBI values compared to females and this was independent of dietary treatment (RM-ANOVA, $F_{(1, 24)} = 49.03$, $p < 0.001$). There was no significant interaction between diet and time for females (RM-ANOVA, $F_{(24, 96)} = 0.79$, p = 0.74; Figure 2.3.6) and males (RM-ANOVA, $F_{(24, 96)} = 1.08$, p = 0.39; Figure 2.3.6) with mean GBI values showing similar trends for both sexes, increasing from the beginning of the study to sampling days 133 and 182, followed by a gradual decrease towards the end of the study (Figure 2.3.6).

There was a significant difference in the overall GBI values between treatments for females (RM-ANOVA, $F_{(3, 12)} = 5.60$, p = 0.01) and males (RM-ANOVA, $F_{(3, 12)} = 9.72$, p = 0.002). Female abalone fed the FM S diet (15.92 ± 1.88 mm^3 g^{-1}) had significantly higher GBI values than abalone fed S (9.76 ± 1.08 mm^3 g^{-1}), while abalone fed FM (11.96 ± 1.71 mm^3 g^{-1}) and FM SM (11.90 ± 0.80 mm^3 g^{-1}) were similar to abalone from all other treatments (Tukey's

HSD, $p < 0.05$). Male abalone fed the FM S ($21.59 \pm 2.10\ \text{mm}^3\ \text{g}^{-1}$) and FM SM diet ($19.30 \pm 2.63\ \text{mm}^3\ \text{g}^{-1}$) had similar GBI values and were significantly higher than abalone fed the S diet ($14.74 \pm 1.27\ \text{mm}^3\ \text{g}^{-1}$), while abalone fed FM S had significantly higher GBI values than abalone that consumed the FM ($15.08 \pm 1.63\ \text{mm}^3\ \text{g}^{-1}$) dietary treatment (Tukey's HSD, $p < 0.05$). These patterns were observed throughout the study (Figure 2.3.6).

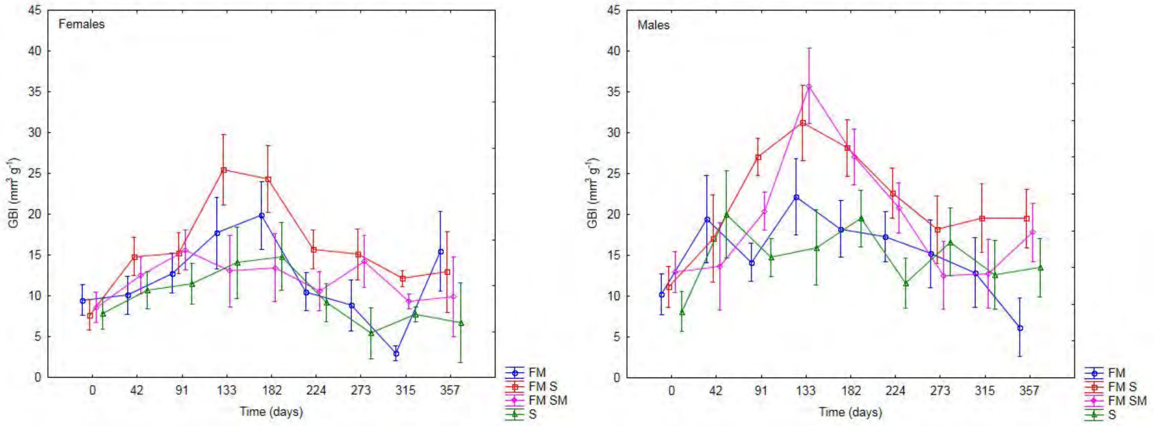


Figure 2.3.6: Mean (± SE) gonad bulk index (GBI; $\text{mm}^3\ \text{g}^{-1}$) of female and male *Haliotis midae* that were fed diets that included fishmeal (FM), fishmeal and soya (FM S), soya (S) and fishmeal and sunflower meal (FM SM) as the main protein sources (RM-ANOVA, $p > 0.05$).

Digestive gland (DG) index

Female abalone had significantly higher DG index values compared to males and this was independent of dietary treatment (RM-ANOVA, $F_{(1,24)} = 4.73$, $p = 0.04$). There was no interaction between time and treatments for both female (RM-ANOVA, $F_{(24,96)} = 1.37$, $p = 0.15$; Figure 2.3.7) and male abalone (RM-ANOVA, $F_{(24,96)} = 1.12$, $p = 0.34$; Figure 2.3.7), with values from animals from all treatments changing similarly over time.

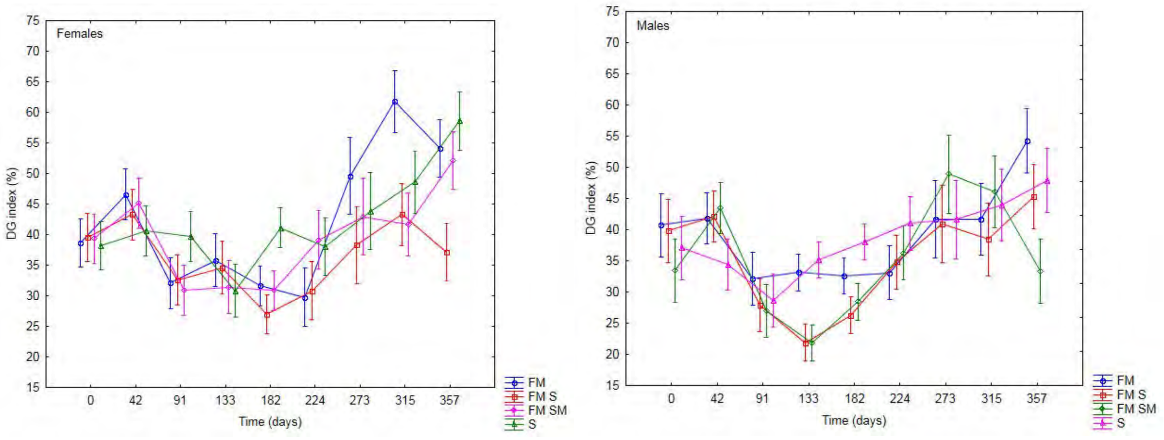


Figure 2.3.7: Mean (± SE) digestive gland (DG) index (%) of *Haliotis midae* fed diets that included fishmeal (FM), fishmeal and soya (FM S), soya (S) and fishmeal and sunflower meal (FM SM) as the main protein sources (RM-ANOVA, $p > 0.05$).

Relationship between GBI and DG index

There was a significant negative relationship between GBI and DG index of females that were fed FM and S ($p = 0.001$ and $p = 0.001$, respectively; Table 2.3.2), while there was no relationship between GBI and DG index for females that were fed FM S and FM SM ($p > 0.05$; Table 2.3.2). There was a significant negative relationship between GBI and DG index in male abalone from all dietary treatments ($p < 0.05$; Table 2.3.2).

Table 2.3.2: Regression analysis of gonad bulk index as a function of digestive gland index in female and male *Haliotis midae* that were fed fishmeal (FM), soya (S), combination of fishmeal and soya (FM S) and fishmeal and sunflower meal (FM SM) based diets.

Abalone sex	Treatment	Regression	F	r^2	p
Female	FM	y = 15.37 – 0.14x	11.75	0.07	0.001
	FM S	-	3.20	0.02	0.08
	FM SM	-	1.64	0.01	0.20
	S	y = 20.28 – 0.17x	12.72	0.08	0.001
Male	FM	y = 21.10 – 0.21x	18.99	0.11	<0.0001
	FM S	y = 28.97 – 0.28x	14.59	0.09	0.0002
	FM SM	y = 26.36 – 0.26x	14.65	0.09	0.0002
	S	y = 20.28 – 0.17x	9.22	0.06	0.003

Feed conversion ratio

There was no significant interaction between diets for feed conversion ratio (FCR) over time (RM-ANOVA, $F_{(3, 12)} = 2.81$, $p = 0.08$; Table 2.3.3). From July 2017 to January 2018, abalone that consumed the FM diet had a significantly higher FCR (1.66 ± 0.09) than abalone that consumed the FM S diet (1.33 ± 0.09, Tukey's HSD, $p < 0.05$), while abalone in the FM SM (1.56 ± 0.09) and S (1.51 ± 0.09) had similar FCR to the other treatments (Tukey's HSD, $p > 0.05$; Table 2.3.3). From January 2018 to July 2018 abalone in the FM S treatment had significantly lower FCR compared to the other three treatments (Tukey's HSD, $p < 0.05$). There was a significant difference in the 12-month average FCR (RM-ANOVA, $F_{(3, 12)} = 12.30$, $p = 0.001$), where abalone that were fed FM S had significantly lower FCR than abalone in the other three dietary treatments (Tukey's HSD, $p < 0.05$).

Table 2.3.3: Mean (± SD) feed conversion ratio (FCR) of *Haliotis midae* fed a fishmeal-only (FM) based diet, combination of fishmeal and soya (FM S), fishmeal and sunflower meal (FM SM) and soya-only based diet (S). Significant differences are indicated by different superscripts within each row and column from July 2017 – July 2018 (Tukey's HSD, $p < 0.05$). Six and 12-month FCRs were analysed separately for multiple comparisons analysis (Tukey's HSD, $p < 0.05$).

	FCR		
Treatment	July 2017- January 2018	January 2018 – July 2018	12-month average
FM	1.66 ± 0.31^{a}	1.73 ± 0.25^{a}	1.70 ± 0.27^{a}
FM S	1.33 ± 0.07^{bc}	1.25 ± 0.17^{b}	1.30 ± 0.13^{b}
FM SM	1.56 ± 0.11^{abc}	1.75 ± 0.11^{a}	1.65 ± 0.14^{a}
S	1.51 ± 0.08^{abc}	1.94 ± 0.10^{a}	1.72 ± 0.24^{a}

Stable isotope analysis

Isotopic incorporation and discrimination factors

The fishmeal and soya ingredients that were used for diet formulation had contrasting δ^{13}C (-15.61 ± 0.04 and -25.20 ± 0.14 ‰, respectively) and δ^{15}N values (11.31 ± 0.18 and 0.49 ± 0.07 ‰, respectively), while the sunflower meal ingredient had δ^{13}C ratio (-23.36 ± 0.09 ‰) that was similar to soya and δ^{15}N ratio (4.42 ± 0.16 ‰) that was in between fishmeal and soya. Consequently, the FM-only and S-only based diets had contrasting δ^{13}C (-14.57 ± 0.19 and -21.69 ± 0.34 ‰, respectively) and δ^{15}N values (9.49 ± 0.30 and 0.84 ± 0.17 ‰, respectively), while FM S and FM SM diets had similar δ^{13}C (-17.47 ± 0.22 and -17.26 ± 0.24 ‰, respectively) and δ^{15}N ratios (7.11 ± 0.09 and 8.34 ± 0.19 ‰, respectively).

A shift in the δ^{13}C and δ^{15}N stable isotope ratios can be seen in the tissues of female and male abalone from all dietary treatments (Figure 2.3.8 and 2.3.9). The δ^{13}C turnover rate varied between treatments for each sex, as well as tissue type (Table 2.3.4). Time taken to reach isotopic equilibrium for meat samples of abalone fed the single protein-based diets ranged between 67 – 343 and 209 – 346 days for δ^{13}C and δ^{15}N, respectively (Table 2.3.4). The gonad δ^{13}C and δ^{15}N ratios of females fed the single protein diets took fewer days to reach isotopic equilibrium with its respective diet compared to the meat, ranging from 85 – 90 and 47 – 59 days, respectively. Similarly, δ^{13}C and δ^{15}N ratios in the gonads of males fed the single protein-based diets reached isotopic equilibrium faster than the meat samples, except for δ^{13}C of males fed FM (Table 2.3.4).

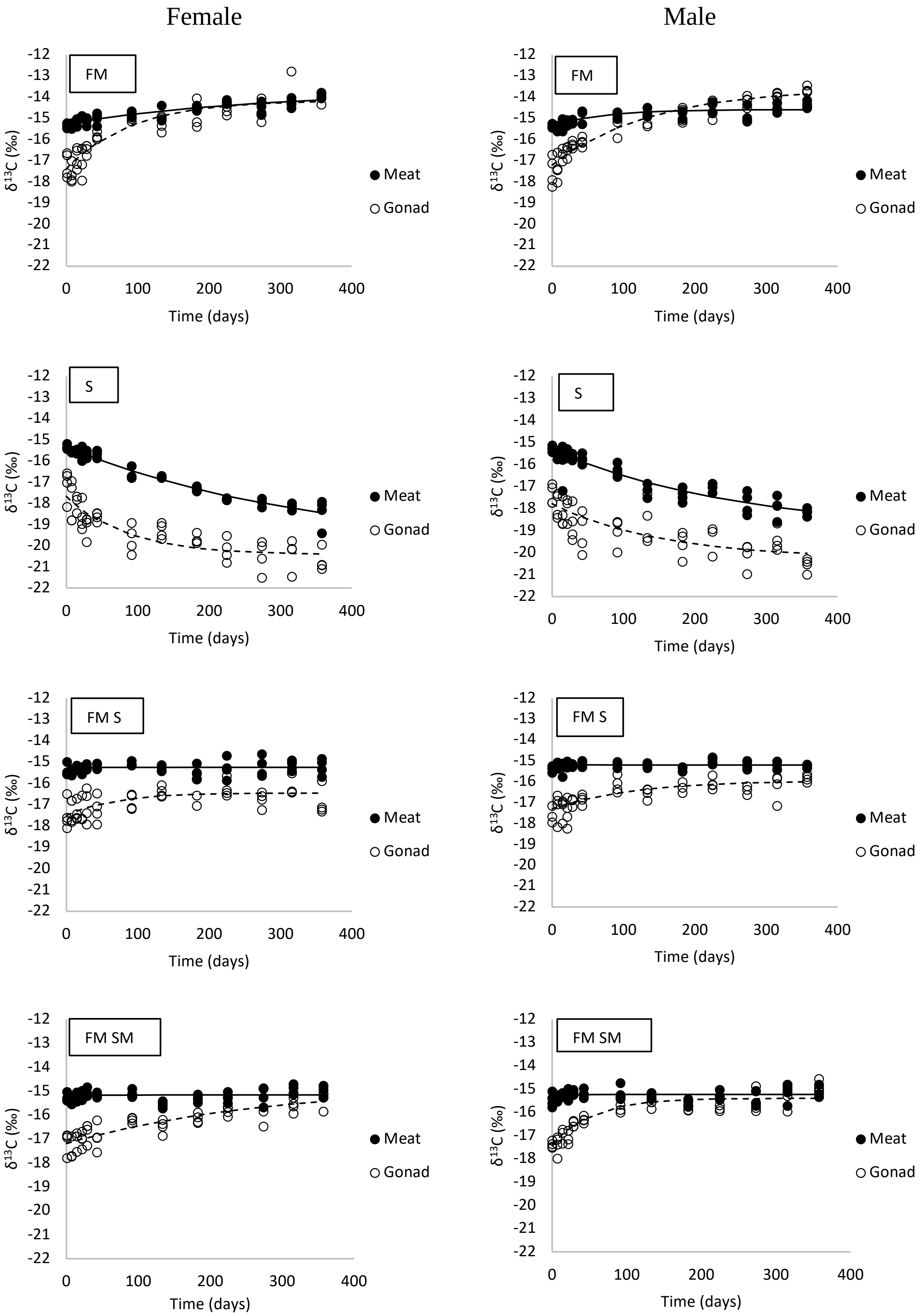


Figure 2.3.8: Change in carbon isotope ratios in the meat and gonad tissue of female and male *Haliotis midae* fed fishmeal (FM), soya (S), fishmeal and soya (FM S) and fishmeal and sunflower meal (FM SM) based diets. Dashed and solid lines were fitted using the one-compartment model ($\delta X_t = \delta X_\infty - (\delta X_\infty - \delta X_0)e^{-t/\tau}$) for gonad and meat samples, respectively.

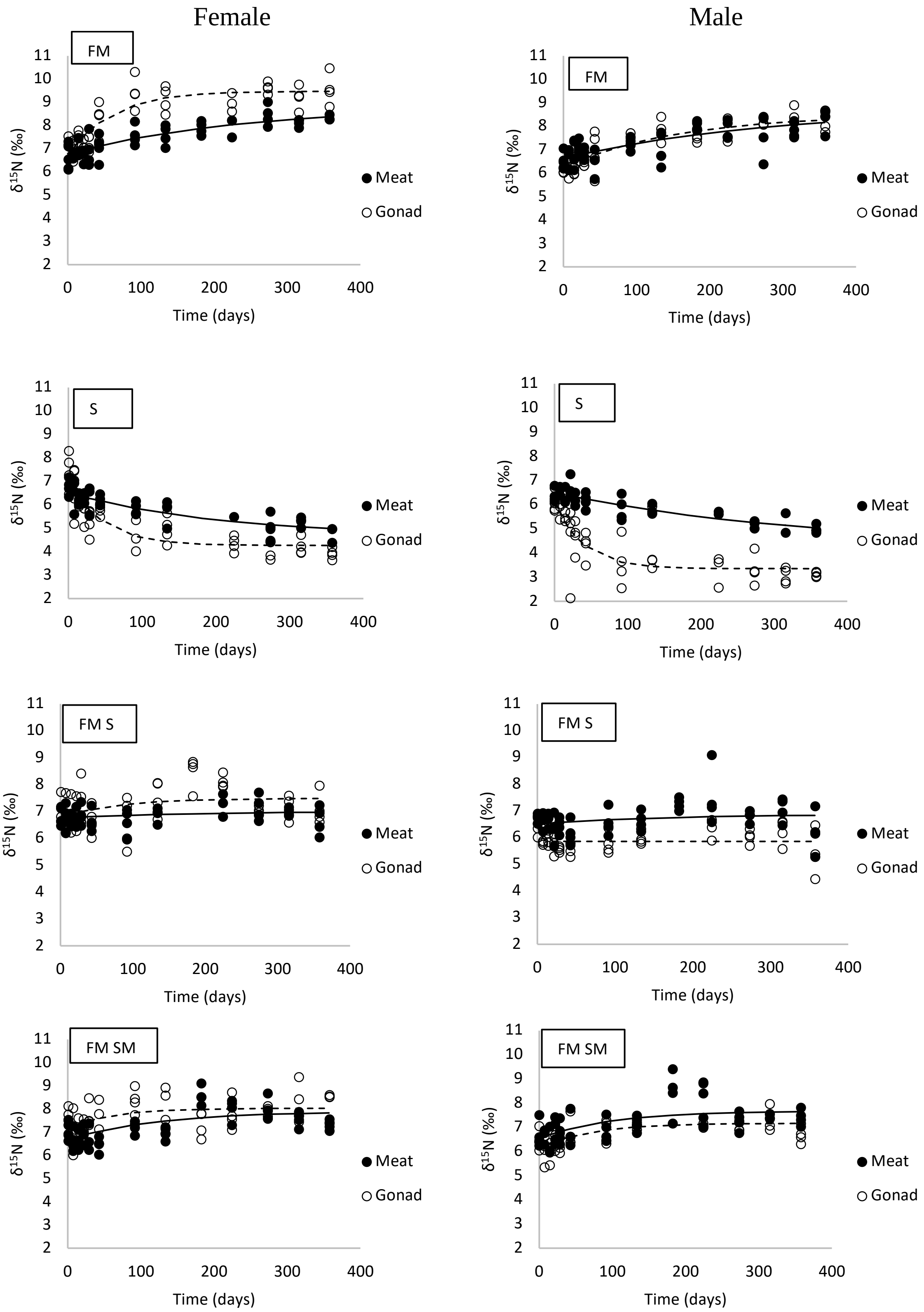


Figure 2.3.9: Change in nitrogen isotope ratios in the meat and gonad tissue of female and male *Haliotis midae* fed fishmeal (FM), soya (S), fishmeal and soya (FM S) and fishmeal and sunflower meal (FM SM) based diets. Dashed and solid lines were fitted using the one-compartment model ($\delta X_t = \delta X_\infty - (\delta X_\infty - \delta X_0)e^{-t/\tau}$) for gonad and meat samples, respectively.

The gonad $\delta^{13}C$ ratios of females that were fed FM S and FM SM diets took longer than the meat samples to reach isotopic equilibrium, which took 66 and 271 days, respectively, while $\delta^{15}N$ ratios were faster to reach equilibrium with its diet compared to the meat, taking 82 and 59 days, respectively. Similarly, gonad $\delta^{13}C$ ratios of males fed the combination diets took longer to reach isotopic equilibrium with its respective diets compared to the meat, which took 111 and 53 days for abalone fed FM S and FM SM, respectively, while $\delta^{15}N$ turnover took 5 and 71 days, respectively (Table 2.3.4). The time taken for the meat $\delta^{15}N$ ratios of females and males fed the combinations diets ranged between 120 – 175 days and 93 – 149 days, respectively, compared to gonad $\delta^{15}N$ ratios (Table 2.3.4).

The tissue-diet discrimination factors were variable, with $\Delta^{13}C$ ranging 0.22 – 3.54 ‰ and $\Delta^{15}N$ ranging -1.72 – 4.14 ‰ (Table 2.3.4). Discrimination factors of both stable isotopes were the highest in the meat tissue of both female ($\Delta^{13}C$ = 3.27 ‰, $\Delta^{15}N$ = 3.92 ‰) and male ($\Delta^{13}C$ = 3.54 ‰, $\Delta^{15}N$ = 4.14 ‰) abalone that were fed the S-only based diet, contrasted with the lowest discrimination factors in the meat tissues of females ($\Delta^{13}C$ = 0.62 ‰, $\Delta^{15}N$ = -1.20 ‰) and males ($\Delta^{13}C$ = 0.22 ‰, $\Delta^{15}N$ = -1.22 ‰) fed the FM-only based diet (Table 2.3.4). Gonad $\Delta^{13}C$ values were highest for females (1.96 ± 0.35 ‰) and males (2.40 ± 0.23 ‰) that were fed FM SM, while $\Delta^{15}N$ values were the highest in the gonads of females (3.07 ± 0.23 ‰) and males (2.27 ± 0.11 ‰) that were fed S-only (Table 2.3.4). The lowest $\Delta^{13}C$ values in the gonads of females and males were seen in those fed FM S (0.59 ± 0.67 ‰) and FM (0.92 ± 0.15 ‰), respectively, while the lowest gonad $\Delta^{15}N$ values were found in females and males fed FM SM (-0.06 ± 0.57 ‰) and FM S (1.72 ± 0.82 ‰), respectively (Table 2.3.4).

Table 2.3.4: $\delta^{13}C$ and $\delta^{15}N$ incorporation, turnover rates and mean (± SD) discrimination factors of meat and gonad tissues of female and male *Haliotis midae* fed diets comprising of fishmeal (FM), soya (S), fishmeal and soya (FM S) and fishmeal and sunflower meal (FM SM).

				^{13}C			^{15}N	
Tissue	Sex	Treatment	One-compartment	Turnover	$\Delta^{13}C$	One-compartment	Turnover	$\Delta^{15}N$
Meat	Females	FM	$-13.71 – 1.56e^{-t/278}$	278	0.62 ± 0.11	$8.84 – 2.06e^{-t/240}$	240	-1.20 ± 0.18
		S	$-20.15 + 4.82e^{-t/343}$	343	3.27 ± 0.68	$4.65 + 1.86e^{-t/209}$	209	3.92 ± 0.34
		FM S	$-15.25 – 0.18e^{-t/15}$	15	2.25 ± 0.38	$6.99 – 0.25e^{-t/175}$	175	-0.45 ± 0.53
		FM SM	$-15.16 – 0.16e^{-t/12}$	12	2.32 ± 0.22	$7.89 – 1.22e^{-t/120}$	120	-1.02 ± 0.21
	Males	FM	$-14.60 – 0.80e^{-t/67}$	67	0.22 ± 0.16	$8.75 – 2.14e^{-t/279}$	279	-1.22 ± 0.46
		S	$-19.10 + 3.75e^{-t/269}$	269	3.54 ± 0.16	$4.22 + 2.27e^{-t/346}$	346	4.14 ± 0.16
		FM S	$-15.20 – 0.21e^{-t/7}$	7	2.22 ± 0.08	$6.86 – 0.36e^{-t/149}$	149	-0.91 ± 0.08
		FM SM	$-15.23 – 0.24e^{-t/7}$	7	2.23 ± 0.28	$7.67 – 1.15e^{-t/93}$	93	-0.92 ± 0.32
Gonad	Females	FM	$-14.16 – 3.24e^{-t/90}$	90	0.59 ± 0.26	$9.47 – 2.80e^{-t/59}$	59	0.09 ± 0.69
		S	$-20.44 + 2.76e^{-t/85}$	85	0.98 ± 0.52	$4.26 + 2.79e^{-t/47}$	47	3.07 ± 0.23
		FM S	$-16.45 -1.05e^{-t/66}$	66	0.59 ± 0.67	$7.49 – 0.70e^{-t/82}$	82	-0.01± 0.60
		FM SM	$-14.80 – 2.40e^{-t/271}$	271	1.96 ± 0.35	$8.03 – 0.89e^{-t/59}$	59	-0.06 ± 0.57
	Males	FM	$-13.60 – 3.62e^{-t/136}$	136	0.92 ± 0.15	$8.48 – 2.2e^{-t/155}$	155	-1.21 ± 0.47
		S	$-20.27 + 2.49e^{-t/151}$	151	1.14 ± 0.31	$3.35 + 2.80e^{-t/39}$	39	2.27 ± 0.11
		FM S	$-15.95 -1.35e^{-t/111}$	111	1.63 ± 0.17	$5.85 + 0.56e^{-t/5}$	5	-1.72 ± 0.82
		FM SM	$-15.40 – 2.03e^{-t/53}$	53	2.40 ± 0.23	$7.17 – 1.02e^{-t/71}$	71	1.63 ± 0.35

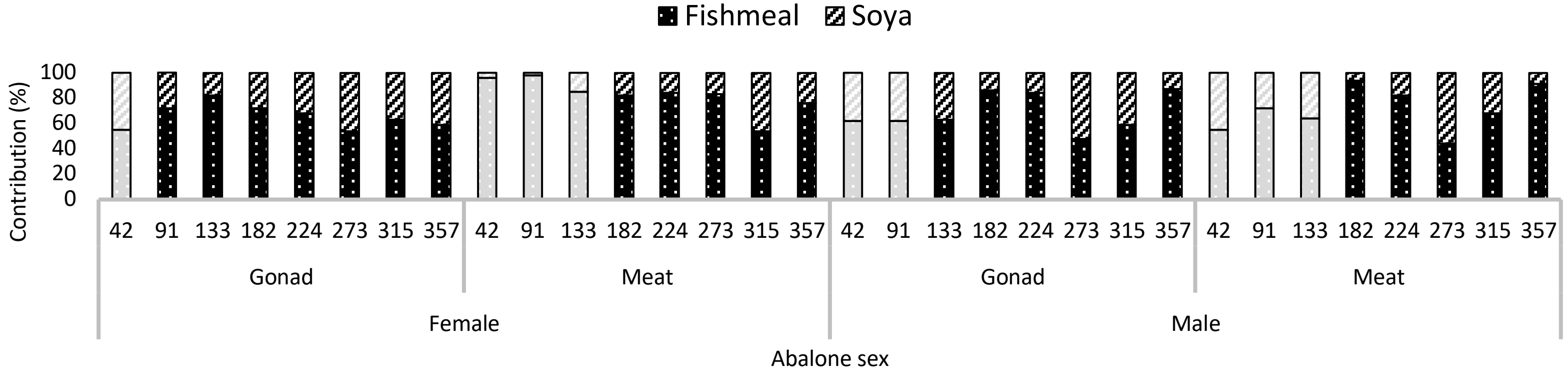
Relative contribution from fishmeal, soya and sunflower meal

One of the assumptions when using isotopic mixing models to estimate nutritional contributions is that isotopic equilibrium has been reached between the consumer and its diet (Gamboa-Delgado, 2014), thus some of the results should be considered with caution as some tissues reached equilibrium faster than others (Figure 2.3.10). As such, samples that had not reached isotopic equilibrium with its diet will not be included in the interpretation of the findings. The relative contribution of fishmeal, soya and sunflower meal to the tissue growth of female and male abalone fluctuated over the one-year study (Figure 2.3.10). The contributions of protein sources to the gonad and meat tissue growth of females and males followed similar patterns over the year, with the relative contribution of fishmeal peaking at either day133 or 182, followed by a gradual decrease until day 273 and increasing towards the end of the study (Figure 2.3.10a). Fishmeal contributed over 50 % of gonad (54 – 82 %) and meat (54 – 84 %) tissue growth in females throughout the study (Figure 2.3.10a). Similarly, fishmeal contributed over 50 % of gonad tissue growth of male abalone, ranging from

59 – 87 % over the one-year period, except on day 273 (48 %; Figure 2.3.10a), while the contribution to meat tissue growth in males ranged between 44 – 94 % (Figure 2.3.10a).

Female and male abalone that were fed FM SM had very little protein contribution from sunflower meal to both gonad and meat tissue growth with the exception of day 273 for gonad tissues (Figure 2.3.10b). Excluding the anomaly on day 273 where sunflower meal contributed approximately 65 and 58 % to the gonad growth of females and males, respectively, the contribution of fishmeal to gonad tissue growth in females and males ranged from 78 – 96 % (Figure 2.3.10b). Similarly, fishmeal contributed between 89 and 97 % of meat tissue growth in both female and male abalone, over the one year (Figure 2.3.10b).

a



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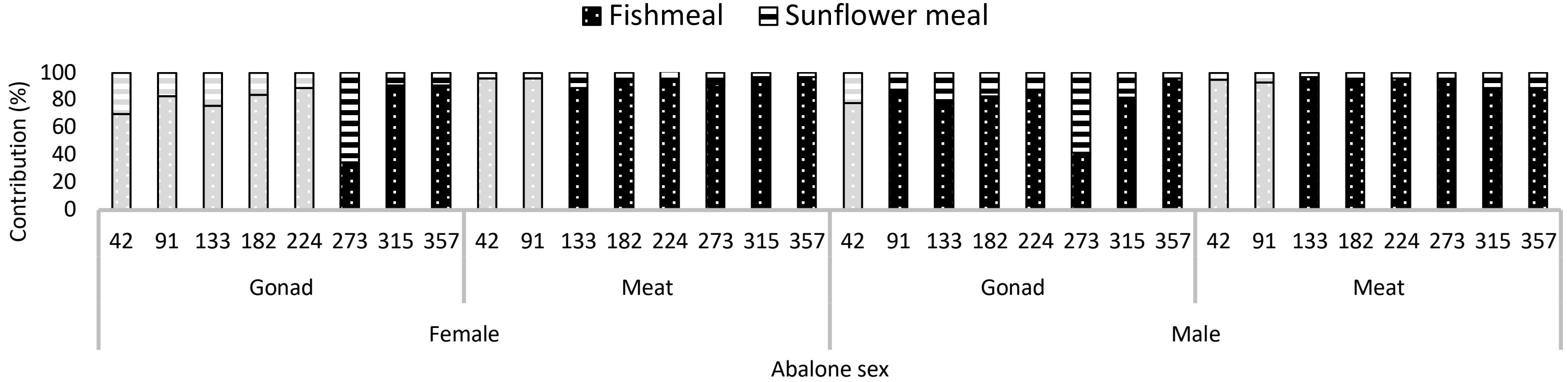


Figure 2.3.10: (a) Mean relative contribution of dietary fishmeal and soya ingredients to the gonad and meat tissue growth of female and male *Haliotis midae* tissue fed a fishmeal and soya combination diet; and (b) mean relative contribution of dietary fishmeal and sunflower meal to the gonad and meat tissue growth of female and male *H. midae* fed a fishmeal and sunflower meal combination diet over one year. Bars that have been discoloured (i.e. shaded lightly) should be interpreted with caution as tissues had not reached isotopic equilibrium with its diet.

Water quality

There was no significant difference in water temperature, dissolved oxygen and pH between treatments over time (RM-ANOVA, $p > 0.05$; Table 2.3.5).

Table 2.3.5: Mean (± SD) water temperature, oxygen (O_2) and pH of *Haliotis midae* fed a fishmeal-only (FM) based diet, a combination of fishmeal and soya (FM S), fishmeal and sunflower meal (FM SM) and soya-only (S) based diet (RM-ANOVA, $p > 0.05$).

	Treatment	Mean	Minimum	Maximum
Temperature (°C)	FM	15.08 ± 1.54	11.80	18.70
	FM S	15.09 ± 1.54	11.80	18.70
	FM SM	15.08 ± 1.54	11.70	18.70
	S	15.08 ± 1.53	11.70	18.70
O_2 (mg L^{-1})				
	FM	7.98 ± 0.41	6.70	9.00
	FM S	7.98 ± 0.40	6.82	8.99
	FM SM	7.97 ± 0.39	6.92	8.97
	S	7.97 ± 0.39	6.91	8.99
O_2 saturation (%)				
	FM	97.58 ± 3.19	86.20	104.00
	FM S	97.50 ± 2.96	88.80	103.90
	FM SM	97.34 ± 2.93	88.10	103.60
	S	97.54 ± 2.91	89.80	103.80
pH				
	FM	7.71	6.90	7.94
	FM S	7.71	6.93	7.96
	FM SM	7.71	6.93	7.97
	S	7.71	6.94	7.96

2.4 DISCUSSION

The combination of fishmeal and soya as the main protein sources in the formulated diets of farmed *Haliotis midae* have been found to produce superior growth compared to those fed single-source protein diets (Guzmán and Viana, 1998; Dlaza *et al*., 2008; Ayres, 2013; Riddin, 2013; Wu *et al*., 2019). This was also seen in the present study. Abalone fed these combination diets exhibited higher weight and length gain, while also producing gonads with more oocytes in the more advanced stages (Ayres, 2013; Riddin, 2013; Wu *et al*., 2019). As such, the replacement of soya with an alternate plant protein source, such as sunflower meal was considered beneficial for the understanding and elimination of the potential hormonal and/or nutritional effects of soya that has been attributed to the enlarged gonads.

Stable isotope analysis has not only been used as a means of identifying food sources of animals and its trophic level within an ecosystem, but it has also been increasingly implemented into aquaculture nutrition research over the past decade. Only one study has recently implemented stable isotope analysis in distinguishing the relative contribution of formulated feed and macroalgae on the growth of farmed, *H. midae* (Kemp, 2018). The use of mixing-models estimates the relative proportion of nutrients that have been derived from different food sources that have been retained in the tissue of the consuming organism (Gamboa-Delgado *et al.*, 2008). The allocation of the commonly used protein sources, namely fishmeal and soya to tissue growth and the potential temporal changes over the course of a year has not been explored.

The aim of this study was to use stable isotope analysis as a tool to trace and follow the fate of the two most important dietary components, namely fishmeal and soya, as well as an alternative plant protein source, sunflower meal, in abalone growth and reproduction over a one-year period, thereby encompassing a full reproductive cycle. This was achieved by the quantification of abalone growth and reproduction that were fed single-protein based diets and combination diets. The prerequisites for stable isotope mixing models, namely the time taken to reach isotopic equilibrium and discrimination factors were evaluated for somatic and gonadal tissue of abalone from each dietary treatment. Stable isotope mixing models were implemented and the temporal variation in dietary feed ingredient contribution to foot muscle and gonad tissue growth was estimated.

Growth

The mean monthly weight and length gain of female abalone were significantly higher compared to male abalone, and this was independent of dietary treatment. This could be explained by the significant interaction between time and sex of abalone for the average whole-body mass and shell length, particularly at the end of the study, where females grew faster than males, independently of dietary treatment. Within sex, dietary treatment had no effect on the mean monthly weight gain of females, while male abalone that were fed FM S had significantly higher weight gain than those that were fed FM. Wu *et al*. (2019) found that male abalone exhibited increasing mean monthly weight gain with increasing rates of soya inclusion levels in a fishmeal-based diet, while females were not influenced by inclusion levels of soya in the diet. Similarly, Wu *et al*. (2019) found that male abalone had a significantly higher mean monthly length gain compared to females, with no influence of soya inclusion. Although the weight and length gain reported by Wu *et al*. (2019) fell within the range of the present study, the discrepancies in the sex related differences could have been attributed to the differences in the scale of the studies. For example, Wu *et al*. (2019) conducted a small-scale trial, using fewer animals (1960 versus 3392 abalone) in a much smaller system (85 L versus 2800 L) over a shorter period of time (six months versus one year). On the contrary, Ayres (2013) reported that abalone fed a combination diet of fishmeal and soya grew faster than abalone that were fed a fishmeal-only based diet with no observed sex-related differences.

Abalone that were fed FM S and FM SM had similar whole-body mass averages over the one-year period, while only animals fed FM S were significantly heavier than those that were fed the single-protein based diets. The improved growth of abalone fed the combination of fishmeal and soya support studies conducted on other haliotid species (Bautista-Teruel *et al*., 2003; Tung and Alfaro, 2012). There was a significant interaction between time and abalone sex, where female abalone appear to outgrow male abalone towards the end of the study and this occurred for both the average whole-body mass and shell length. Temperature is the most important external variable that affects growth in aquatic invertebrates, including most abalone species (Kikuchi and Uki, 1974a, 1974b) and since water quality was similar between all treatments throughout the study, differences were likely due to dietary effects. The average feed conversion ratio (FCR) over the 12-months was lower for abalone that were fed the FM S diet compared to those in the other three treatments used in the present study. No differences in FCR values have been previously reported for abalone fed a fishmeal and soya diet compared to those fed a fishmeal-only based diet (Ayres, 2013; Riddin, 2013; Wu *et al*., 2019), while abalone fed a soya-only based diet had higher FCR values than those fed a fishmeal and soya diet (Riddin, 2013). Interestingly, the FCR of abalone fed the FM SM and single-protein based diets were similar to the above-mentioned studies, with an FCR of approximately 1.70 (Ayres, 2013; Riddin, 2013; Wu *et al.*, 2019), indicating that abalone fed FM S in the present study performed particularly well with lower FCRs (1.30 ± 0.13) than the ratio typically reported for this species that have been fed a formulated diet comprised of either fishmeal as the main protein source or a combination of fishmeal and soya. Although the formulated diets used in the present study and the above-mentioned studies were manufactured by the same company

(Marifeed (Pty) Ltd, Hermanus, South Africa), the soya and fishmeal ingredients used were not from the same batch and may have differed in protein content or any other dietary parameter. Consequently, the composition of the diets differed in order to maintain isonitrogenous and isoenergetic levels. The proximate analyses of the diets from the present study and the ones mentioned above were similar, except the protein content in the present study was approximately 4 – 8 % less than that of the previous studies, which ranged between 33 and 36 % (Ayres, 2013; Riddin, 2013; Wu *et al.*, 2019). However, it is unlikely that the protein content (%) was responsible for the differences in FCR in the present study since the FM SM diet had the lowest protein content (26.18 %), but produced similar FCRs compared to those that were fed S (30.91 %), while FM (27.72 %) and FM S (28.61 %) were intermediate. Furthermore, the FCR for abalone fed FM S in the present study (1.30 ± 0.13) was the closest to that reported by industry (approximately 1.2) when abalone are fed a fishmeal and soya combination diet (N. Ketse-Matiwane, Whale Rock Abalone Farm (Pty) Ltd, pers. comm., December 2019); thereby, validating the strength of the results of the present commercial-scale trial.

The meat mass as a proportion of whole-body mass differed between treatments over time, but overall, abalone fed FM SM was significantly higher than those that were fed the S-only diet, while abalone fed FM S and FM-only performed similarly to abalone in the other two treatments. Similar studies have also found that a fishmeal-based diet as well as a fishmeal and soya combination diet had no influence on the meat mass index (Ayres, 2013; Wu *et al.*, 2019). The present study corroborates these findings; however, the replacement of soya with sunflower meal yielded relatively high meat mass indices, but only when compared to animals that were fed a S-only based diet. Fishmeal diets generated more muscle tissue in abalone, *Haliotis iris* (Tung and Alfaro, 2012), but abalone fed FM-only and S-only in the present study had similarly lower meat indices, therefore suggesting that the combination of fishmeal and sunflower meal was able to provide additional nutrients that favoured investment into meat tissue growth.

The overall visceral mass as a proportion of the whole-body mass was significantly higher in animals when soya was included in the diet compared to abalone that were fed a FM-only based diet, while abalone that were fed FM SM performed similarly to abalone in all other treatments. Studies reported an effect of diet on visceral index values in male abalone and not females, and in both instances, male *H. midae* that were fed a FM S combination diet had higher visceral index values than those fed a FM-only based diet (Ayres, 2013; Wu *et al.*, 2019). However, the meat visceral ratio indicated that the presence of soybean meal resulted in a significantly greater proportion of visceral mass when only looking at the soft tissue mass (meat and viscera). Similarly, Tung and Alfaro (2012) observed higher gonad to soft body indices in abalone fed diets that included soya and attributed this to the relatively high amount of polyunsaturated fatty acids provided by the diets. Mixed protein sources are commonly used to meet the nutrient balance of animals (Tung and Alfaro, 2012) and therefore, the inclusion of soya likely provided more nutrients, such as essential amino acids and fatty acids that are needed for reproductive tissue growth.

The dietary needs of abalone changes depending on its developmental stage, and slow growth of abalone that have been provided with a formulated feed may be related to factors contributing to the timing of dietary cues that influence their digestive enzymes (Kemp, 2018). Therefore, the time at which weaning animals are switched onto formulated feeds could possibly play a role in their ability to utilize these compound diets later on in development (Kemp, 2018). The animals used in the present study were spawned two months apart and it is possible that they were switched onto formulated feed around the same time. Since abalone from each spawning time and cohort were equally distributed amongst the dietary treatments, the effect of diet switch was unlikely to be a confounding factor. However, the changes in the digestive enzymes after the switch onto the new dietary treatments may have altered the gut bacteria and digestive enzymes of the animals and subsequently played a role in the differences seen in growth throughout the one-year trial (Zhao *et al*., 2012; Nel *et al*., 2017a, 2017b, 2018). Research on the changes in gut bacteria and digestive enzymes after the switch onto new dietary treatments was undertaken by a Masters student at the Department of Ichthyology and Fisheries Science, Rhodes University.

Reproduction

The gonad tissue of abalone was significantly influenced by dietary treatments as well as the sex of the abalone. Reproductive tissue of abalone fed the combination diets were heavier, while feed was more efficiently utilized only by those fed FM S and could potentially be explained by the resorption of the relatively larger gonads. Male abalone had higher GBI values compared to females and this was independent of dietary treatment. Female abalone had a significantly higher average digestive gland index compared to males and male abalone had more gonad tissue compared to females. This sex-effect has also been reported in previous studies on the same species (Ayres, 2013; Wu *et al*., 2019), while female and male gonad sizes have also been found to be similar in the same species (Newman, 1967; Riddin, 2013). Male abalone that were fed the two combination diets had similar GBI values that were significantly higher than males that were fed the single-protein based diets. Similarly, female abalone that were fed the combination diets had the highest overall GBI values, but only females that were fed FM S had significantly higher GBI values than female abalone that were fed S-only. In a similar study, both female and male *H. midae* that were fed a fishmeal-soya combination diet had higher GBI values than those fed a FM-only based diet (Ayres, 2013). Riddin (2013) included a S-only based diet and found that *H. midae* fed a FM S and S-only based diet had higher GBI values than those that were fed a FM-only based diet. Abalone from all treatments followed similar changes in mean GBI values, increasing from the start of the study, peaking at day 133 and 182, followed by a gradual decrease towards the end of the study. The gonad volume increased as animals matured due to the increase in oocyte size and sperm numbers (Setyono, 2006), while a gradual decrease in GBI is indicative of an asynchronous spawning event as opposed to a synchronous spawning event where GBI values drop and rise significantly (Tutschulte and Connell, 1981; Wood and Buxton, 1996; Gurney and Mundy, 2004). A number of stimulus have been recognized to cause spawning in molluscs, including water temperature, food availability and photoperiod (Webber and Giese, 1969; Young and De Martini, 1970; Shepherd and Laws, 1974; Wayne, 2001). Since water temperature and photoperiod were consistent between treatments in the current study and the only variation was dietary protein, differences in gonad volume observed here were likely due to the difference in availability of nutrients from the respective protein sources. The FCR during the first half of the study was similar for abalone that were fed S-only and the combination diets, while those fed FM-only had significantly higher FCRs than those fed FM S, suggesting that during the period of increasing reproductive investment (i.e. increased GBI values), abalone that were fed FM-only required more feed to meet their nutritional requirements. However, during the second half of the study, abalone fed FM S had lower FCRs than those in the other three treatments, suggesting that abalone fed FM S required less feed to maintain growth during the apparent spawning period. Therefore, abalone fed the two combination diets resulted in the largest gonads with the FM S diet being most efficiently utilized during the spawning period and over the 12-month trial.

Meat indices were similar between females and males, but male abalone had significantly higher GBI values, suggesting that male abalone were investing more into gonad growth and this pattern has been seen in a similar study on *H. midae* (Ayres, 2013). Since both females and males that were fed FM S had significantly higher gonad tissue than abalone fed the S-only based diets, it is unlikely that the higher visceral index and GBI values in abalone that were fed FM S were due to the presence of phytoestrogens in soya, as had been hypothesised by authors who conducted similar studies on the same species (Ayres, 2013; Riddin, 2013). Furthermore, similar reproductive outputs could not be verified with the use of crystalline isoflavones at rates similar to those found in FM-based diets with graded levels of soya (Wu *et al.*, 2019). However, the verification of an abalone's reproductive state through gonad histological assessment, coupled with hormone assays, would be useful in substantiating the possible hormonal effects of dietary protein source and will be investigated in Chapter 4. It is likely that the various nutrients in each diet made available from the respective dietary treatments were the cause for the variations in gonad tissue; and the fatty acid content will be explored in Chapter 3.

The relationships between GBI and DG index were significant, but r^2 values were very low, which may assist in explaining why there was no significant relationship between the two variables in females that were fed the two combination diets. Particularly since females from all treatments changed similarly over time, for both GBI and DG index. Therefore, although the relationships were weak, some trends between GBI and DG index were observed. Energy transformation occurs in the digestive gland and its metabolic activity would be expected to correspond with gametogenesis (Carefoot *et al.*, 1998). The relationship between the gonad and digestive gland is due to the digestive gland functioning as a nutrient store, transporting nutrients to the gonad for maturation and reproductive purposes (Soudant *et al.*, 1996; Litaay and De Silva, 2003; Najmudeen, 2007). Measuring these nutritive compounds and monitoring their changes between the digestive gland and gonad would give us a better understanding of the pertinent essential nutrients that are involved in the reproductive cycle of farmed *H. midae.* Protein sources that are linked with essential fatty acids and highly unsaturated fatty acids are used for gonad development in abalone (Bautista-Teruel *et al*., 2001). Understanding which essential fatty acids are utilized during reproduction would be useful in forming diets for broodstock for abalone aquaculture facilities. The significance of essential fatty acids in reproduction will be addressed in Chapter 3.

Stable isotope incorporation and discrimination factor

The diet composition had an effect on the $\delta^{13}C$ and $\delta^{15}N$ incorporation rates in the gonad and meat tissues of abalone and this differed between females and males. The variable incorporation rates are an indication of isotopic routing, where dietary elements were differentially allocated to the muscle and gonad tissue. Isotopic incorporation is reached through tissue accretion, protein turnover or both and the amount of time taken for an animal to reach isotopic equilibrium with its diet depends on biotic factors such as the animal’s growth rate, metabolic rates, age of the animals, quality of the protein, as well as abiotic factors such as water temperature (Won *et al*., 2008; Gamboa-Delgado and LeVay, 2009; Won *et al*., 2010a; Bloomfield *et al*., 2011; Le Vay and Gamboa-Delgado, 2011; Gamboa-Delgado, 2014). The animals used in the present study were of similar age and the water temperature did not differ between treatments, therefore these two variables were unlikely contributing factors to the differences in the isotopic incorporation rates. Since growth rates in female abalone were similar between treatments, it can be assumed that isotopic equilibrium was reached through metabolic turnover rates. Male abalone that consumed the FM-only based diet grew significantly slower than those that consumed the FM S diet, while male abalone that were fed S-only and FM SM performed similarly to animals in both treatments. Therefore, turnover rates in male abalone were also likely attributed to metabolic turnover. Typically, fast growth results in the rapid accretion of tissue, which causes a fast transfer of dietary $\delta^{15}N$ ratios into the tissue of the animal compared to that of slow growing animals (MacAvoy *et al.*, 2005; Gamboa-Delgado *et al.*, 2016). For example, the carbon and nitrogen isotopic changes in larvae are typically due to tissue accretion, while isotopic incorporation in adults are typically due to metabolic turnover (Martínez del Rio *et al.*, 2009). Abalone have very slow growth rates and since the abalone used in the present study were mature adults, the very high incorporation rates (5 – 15 days) were likely due to metabolic turnover, which is the replacement of old tissue with new, despite no net growth (Jardine *et al.*, 2003). Juvenile *Haliotis discus hannai* that have been introduced to formulated feed have been estimated to take at least 120 days to reach equilibrium with its diet (Won *et al*., 2008), therefore further pointing to metabolic turnover as the main driver for incorporation rates in the present study. The differences in developmental stages make the comparison of incorporation rates difficult as animal size is one of the key factors that contribute to the dynamics of isotopic incorporation (Martínez del Rio *et al*., 2009). The present study was conducted on sexually mature abalone, while previous studies were conducted on rapidly growing young abalone (Won *et al.*, 2008, 2010a; Kemp, 2018).

Diet composition had an effect on the $\Delta^{13}C$ and $\Delta^{15}N$ in the meat and gonad tissue of female and male farmed *H. midae*. Findings from the present study contribute to the increasing evidence that propose that isotopic discrimination is species- and tissue-specific and therefore the frequently used carbon (1 ‰) and nitrogen (3.4 ‰) discrimination factors could result in inaccurate estimations of nutrient contribution when used incorrectly (DeNiro and Epstein, 1981; Bloomfield *et al*., 2011; Le Vay and Gamboa-Delgado, 2011). The large range in $\Delta^{13}C$ represents isotopic routing, where dietary elements are selectively metabolised and incorporated into different tissues (Gannes *et al*., 1997; Le Vay and Gamboa-Delgado, 2011).

The routing of elements between the digestive gland and gonad was eliminated by pooling these two tissue samples, giving an indication of the allocation of nutrients into the bulk of the visceral mass (i.e. the gonad and digestive gland). Variations in $\Delta^{15}N$ between tissue type may be attributed to the diet quality, protein content, variation in amino acids of the tissue and different nutrient assimilation rates (Gamboa-Delgado and Le Vay, 2009; Le Vay and Gamboa-Delgado, 2011; Gamboa-Delgado, 2014). The high $\Delta^{15}N$ for S-only fed abalone within each tissue type and abalone sex, suggest an imbalance in the necessary dietary nutrients, such as a lower availability of essential amino acids found in soya and this could be corroborated by the slower growth, including the lowest mean GBI values and lowest meat index values. Previous studies on *H. midae* have found that S-only diets produce slower growth compared to fishmeal-based diets (Britz, 1996b). Higher $\Delta^{15}N$ values have been speculated to be due to metabolic cycling of nutrients (Martínez del Rio and Wolf, 2005), while small $\Delta^{15}N$ values are considered to infer a fast incorporation of nutrients (Gamboa-Delgado *et al*., 2014). The quality of a protein source is primarily determined by its amino acid composition in comparison to the amino acid requirements of the consuming organism (Kies, 1981). The lower $\Delta^{15}N$ in abalone fed the two combination diets would support the protein quality hypothesis, which suggests that $\Delta^{15}N$ will decrease as the biological value of the protein source increases (Roth and Hobson, 2000). Both tissues of abalone that were fed diets that included fishmeal had very small or negative values, which suggest the favourable nutritional profile of the fishmeal ingredient as a protein source as well as the combination of fishmeal and a plant-protein source.

The use of stable isotope analysis on abalone has mostly been used in ecological studies, exploring variations in the diets of wild populations (Guest *et al*., 2008; Vega-García *et al*., 2015; Won *et al*., 2007, 2008, 2010b). Furthermore, one laboratory study has provided discrimination factors ($\Delta^{13}C$ – 1.6 and $\Delta^{15}N$ – 2.2) in the muscle tissue of abalone, *H. discus* *hannai* (27 – 35 mm) that were fed a formulated feed (Won *et al*., 2008) and were subsequently used in ecological studies (Won *et al*., 2010b). Won *et al*. (2010a) determined discrimination factors of post-larval *H. discus hannai* (< 2mm) that were fed benthic diatoms (Δ^{13}C – 1.2 ‰ and Δ^{15}N – 2.9 ‰) and these fell within the range of discrimination factors in the present study. In addition, post-larvae fed gametophytes had Δ^{13}C (8.4 ‰) that was much higher and Δ^{15}N (3.7 ‰) that was within the range of this study (Won *et al*., 2010a). Kemp (2018) recently conducted a study on *H. midae* (± 37 mm shell length) and calculated discrimination factors using the diet dependent discrimination factor approach as described by Caut *et al*. (2008, 2009) and this study only revealed Δ^{15}N for muscle tissue of abalone fed on macroalgae diets (*Gracilaria* Δ^{15}N – 5.03; *Ulva* Δ^{15}N – 4.28), which were higher than the highest group of discrimination factors in the present study (S diet; Δ^{15}N = 2.27 – 4.14 ‰). This suggests that discrimination factors for abalone vary considerably and that laboratory studies are essential to validate the use of discrimination factors, whether for ecological studies or studies that are applied in aquaculture nutrition.

Nutrient contribution from fishmeal, soya and sunflower meal

Temporal variations in the relative contribution of protein sources to tissue growth were observed in this study. Similar patterns of fishmeal and soya contributions were seen in both the meat and gonad tissue samples, while the proportions differed between sexes. The contribution of fishmeal appeared to reflect the temporal changes in GBI values, increasing and peaking at day 133 and 182, followed by a gradual decrease up to day 273 and then increasing at the end of the one year. Therefore, during periods of increased reproductive investment, fishmeal was utilised more in both the gonad and meat tissue, and as GBI decreased, soya utilization gradually increased. Differences in contribution were unlikely due to digestibility, since fishmeal, soybean meal and sunflower meal have been shown to be highly digestible by *H. midae* (76, 85 and 99 %, respectively; Sales and Britz, 2003). Contrary to studies that illustrated good growth in abalone that have been fed fishmeal in a formulated diet (Britz, 1996b; Fleming *et al*., 1996; Sales and Janssen, 2004; Tung and Alfaro, 2012), Kemp (2018) found poor utilization of fishmeal as a protein source in foot muscle tissue growth, while soya was better utilized. The majority of the studies on *H. midae* relating to diet, particularly formulated feed and growth, have used juvenile abalone (Britz, 1996a, 1996b; Britz and Hecht, 1997; Shipton and Britz, 2001a; Shipton *et al*., 2002; Sales *et al*., 2003; Naidoo *et al*., 2006; Dlaza *et al*., 2008; Green *et al*., 2011a; Kemp, 2018), inferring that the animal's nutritional requirements would be different in mature, reproducing animals such as those used in the present study. Older, mature abalone would be diverting energy into reproductive development, whereas young animals may exclusively invest energy into somatic growth (Barkai and Griffiths, 1988). For example, juvenile and young adult *H. midae* have been shown to have different protein requirements (Shipton and Britz, 2001a). Furthermore, variables such as culture systems, study period, study season, feeding protocol, diet formulation differed between studies, making comparisons challenging.

The higher proportion of fishmeal utilisation suggest that it is more nutritionally suitable, but the better growth performance of abalone fed FM S in terms of better overall whole-body mass and gonad volume suggest that the combination of fishmeal and soya supplied a better nutritional profile, such as increased availability of essential amino acids and fatty acids. For example, Gamboa-Delgado and Le Vay (2009) reported that fishmeal contributed more to muscle tissue growth than soy protein isolate in shrimp *L. vannamei*, and this was likely due to a restriction of the essential amino acids. Similarities in the growth of abalone fed FM S and FM SM, but lower utilisation of sunflower meal suggest that sunflower meal was able to complement the fishmeal ingredient to some extent, but was an inferior protein source compared to soya. In addition, the little contribution of sunflower meal to both meat and gonad may suggest that sunflower meal was used in shell growth, a suggestion that is corroborated by the relatively higher mean monthly length gain, but further research would be required to elucidate the allocation of ingredients into shell growth. However, more fishmeal ingredient was required in the FM SM diet in order to standardise the proximate composition between the dietary treatments, which makes this treatment less sustainable. Similar patterns of fishmeal contribution to the meat and gonad suggest fishmeal was similarly utilised for both somatic and gonad tissue growth. Differences in nutritional contribution of protein sources can possibly be explained by the differences in amino acid profiles of the fishmeal, soya and sunflower meal (Gamboa-Delgado *et al.*, 2013). Abalone require the same 10 essential amino acids as other animal groups (Allen and Kilgore, 1975), which are mostly found in higher proportions in fishmeal compared to soybean and sunflower meal (Sanz *et al.*, 1994). Furthermore, Shipton and Britz (2001b) found that sunflower meal was a good candidate as a partial replacement for fishmeal, "although it may be limiting in the available essential amino acids". Differences in the overall growth may have also been due to differences in fatty acid profiles of the diets and will be discussed in Chapter 3. The only significant differences seen in growth between abalone fed the combination diets was in the meat viscera ratio, where abalone fed FM SM had significantly higher proportions of meat. Although sunflower meal contributed very little to the nutrient allocation into tissues, it was enough to complement the routing of nutrients to produce a higher proportion of meat tissue when only looking at the soft body mass (meat and viscera). Thus, sunflower meal may support a greater proportion of meat growth, even though its relative contribution was much lower than that of the soya ingredient. By implementing stable isotope analysis into abalone nutrition, the utilisation of whole-ingredients can be assessed, however, the use of compound specific stable isotope analysis may provide a greater clarification by tracing the allocation of macro-molecules, including amino acids and fatty acid methyl esters from its source ingredient (Boecklen *et al.*, 2011; Gamboa-Delgado *et al.*, 2016). The identification of individual essential amino and fatty acids in abalone somatic and reproductive tissue from its source would be valuable in feed formulation.

Conclusion

The prerequisites required for the use of stable isotope mixing models were determined, which allowed for the estimation of protein ingredient utilisation in the tissues of abalone over the one-year feeding trial. Both the $\delta^{13}C$ and $\delta^{15}N$ discrimination factors and turnover rates were found to be influenced by diet composition and demonstrated tissue-specific and sex-specific differences. These findings support the importance of laboratory studies when using stable isotope analysis and mixing models for nutrient allocation.

Abalone that were fed the FM-only and S-only based diets exhibited inferior growth compared to abalone that were fed FM S in terms of whole-body mass and gonad volume. Abalone that were fed the two combination diets (fishmeal-soya and fishmeal-sunflower meal) grew similarly over the one-year period, however, feed utilisation was most efficient in abalone fed the combination of fishmeal and soya. Female abalone grew faster than male abalone, while male abalone had larger gonads compared to females and these were both independent of dietary treatment.

The utilisation of fishmeal and plant protein sources showed temporal changes, but fishmeal was relatively more utilised in somatic and reproductive tissues and allocation of fishmeal increased during increased reproductive development. The utilisation of sunflower meal was relatively low compared to that of soya, but appeared to be sufficient to maintain similar growth to soya inclusion. The larger gonad volume in female and male abalone fed fishmeal with a plant protein source compared to single-protein based diets was likely due to the provision of more available essential amino acids and fatty acids (Chapter 3). The possible hormonal effects of the phytoestrogens present in soya were unlikely, since both females and males fed the S-only diet had the smallest gonad volume, but this remains to be tested. The validation of the reproductive state of an animal through gonad histological assessment, coupled with hormone assays would be useful in corroborating this assumption (Chapter 4).

CHAPTER 3

THE EFFECTS OF DIETARY PROTEIN SOURCES ON THE FATTY ACID PROFILE OF FARMED ABALONE TISSUE

3.1 INTRODUCTION

Lipid content, particularly lipid class and fatty acid profiles are vital to abalone nutrition and play a crucial role in growth and gametogenesis in abalone (Webber 1970; Uki *et al.,* 1986; Mai *et al*., 1996; Bautista-Teruel *et al.,* 2001; Nelson *et al.*, 2002; Durazo-Beltrán and Viana, 2013). Haliotids cannot synthesize all fatty acids *de novo*, such as 20:4n-6 (arachidonic acid, ARA), 20:5n-3 (eicosapentaenoic acid, EPA), 22:6n-3 (docosahexanoic, DHA), 18:2n-6c (linoleic acid, LA) and 18:3n-3 (α-linolenic acid, ALA), which are required for normal cellular function and growth and abalone therefore rely on their diet to fulfil these nutritional requirements (Uki *et al*., 1986; Mai *et al*., 1996; Durazo-Beltrán *et al*., 2003a, 2003b, 2004; Grubert *et al*., 2004; Bautista-Teruel *et al*., 2011; Bilbao *et al*., 2012; Durazo-Beltrán and Viana, 2013; Li *et al*., 2013). These are essential fatty acids (EFAs). In addition to food supply, fatty acid requirements in invertebrates depend on: environmental conditions and developmental and physiological conditions of the animal (Chiou *et al*., 2001; Kube *et al*., 2007). They can also be affected by anaerobic energy metabolism, parasitism, reproduction and pollutants (Kube *et al*., 2007). Feeding abalone diets low in EFAs resulted in suboptimal growth (Uki *et al*., 1986; Floreto *et al*., 1996; Mai *et al*., 1996).

Fatty acids serve as reserve constituents such as glycogen, lipid and protein. They also serve as energy sources during reproduction, growth and hypoxic conditions (Kube *et al*., 2007). Fatty acids with two or more double bonds are termed polyunsaturated fatty acids (PUFA) and are important in animal growth. Both the n-3 and n-6 PUFAs have been determined to be essential to abalone growth (Nelson *et al.*, 2002). Highly unsaturated fatty acids (HUFA) are PUFAs that contain more than 20 carbon atoms and four double bonds and have specific roles in controlling and regulating cellular metabolism in organisms (Bautista-Teruel *et al*., 2011).

Dietary lipid is a major nutrient that plays a key role in abalone nutrition. It not only provides energy and EFAs, but also fat-soluble nutrients for normal growth (Bautista-Teruel *et al*., 2011; Venter *et al*., 2016). Lipids also provide compounds such as sterols and polar lipids which form structural components of cell membranes (Bautista-Teruel *et al.*, 2011). Fatty acids are stored in the form of lipids in the body. However, abalone are herbivorous and their natural diet usually consists of a low lipid, but a high carbohydrate content (Thongrod *et al*., 2003) and as such, abalone store energy as carbohydrates, which appears to be the preferred energy source in these animals (Moñtano-Vargas *et al*., 2005). The primary role for lipids in abalone is for growth and gonad maturation (Durazo-Beltrán and Viana, 2013). Reservoirs for lipids include organs such as the liver, muscle, gonads or glands. The importance of organs for the storage of lipids changes according to life stages and is affected by environmental variables. Fatty acid content not only differ between organs, but the abundance of fatty acid types varies as well (Hu *et al*., 2009).

The natural diet of abalone consists of marine algae, which vary in lipid composition, therefore, the consumers' growth may be affected by the species of macroalgae consumed as well as the nutritional status of the macroalgae (Uki *et al*., 1986). Similarly, dietary ingredients used in formulated feed production differ in lipid composition. Fish and/or plant oils are added into formulated feeds in order to increase their energy content and provide EFAs (Alexis, 1997). However, the combination of protein sources in compound feeds, such as fishmeal, soya (*Glycine max*) and sunflower (*Helianthus annuus*) meal also contain EFAs that also contribute to the fatty acid profiles of the diets. The differences in the availability of EFAs from different protein sources and their subsequent effect on growth and reproductive development play a crucial role in diet development and understanding of nutrient allocation in farmed *Haliotis midae*.

Numerous studies have been conducted on the effects of formulated feed and macroalgae on the lipid and fatty acid composition of abalone (Uki *et al*., 1986; Dunstan *et al*., 1996; Floreto *et al*., 1996; Nelson *et al*., 2002; Durazo-Beltrán *et al*., 2003a, 2003b; Thongrod *et al*., 2003; Su *et al*., 2004, 2006; Bautista-Teruel *et al*., 2011; Hernández *et al*., 2013; Li *et al*., 2015; Mulvaney *et al*., 2015; de la Peña *et al*., 2016; Pan *et al*., 2018), but few have monitored the influence of fatty acids on reproductive development in haliotids (Bautista-Teruel *et al*., 2001; Grubert *et al*., 2004; Litaay *et al*., 2007; Najmudeen, 2007; Mateos *et al*., 2010). Fatty acid analysis has also been used to determine the food source of abalone (Guest *et al*., 2008; Pan *et al*., 2018). Protein requirements in haliotids are highly species specific and inclusion of lipids as well as n-3 and n-6 inclusion levels in the feed vary among species (Glencross, 2009; Tung and Alfaro, 2011). For example, Mai *et al*. (1996) presented evidence suggesting n-3 and n-6 are essential for growth in *Haliotis discus hannai*, while *Haliotis tuberculata* were largely dependent on n-3 PUFAs. *Haliotis fulgens* that were fed macroalgae high in LA and ALA grew slowly (Nelson *et al*., 2002), but *H. discus hannai* and *H. tuberculata* fed a diet high in LA and ALA had high growth rates (Mai *et al*., 1996). A study was conducted on two abalone species (*H. discus hannai and Haliotis diversicolor*) of similar size, fed the same feed and the lipid and fatty acid composition in the muscle and viscera of both species were similar, except for the presence of DHA in the tissues of *H. discus hannai* and not in the tissues of *H. diversicolor* (Lou *et al*., 2013). Litaay *et al.* (2007) observed changes in EFAs (ARA, ALA and EPA) in the gonads of female blacklip abalone, *Haliotis rubra*, increasing with oocyte maturity and decreasing as animals spawned. A similar study found a positive correlation between ovarian lipid content and gonadosomatic index, with the highest lipid content in mature ovaries and decreasing towards the spent stage (Najmudeen, 2007). However, a study conducted on blacklip abalone and greenlip abalone, *Haliotis laevigata*, found similar fatty acid composition between gravid and spent ovaries (Grubert *et al*., 2004). Elevated levels of particular EFAs have been observed in the foot muscle, digestive gland and gonad of Haliotids and have been found to differ in abundance between females and males (Nelson *et al*., 2002; Grubert *et al*., 2004; Njamudeen, 2007; Mateos *et al*., 2010; Lou *et al*., 2013). Improved growth and reproductive development have been attributed to high abundance of EFAs in abalone (Nelson *et al*., 2002; Bautista-Teruel *et al*., 2011). The allocation and utilisation of these EFAs in the tissues of abalone are important as they illustrate the metabolism of these compounds in relation to growth and reproduction (Nelson *et al*., 2002; Mau and Jha, 2018).

Although tissue fatty acid composition of abalone was dependent on the diet consumed (Nelson *et al*., 2002; Grubert *et al*., 2004; Guest *et al*., 2008; Bautista-Teruel *et al*., 2011; Durazo-Beltrán and Viana, 2013), no studies relating to both growth and reproduction have been conducted on the commercially important South African abalone species, *H. midae*. Understanding how protein sources used in the formulation of compound feed influence the fatty acid composition of farmed *H. midae* and its subsequent effect on reproduction will contribute to the understanding of nutrient requirements in this species.

The aim of this study was to determine the effects of dietary protein sources, specifically soybean meal, sunflower meal and fishmeal, on the fatty acid utilisation of farmed *H. midae* during the apparent peak spawning period and the period of the lowest reproductive investment, and to determine the differences between these two periods. The allocation of particular fatty acids into specific tissues, namely the foot muscle, digestive gland and gonad during increased reproductive investment will pave the way for future studies on better diet formulation for the South African abalone, *H. midae*. Furthermore, a better understanding of *H. midae* nutrition will be developed. The aim of this study was addressed by carrying out the following objectives:

1) quantify the dietary fatty acid concentration and composition (% total fatty acid) in the diets containing fishmeal and soya as the sole protein sources, as well as combination diets of fishmeal and soya and fishmeal and sunflower meal;
2) determine the periods of peak and least reproductive investment based on gonad bulk indices observed in chapter 2;
3) determine the effect of dietary treatment, time of sampling (highest versus lowest gonad bulk index values), abalone sex and tissue type on the fatty acid composition;
4) identify relationships between dietary treatment, sampling time, abalone sex and tissue type on the fatty acid composition;

5) determine the EFAs that most contribute to the differences between dietary treatment, sampling time, abalone sex and tissue type and compare the concentrations of the identified EFAs; and
6) quantify the fatty acid composition of the muscle, gonad and digestive gland tissues of female and male abalone that have been fed the respective diets during the two identified periods;

3.2 MATERIALS AND METHODS

Experimental system and animals

Animals that were used for fatty acid analysis were the same animals, subject to the same dietary treatments (Chapter 2, Table 2.2.1) and housed in the same system as described in Chapter 2. Dietary treatments included a fishmeal only (FM), soya only (S), combination of fishmeal and soya (FM S) and combination of fishmeal and sunflower meal (FM SM) based diets.

Sampling protocol

As described in Chapter 2, one of the two males and one of the two females that were randomly collected from each replicate tank were used for fatty acid methyl ester analysis at day 133 (4 December 2017) and day 315 (4 June 2018) of the trial. These samples were selected for fatty acid methyl ester analysis based on the gonad bulk index values (Chapter 2), with day 133 and 315 representing the highest and lowest gonad tissue volume relative to the soft tissue of the abalone. This was based on the observation that abalone gonad volume increases due to the increase in oocyte size and sperm numbers and the reduction in gonad volume is due to spawning. In addition, maturation and breeding have been show to coincide with maximum gonad weight (Setyono, 2006; Najmudeen, 2007). Collected abalone were placed in labelled mesh bags that were carried in a crate and immediately transported to SPP Canning (Pty) Ltd, Hermanus, where animals were stored in a chiller room at approximately 10 °C. Abalone were randomly removed and placed in numbered 350 ml plastic tubs and subsequently shucked by staff in the facility.

Tissue collection

A transverse section was taken through the adductor muscle and foot and a vertical slice of approximately 2.0 g was cut, rinsed with distilled water and placed in a 1.5 ml Eppendorf vial. In addition, a cross section was taken through the conical appendage and approximately 2.0 g of the digestive gland and gonad tissue was rinsed with distilled water and samples were placed in 1.5 ml Eppendorf vials and stored at -20 °C until further analysis. The visceral samples were removed from the -20 °C freezer to separate the digestive gland from the gonad tissue, and this took place in a 4.0 °C chiller room to reduce the rate of thawing and thus maintain the integrity of the samples during the procedure.

Sample preparation

Approximately 100 – 300 mg of the meat samples were weighed with a fine scale (Mettler-Toledo XP26, Mettler-Toledo AG, Laboratory & Weighing Technologies, Greifensee, Switzerland) and placed into a 15 ml glass tube. Due to the practical difficulties in obtaining large samples of the digestive gland and gonad, the maximum mass of tissue that was excised was kept and this ranged between approximately 1 – 157 mg. In addition, the four diets were crushed and approximately 1.0 mg of each diet was weighed using a fine scale into glass tubes. For the tissue and feed samples, 1.0 ml of hexane was added into each tube, followed by 100 μl of an internal standard of heptadecanoic acid (1000 ppm in hexane). For the ingredients, 2.0 ml of hexane and 200 μl of the internal standard were added into the tubes. An additional 1.0 ml and 2.0 ml of 20 % sulfuric acid in methanol was added for the tissue/feed samples and ingredients, respectively. The tubes were then vortexed for approximately 5.0 s, placed in a beaker with water and placed in an oven at 80 °C for an hour. Once removed, the tubes were allowed to cool, before 2.0 ml of a 20 % sodium chloride solution was added. The tubes were centrifuged at 3000 RPM for 1.0 min. All samples were concentrated by transferring approximately 500 μl of the top hexane layer into a 2-ml gas chromatography vial and evaporated to approximately 200 μl, using nitrogen gas that was introduced into the vials through a pipe. The remaining hexane was transferred into an insert and placed back into the same vial and capped.

Standards were prepared using Supelco 37 Component fatty acid methyl ester mix (Sigma Aldrich) and diluted to appropriate concentrations. Concentrations of the standards were calculated using the calibration standards to construct a calibration curve using the internal standard method of 1000 ppm C17 (100 μl). The mass:volume ratio of the samples (g ml^{-1}) was divided by the concentration (in μg ml^{-1} = mg l^{-1} = ppm) to convert it to μg g^{-1}.

Fatty acid methyl esters analysis

Separation was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics

PAL autosampler. Separation of fatty acids methyl esters was performed on a Stabilwax (60 m, 0.25 mm ID, 0.25 µm film thickness) capillary column. Helium was used as the carrier gas at a flow rate of 2 ml min^{-1}. The injector temperature was maintained at 250 °C. One microlitre of the sample was added using spitless injection. The oven temperature was programmed as follows: 50 °C for 2 min; and ramped up to 180 °C at a rate of 25 °C min^{-1} for 5 min; followed by a ramping rate of 3 °C min^{-1} and held for 15 min at 260 °C. Individual fatty acid methyl esters were identified by comparison of retention time with the fatty acid standards.

Seasonal sea water temperature

The sea water temperature in the header tank (i.e. tank into which sea water was pumped from the ocean, before being gravity fed to all abalone raceways on the farm; Chapter 2) was monitored using a temperature logger (HOBO UX100, Onset Computer Corporation, Bourne, Massachusetts, Unites States) every 10 min for the duration of the study.

Statistical analysis

A permutational multivariate analysis of variance (PERMANOVA) was used to test the effects of day, treatment, tissue type and abalone sex on the fatty acid composition. Analysis was conducted using Bray-Curtis dissimilarities with 999 permutations.

Based on the PERMANOVA results, dietary treatment was excluded as a variable and a non-metric multidimensional scaling analyses (NMDS) was conducted on the log-transformed fatty acid concentrations of abalone digestive gland, gonad and meat samples at each sampling time for female and male abalone. Analysis of similarity (ANOSIM) was conducted to test the level of significance between tissue type, abalone sex and sampling day based on the fatty acid composition, with R values close to one having a high degree of separation between levels of factors. All ANOSIM analyses used the Bray-Curtis index with 999 permutations. Similarity percentage analyses (SIMPER) were used to identify the most prominent fatty acids in the NMDS ordination plots. Of the fatty acids that contributed to approximately 75 % of the ordinations, the essential fatty acids (EFAs) were isolated and discussed. All analysis was conducted in R. Fatty acid concentrations were reported as means ± standard error.

The mean, standard deviation, minimum and maximum sea water temperatures from the header tank were calculated for autumn (March – May), winter (June – August), spring (September – November) and summer (December – February).

3.3 RESULTS

Dietary fatty acid composition

The combination diets had higher concentrations of total saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) compared to the single protein diets, with fishmeal-sunflower (FM SM) meal containing the highest concentration of each group (Table 3.3.1). However, the percentage composition of total SFA and MUFA were similar between the combination diets and FM-only diet, ranging between 17.03 – 18.54 and 9.57 – 11.82 %, respectively, while total SFA and MUFA were lowest in the S-only diet at 15.05 and 5.79 %, respectively (Table 3.3.1). In contrast, the total PUFA was highest in the S-only diet, due to the high abundance of linoleic acid (C18:2n6c), while the total PUFA ranged between 69.65 – 73.09 % for the other three dietary treatments (Table 3.3.1).

The most abundant SFA in all treatments was palmitic acid (PA, C16:0), while the most abundant MUFA in all treatments, except the S-only diet was palmitoleic acid (C16:1). The most abundant MUFA in the S-only diet was oleic acid (OA, C18:1n9c). Three essential fatty acids (EFAs), namely arachidonic acid (ARA, C20:4n6), eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3) were non-detectable in the soya-only diet, while these EFAs were highest in the FM SM combination diet at 14.27, 211.10 and 264.23 $\mu g\ g^{-1}$, respectively (Table 3.3.1). However, as a percentage of total FAs, ARA (1.04 – 1.46 %), EPA (17.19 – 21.67 %) and DHA (26.21 – 31.21 %) concentrations were similar in all three treatments. Both α-linolenic acid (ALA, C18:3n3) and eicosadienoic acid (EDA, C20:2n6) were not detectable in any of the dietary treatments (Table 3.3.1).

Dietary treatment had no influence on the fatty acid composition (PERMANOVA, $p = 0.21$; Table 3.3.2), while fatty acid composition was influenced by day, tissue and abalone sex

($p < 0.05$; Table 3.2.2).

Table 3.3.2: Effect of treatment, day, tissue and sex on the fatty acid composition (Permutational multivariate analysis variance, $p < 0.05$).

Variable	df	SS	MS	F	p
Treatment	3	0.10	0.03	1.37	0.21
Day	1	1.49	1.49	59.77	0.001
Tissue	2	2.92	1.46	58.33	0.001
Sex	1	0.08	0.08	3.19	0.04

Table 3.3.1: Mean fatty acid (FA) concentration ($\mu g\ g^{-1}$) and composition (% total FA) in fishmeal-only based diet (FM), combination of fishmeal and soya (FM S), fishmeal and sunflower meal (FM SM) and soya-only (S) based dietary treatments. nd, not detected.

		Treatment ($\mu g\ g^{-1}$)				Treatment (% total FA)			
FAMEs		FM	FM S	FM SM	S	FM	FM S	FM SM	S
Tridecanoic acid	C13:0	0.12	0.10	0.10	nd	0.02	0.01	0.01	nd
Myristic acid	C14:0	22.80	27.66	38.92	2.71	4.12	3.01	4.00	0.38
Pentadecanoic acid	C15:0	4.46	5.68	6.19	nd	0.81	0.62	0.64	nd
Palmitic acid	C16:0	74.46	115.50	101.64	87.20	13.44	12.57	10.44	12.26
Stearic acid	C18:0	0.81	10.42	19.05	17.18	0.15	1.13	1.96	2.42
Total saturated FA		**102.65**	**159.36**	**165.91**	**107.09**	**18.54**	**17.34**	**17.03**	**15.05**
Palmitoleic acid	C16:1	37.55	46.23	69.79	2.32	6.78	5.03	7.17	0.33
Heptadecanoic acid	C17:1	0.34	0.27	1.51	nd	0.06	0.03	0.16	nd
Oleic acid	C18:1n9c	24.22	37.05	34.13	38.84	4.37	4.03	3.50	5.46
Eicosanoic acid	C20:1	3.34	4.35	2.88	nd	0.60	0.47	0.30	nd
Total monounsaturated FA		**65.44**	**87.91**	**108.32**	**41.16**	**11.82**	**9.57**	**11.12**	**5.79**
Linoleic acid	C18:2n6c	82.21	232.22	195.40	484.65	14.84	25.27	20.06	68.12
α-Linolenic acid	C18:3n3	nd	nd	nd	nd	nd	nd	nd	nd
γ-Linolenic acid	C18:3n6	7.91	31.15	14.71	78.52	1.43	3.39	1.51	11.04
Eicosadienoic acid	C20:2n6	nd	nd	nd	nd	nd	nd	nd	nd
Arachidonic acid	C20:4n6	7.30	9.52	14.27	nd	1.32	1.04	1.46	nd
Eicosapentaenoic acid	C20:5n3	115.49	157.99	211.10	nd	20.85	17.19	21.67	nd
Docosahexaenoic acid	C22:6n3	172.82	240.83	264.23	nd	31.21	26.21	27.13	nd
Total polyunsaturated FA		**385.73**	**671.72**	**699.71**	**563.17**	**69.65**	**73.09**	**71.84**	**79.16**
Total n-6 fatty acid		**97.42**	**272.89**	**224.38**	**563.17**	**17.59**	**29.70**	**23.04**	**79.16**
Total n-3 fatty acid		**288.31**	**398.82**	**475.33**	**nd**	**52.06**	**43.40**	**48.81**	**nd**
n-6/n-3 ratio		**0.34**	**0.68**	**0.47**	**nd**				
Total fatty acid		**553.82**	**918.99**	**973.93**	**711.42**				

Relationships; between dietary treatment, time of sampling, abalone sex and tissue type on the fatty acid composition and identifying the most prominent essential fatty acids

The tissues of female (ANOSIM R = 0.772, p = 0.001; Figure 3.3.1a) and male (ANOSIM R = 0.733, p = 0.001; Figure 3.3.1b) abalone at day 133 had different fatty acid concentrations that were statistically different in overall fatty acid profiles. Three distinct clusters were formed for each tissue in both females and males, supported by the low stress values of 0.0253 and 0.0477, respectively (Figure 3.3.1a and b). Using similarity percentages (SIMPER) analysis, the main essential fatty acids (EFAs) contributing to the differences in the gonad and digestive gland (DG) in females on day 133 included ALA (9 %), LA (8 %), γ-LA (7 %), EPA (6 %) and EDA (5 %), while the gonad and DG in males were separated by LA (10 %), ARA (9 %), γ-LA (8 %) and ALA (6 %), with ARA only contributing to the males.

The EFAs that contributed to the differences in female and male DG and meat clusters on day 133 were similar and included LA (9 %), γ-LA (Females 7 %, males 8 %), ALA (Females 6 %, males 7 %) and EPA (6 %) (Figure 3.3.1a and b). The EFA that contributed to the distinct clusters in both female and male gonad and meat samples were EDA (Female, 7 %, male 9 %), EPA (Female 7 %, male 11 %) and γ-LA (6 %), while LA (6 %) only contributed to the separation of gonad and meat in females and ALA (7%) only contributed to the separation in males (Figure 3.3.1a and b).

Similarly, the tissues of female (ANOSIM R = 0.501, p = 0.001; Figure 3.3.1c) and male (ANOSIM R = 0.808, p = 0.001; Figure 3.3.1d) abalone at day 315 had statistically significant different fatty acid profiles. Three distinct clusters were formed for each tissue in both females (Stress = 0.0431) and males (Stress = 0.0354). Similar EFA contributed to the separation of DG and gonad clusters in female and male abalone and these included LA (9 %), ARA

(Females 8 %, males 9 %), γ-LA (7 %) and ALA (7 %), while EPA (8 %) only contributed to the separation in female DG and gonad clusters. Similar EFAs that contributed to the separation in DG and meat clusters in females and males and these include LA (females 8 %, males 9 %), γ-LA (8 %), ALA (females 6 %, males 7 %), EPA (females 8%, males 7 %) and ARA (6 %) (Figure 3.3.1c and d). Two EFAs contributed to the separation in meat and gonad clusters in both sexes and that includes ARA (females 9 %, males 12 %) and LA (7 %), while EPA (7 %) and ALA (6 %) only contributed to the cluster separation in females on day 315 (Figure 3.3.1c and d).

The fatty acid profiles of female and male gonads on day 133 were significantly different from each other (ANOSIM R = 0.441, p = 0.001) and day 315 (ANOSIM R = 0.385, p = 0.001). Two distinct clusters were recorded for the two sexes on both days. Female and male gonads on day 133 (Stress = 0.0528) were separated by the EFAs ARA, (8 %) γ-LA (6 %) and LA (6 %) (Figure 3.3.1e), while the EFAs that contributed to the separation on day 315 (Stress = 0.0751) included EPA (10 %) and ARA (7 %) (Figure 3.3.1f).

The gonads of female (ANOSIM R = 0.697, p = 0.001) and male (ANOSIM R = 0.879, p = 0.001) abalone between day 133 and 315 had significant different fatty acid profiles. Two distinct clusters were formed for the two separate days in both females (Stress = 0.0189) and males (Stress = 0.0299; Figure 3.3.g and h). The EFAs that contributed to the cluster separation in female gonads include EPA (7 %), ARA (7 %), LA (6 %) and γ-LA (6%) (Figure 3.3.1g), while the two days were separated by EPA (7 %), ALA (6 %) and EDA (6 %) in male abalone (Figure 3.3.1h).

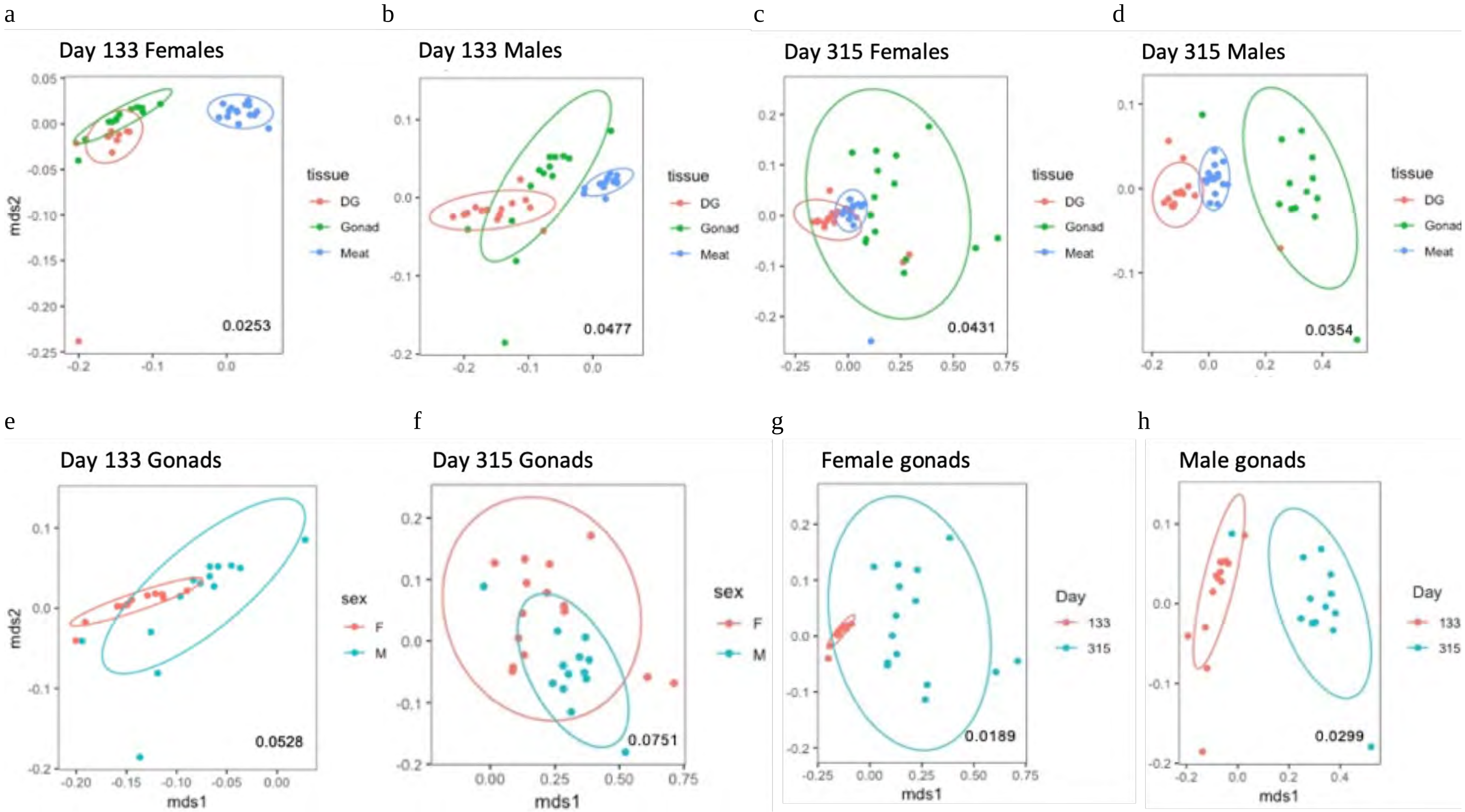


Figure 3.3.1: Nonmetric multidimensional scaling (NMDS) plot based on the Bray-Curtis distances of fatty acid methyl esters in *Haliotis midae* organs, separated by day and sex. Coloured lines surrounding each sample type represent covariance ellipsoids.

Fatty acid concentrations of abalone tissue based on SIMPER analysis

At day 133 EPA and LA were markedly higher in the DG and gonads of both female and male abalone (Figure 3.3.2a and d) compared to day 315, with LA being the highest in the DG of females (1670.37 ± 504.31 µg g^{-1}, Figure 3.3.2a) and males (2131.40 ± 258.48 µg g^{-1}, Figure 3.3.2d) compared to the gonad (462.32 ± 115.66 and 245.73 ± 87.16 µg g^{-1}, respectively). Compared to the meat tissue, the respective EFAs that most contributed to the NMDS plots were higher in the DG and gonad in both sexes at day 133 compared to day 315 (Figure 3.3.2b, c, e and f). Arachidonic acid was highest in the DG (236.23 ± 28.85 µg g^{-1}), followed by the gonad (35.03 ± 18.92 µg g^{-1}) and meat (8.45 ± 2.26 µg g^{-1}) in male abalone while it was not a main contributing EFA in females (Figure 3.3.2d, e, f).

The mean concentrations of the respective EFAs were all lower at day 315 compared to day 133. At day 315 ARA, EPA and LA were notably higher in the tissues of male and female abalone, compared to the other contributing EFAs (Figure 3.3.3). For both females and males, ARA was highest in the DG (157.62 ± 21.88 µg g^{-1} and 193.47 ± 20.03 µg g^{-1}, respectively), followed by the meat (45.25 ± 5.20 µg g^{-1} and 44.98 ± 2.66 µg g^{-1}, respectively) and gonad tissue (5.87 ± 1.25 and 2.09 ± 0.79 µg g^{-1}, respectively; Figure 3.3.3). Similarly, for females and males, LA was highest in the DG (757.86 ± 145.14 and 721.08 ± 123.14 µg g^{-1}, respectively), meat (66.80 ±10.64 and 53.41 ±7.67 µg g^{-1}, respectively) and then the gonad (11.93 ± 2.12 and 6.87 ± 1.76 µg g^{-1}, respectively; Figure 3.3.3). Eicosapentaenoic acid in the tissues of female abalone also followed this pattern, with a mean concentration of 648.01 ± 125.66, 85.34 ± 10.08 and 27.35 ± 5.38 µg g^{-1} for the DG, meat and gonad, respectively (Figure 3.3.3a, b, c).

The main EFAs contributing to the separation in clusters of female and male gonads on day 133 were ARA, γ-LA and LA with higher concentrations in females (173.94 ± 20.44, 107.05 ± 38.74 and 462.32 ± 94.14 μg g^{-1}, respectively) compared to males (35.03 ± 12.63, 57.72 ± 33.96 and 245.73 ± 107.08 μg g^{-1}, respectively). Two EFAs contributed to the variation in female and male gonads on day 315 and these included ARA and EPA (Figure 3.3.4b). Eicosapentaenoic acid was higher in females (5.78 ± 1.25 μg g^{-1}) compared to males (2.09 ± 0.79 08 μg g^{-1}), while EPA was higher in males (96.89 ± 45.96 μg g^{-1}) than in females (27.35 ± 5.38 μg g^{-1}; Figure 3.3.4b).

In the female gonads, the main EFAs that contributed to the variation between the two sampling days were ARA, EPA, γ-LA and LA, as all EFAs were higher on day 133 (172.94 ± 20.44, 1046.22 ± 115.14, 107.05 ± 38.74 and 462.32 ± 94.14 μg g^{-1}, respectively) compared to day 315 (5.78 ± 1.25, 27.35 ± 5.38, 3.43 ± 0.90 and 11.93 ± 2.12 μg g^{-1}, respectively; Figure 3.3.4c). For the male abalone gonads, the main EFAs included EPA, EDA and ALA and the concentration of these EFAs were also higher on day 133 (1307.89 ± 151.52, 60.86 ± 19.13 and 73.54 ± 40.97 μg g^{-1}, respectively) compared to day 315 (96.89 ± 45.96, 3.94 ± 1.85 and 0.99 ± 0.45 μg g^{-1}, respectively Figure 3.3.4d).

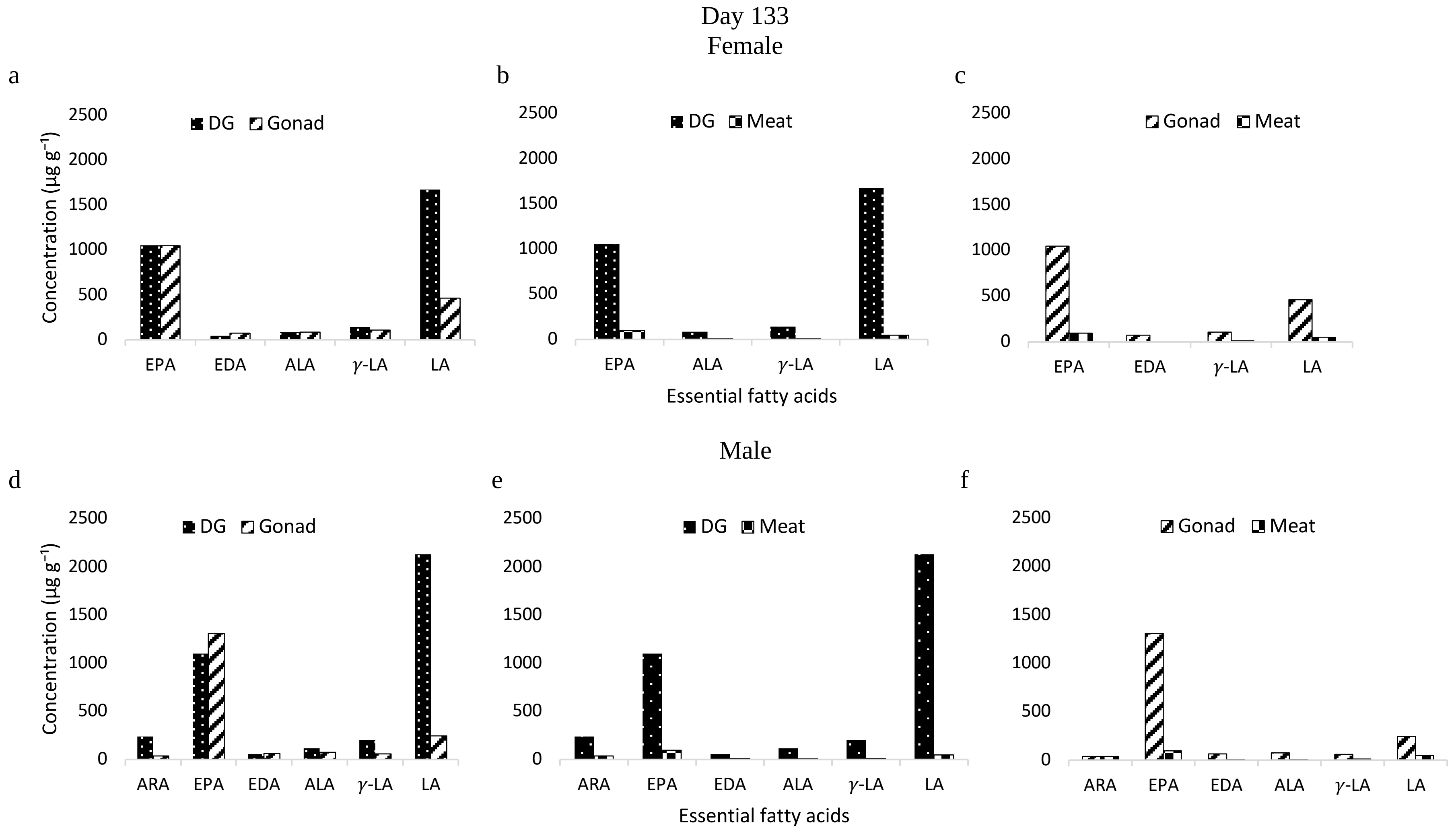


Figure 3.3.2: Mean (± SE) concentration of essential fatty acids (µg g^{-1}; EFAs) in the digestive gland (DG), gonad and meat samples of female *Haliotis midae* at day 133. Each graph includes the EFAs that most contribute to the separation of NMDS clusters of the respective tissues. EPA, eicosapentaenoic acid; EDA, eicosadienoic acid; ALA, α-linolenic acid; γ-LA, γ-linolenic acid; LA, linoleic acid; ARA, arachidonic acid.

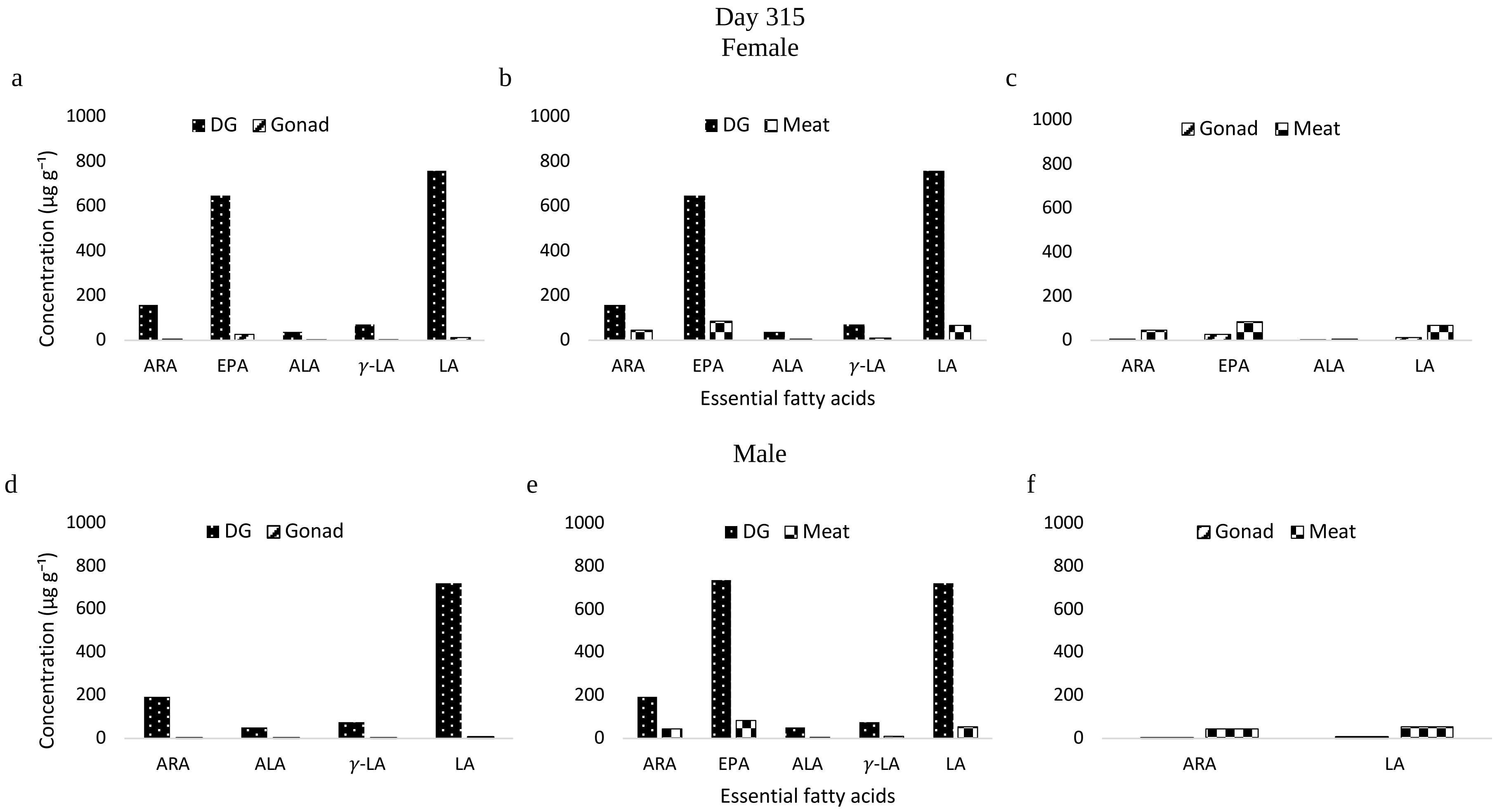


Figure 3.3.3: Mean (± SE) concentration of essential fatty acids (µg g^{-1}; EFAs) in the digestive gland (DG), gonad and meat samples of female *Haliotis midae* at day 315. Each graph includes the EFAs that most contribute to the separation of NMDS clusters of the respective tissues. ARA, arachidonic acid; EPA, eicosapentaenoic acid; ALA, α-linolenic acid; γ-LA, γ-linolenic acid; LA, linoleic acid.

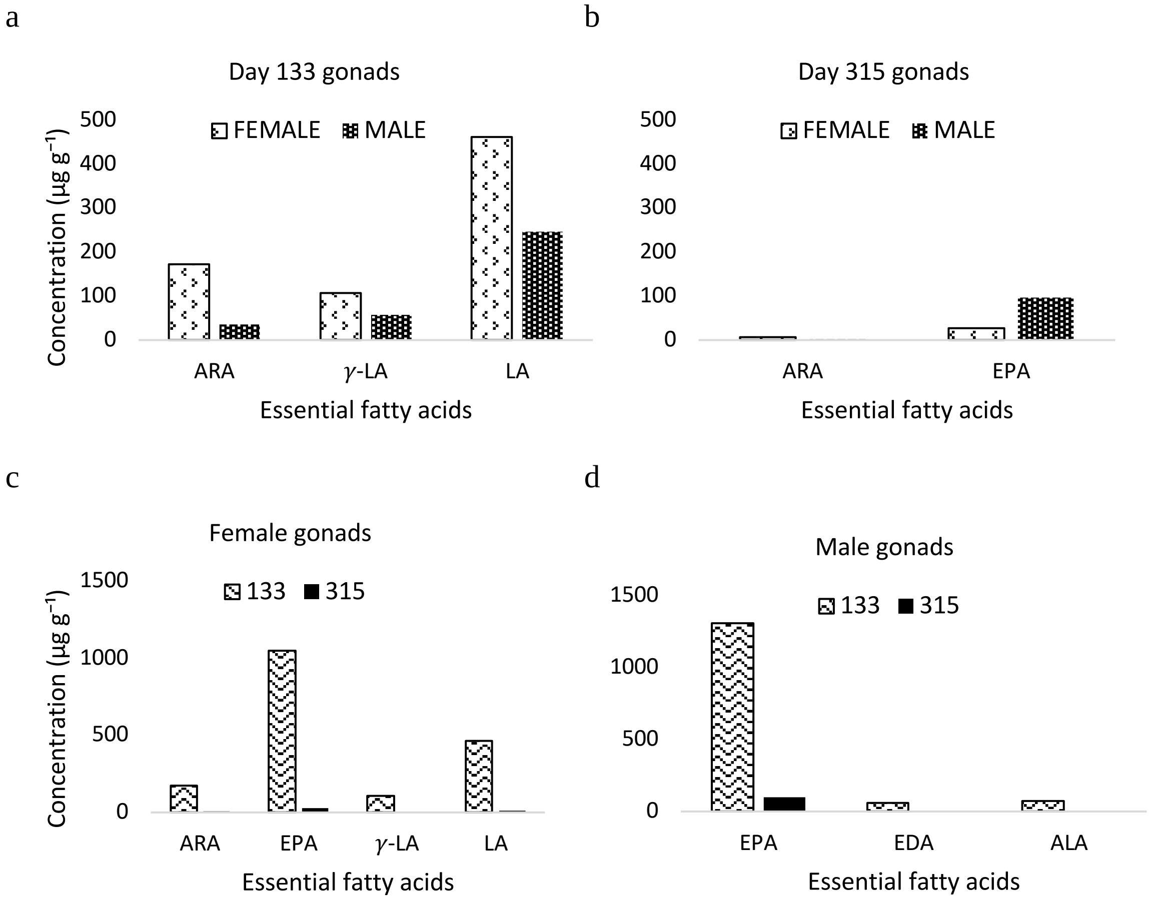


Figure 3.3.4: Mean (± SE) concentration of essential fatty acids (µg g^{-1}) in male and female *Haliotis midae* gonad tissue on day 133 and 315. Essential fatty acids in graphs depict those that most contribute to the separation of NMDS clusters of the respective sexes and sampling days. ARA, arachidonic acid; γ-linolenic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; EDA, eicosadienoic acid.

Tissue fatty acid composition

The overall pattern was a decrease in the proportion of SFA in the DG and an increase in the gonad between the two sampling days. The proportion of MUFA decreased from day 133 to 315 in all tissues for both sexes. For the PUFAs, the overall pattern in the DG was an increase from day 133 to 315 and a decrease in the gonads between the two days. The proportions of SFA, MUFA and PUFA remained relatively similar in the meat samples between the two sampling times (Table 3.3.3).

The most abundant SFA and MUFA in all tissues was PA and OA, respectively at both sampling times for both females and males (Table 3.3.3). The most abundant PUFA in both gonad and meat samples were EPA, while the most abundant PUFA in the DG was LA for both females and males at both sampling times (Table 3.3.3).

Seasonal water temperatures

The mean sea water temperature measured in the header tank between seasons were similar, ranging from 15.45 to 16.65 °C (Table 3.3.4). The sea water temperature range was the smallest during winter, followed by spring, autumn and lastly summer with the largest temperature range (Table 3.3.4).

Table 3.3.4: Mean (± SD), minimum and maximum seasonal sea water temperature from the header tank.

	Sea water temperature (°C)		
Season	Mean (±SD)	Minimum	Maximum
Spring	16.31 ± 1.55	12.20	19.82
Summer	15.45 ± 2.07	10.78	22.95
Autumn	15.94 ± 1.88	11.23	20.34
Winter	16.65 ± 0.32	13.98	18.03

Table 3.3.3: Mean (± SE) of fatty acid (FA) composition (% total FAs) of female and male *Haliotis midae* digestive gland (DG), gonad and meat samples from day 133 and day 315. nd, not detected.

	Female						Male					
	Day 133			Day 315			Day 133			Day 315		
FA	DG (%)	Gonad (%)	Meat (%)	DG (%)	Gonad (%)	Meat (%)	DG (%)	Gonad (%)	Meat (%)	DG (%)	Gonad (%)	Meat (%)
C13:0	0.66±0.07	0.44±0.09	2.4±0.16	3.61±2.23	17.95±8.45	6.08±3.24	0.64±0.07	0.42±0.11	2.29±0.13	2.48±1.61	16.26±4.66	2.72±0.22
C14:0	4.27±0.27	6.72±0.32	7.01±0.32	3.56±0.32	11±1.28	6.8±0.53	3.23±0.32	1.89±0.4	7.09±0.25	2.94±0.28	2.66±0.29	7.45±0.33
C15:0	1.29±0.16	0.93±0.17	2.5±0.09	1.08±0.13	nd	2.34±0.20	1.37±0.16	1.44±0.22	2.54±0.06	1.46±0.13	0.1±0.10	2.42±0.09
C16:0	11.84±2.25	9.66±0.20	15.48±0.25	6.96±0.32	17.94±2.39	14.69±0.75	8.95±0.82	12.47±1.86	15.71±0.25	6.83±0.24	16.21±1.45	15.4±0.35
C18:0	2.66±0.83	3.13±1.24	2.79±0.37	0.49±0.15	2.72±1.15	2.63±0.33	11.72±5.45	4±1.12	2.77±0.31	2.34±0.49	1.38±0.33	3.3±0.45
SFA	**20.74±1.96**	**20.91±1.27**	**30.21±0.49**	**15.68±2.39**	**42.78±3.29**	**32.57±1.98**	**25.92±4.68**	**20.24±2.37**	**30.41±0.63**	**16.05±1.58**	**36.62±3.3**	**31.3±0.60**
C14:1	0.65±0.12	1.98±0.19	nd	0.45±0.08	0.46±0.11	0.01±0.01	0.53±0.13	0.07±0.03	nd	0.26±0.07	0.20±0.20	nd
C16:1	4.11±0.45	11.64±0.62	4.47±0.21	2.94±0.28	5.33±0.61	3.54±0.32	2.49±0.26	1.4±0.30	4.32±0.18	2.39±0.17	0.11±0.05	3.66±0.23
C17:1	4.08±0.93	3.68±0.33	3.22±0.22	2.05±0.26	nd	2.7±0.43	2.9±0.59	5.51±0.71	3.25±0.22	2.78±0.27	0.38±0.38	2.11±0.20
C18:1n9c	9.28±2.01	15.98±0.74	9.65±0.22	6.05±0.22	16.52±1.18	8.87±0.31	5.72±0.4	5.05±0.61	9.6±0.15	5.16±0.17	3.94±0.32	9.07±0.27
C20:1	3.34±0.42	2.54±0.38	1.89±0.15	2.78±0.22	0.63±0.1	1.89±0.25	3.5±0.33	2.16±0.49	1.82±0.18	3.37±0.26	0.27±0.09	1.79±0.22
MUFA	**21.48±2.58**	**35.84±1.16**	**19.24±0.41**	**14.28±0.69**	**22.97±1.77**	**17.02±0.52**	**15.16±1.25**	**14.2±1.61**	**19±0.56**	**13.98±0.55**	**4.68±0.64**	**16.64±0.55**
C18:2n6c	23.17±2.91	8.53±0.91	10.81±0.95	27.92±3.13	8.74±1.94	12.69±1.51	27.4±3.07	4.4±0.77	11.08±1.11	24.35±2.98	4.63±0.47	11.73±1.37
C18:3n6	2.27±0.34	1.73±0.24	1.79±0.12	2.65±0.18	2.06±0.36	1.75±0.21	2.54±0.24	1.24±0.29	1.8±0.11	2.58±0.19	1.17±0.21	1.89±0.16
C18:3n3	1.33±0.25	0.9±0.35	1.17±0.12	1.2±0.15	0.66±0.26	1.19±0.12	1.4±0.27	1.4±0.38	1.2±0.07	1.63±0.16	0.51±0.06	1.3±0.04
C20:4n6	4.22±0.56	3.58±0.20	8.28±0.29	6.77±10	2.94±0.28	9.15±0.71	4.35±0.64	1.02±0.13	8.4±0.33	7.48±0.86	1.15±0.11	10.13±0.32
C20:2n6	0.68±0.08	1.42±0.20	1.15±0.12	0.59±0.06	1.82±0.33	1.3±0.26	0.76±0.06	2.07±0.40	1.5±0.12	0.66±0.07	1.77±0.39	1.32±0.24
C20:5n3	17.59±2.47	22.33±1.62	21.42±0.86	22.39±2.36	12.51±1.11	17.71±1.65	15.47±20	50.1±5.05	21.51±0.81	25.3±2.52	44.73±3.15	19.01±0.92
C22:6n3	8.48±0.69	4.72±0.61	5.88±0.3	8.47±1.05	5.48±0.73	6.56±0.42	6.97±0.95	5.27±0.68	5.05±0.27	7.93±1.08	4.69±0.59	6.62±0.33
PUFA	**57.76±4.39**	**43.24±1.09**	**50.53±0.63**	**70.02±1.94**	**34.24±3.42**	**50.39±1.91**	**58.9±3.82**	**65.54±3.90**	**50.58±0.75**	**69.95±1.16**	**58.69±2.89**	**52.04±0.93**
n-6 (μg g^{-1})	**2096.59±296.12**	**813.64±154.67**	**98.15±6.25**	**1000.99±174.61**	**25.13±5.37**	**127.36±15.14**	**2624.7±508.96**	**399.33±156.41**	**98.4±9.13**	**1008.47±148.69**	**14.85±4.98**	**112.99±11.34**
n-3 (μg g^{-1})	**1925.46±264.16**	**1419.61±247.72**	**126.03±5.78**	**928.63±187.05**	**39.42±7.76**	**123.33±12.72**	**1780.48±354.08**	**1644.71±254.59**	**119.35±9.6**	**1020.36±157.48**	**106.27±49.03**	**119.13±7.55**
n-6/n-3	**1.31±0.33**	**0.59±0.08**	**0.79±0.05**	**2.13±0.76**	**0.96±0.19**	**1.3±0.29**	**2.3±0.62**	**0.19±0.04**	**0.85±0.08**	**1.49±0.44**	**0.2±0.04**	**1±0.12**
TFA (μg g^{-1})	**9215.48±3165.38**	**5295.03±989.68**	**444.34±19.67**	**2704.7±476.62**	**216.39±37.47**	**502.73±40.13**	**9061.86±2406.27**	**3772.2±1000.55**	**428.36±28.06**	**2875.29±382.32**	**190.61±73.45**	**445.55±24.32**

3.4 DISCUSSION

In aquaculture, the protein component of compound feeds is one of the most important and expensive aspects (Tung and Alfaro, 2011). However, formulated feed not only improves growth rates compared to that seen in wild abalone, but it can be manipulated to yield desired outcomes. Haliotids are unable to synthesize all fatty acids required for growth and regular cellular function and rely on their diet to fulfil their requirements (Uki *et al*., 1986; Grubert *et al*., 2004). There is increasing evidence suggesting that lipid and fatty acids play an important role in gonadogenesis in abalone (Webber, 1970; Uki *et al.*, 1986; Uki and Watanabe, 1992; Bautista-Teruel *et al*., 2001; Nelson *et al.*, 2002; Roussel *et al.*, 2019). Polyunsaturated fatty acids (PUFA) are the biggest group of highly unsaturated fatty acids (HUFA), with n-3 and n-6 fatty acids being the most studied. Polyunsaturated fatty acids including arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), eicosadienoic acid (EDA), α-linolenic acid (ALA), γ-linolenic acid (γ-LA) and linoleic acid (LA) are essential for abalone and play a crucial role in metabolism (Floreto *et al.*,1996; Mai *et al.*, 1996; Nelson *et al*., 2002; Hu *et al*., 2009; Bautista-Teruel *et al.*, 2011; Mulvaney *et al.*, 2015). The relative importance of these essential fatty acids (EFAs) to growth have also varied between species. For example, LA and ALA are important for *Haliotis discus hannai* and *Haliotis asinina* (Mai *et al*., 1996; Bautista-Teruel *et al.*, 2011), while ARA and EPA and important for *H. discus hannai* and *Haltios fulgens* (Floreto *et al*., 1996; Mai *et al*., 1996; Nelson *et al.*, 2002; Durazo-Beltrán *et al*., 2004) and DHA is important for *Haliotis tuberculata* and *H. asinina* (Mai *et al*., 1996; Bautista-Teruel *et al*., 2001). Gamete production is energy expensive (Ault, 1985) and since abalone are broadcast spawners, investments into reproductive tissue is high. Understanding how different dietary protein sources affect the allocation of nutrients, particularly essential fatty acids during periods of enhanced reproduction, such as spawning peaks and periods of low reproductive investments, feed formulations could potentially be adapted to either: (1) improve somatic growth during these periods by providing the essential fatty acids that benefit muscle tissue growth; or (2) improve reproductive performance by providing essential fatty acids during both spawning cycles and periods of reduced reproductive investment.

The aim of this study was to quantify the effects of dietary protein sources on the allocation of essential fatty acids in tissues related to somatic growth, nutrient storage and reproduction, by determining the changes in EFAs in muscle tissue, digestive gland and gonad tissues at known periods of increased reproductive investments and periods of least reproductive investment. This was achieved by measuring the essential fatty acids in the respective tissues when the gonad bulk index values were the highest and the lowest (Chapter 2) and by detecting which EFAs were contributing to the differences in fatty acid profiles between the tissues and between the two sampling times for each sex.

Polyunsaturated fatty acids

Highly unsaturated fatty acids are essential for normal bodily functions. The most intensively studied PUFAs include DHA, EPA and ARA (Hu *et al.*, 2009). While only EPA and ARA were identified as contributors to the non-metric multidimensional scaling (NMDS) ordinations, a number of other EFAs were identified as important contributors to the distinct cluster for each tissue, sex and day and these included EDA, ALA, LA and γ-LA. Interestingly, EDA and ALA were not detected in any of the dietary treatments. Floreto *et al*. (1996) found a high presence of PUFAs 16:4n3, EPA and docosapentaenoic acid (22:5n3; DPA) in Japanese disc abalone, despite being undetected or very low in the dietary treatment, thereby suggesting their synthesis from their precursors that were available in the diet. Wild Australian abalone, *Haliotis rubra,* had significantly higher n-6 PUFA concentrations compared to farmed abalone, despite the fact that their natural diet of macroalgae contained a lower lipid content than formulated feed. It is however, unclear if this is due to a higher lipid intake or because of the greater bioavailability of the lipids (Su *et al*., 2004). Li *et al.* (2013) found the presence of long chain PUFAs (LC-PUFA) in the muscle of *H. discus hannai* that were fed diets absent in LC-PUFA, also suggesting their capacity for converting dietary C18 precursors to LC-PUFA and retaining these in the tissue. Mollusks have also been observed to accumulate C20 and C22 PUFAs in the gonads by the end of the gamete maturation process (De Moreno *et al.*, 1980). Evidence suggests that abalone are capable of synthesizing LC-PUFAs from short chain precursors, such as converting LA to ARA and ALA to EPA in order to meet physiological needs (Nelson *et al*., 2002; Durazo-Beltrán *et al.*, 2003a, 2003b). Arachidonic acid was not detected in the S-only diet, but the high abundance of LA in the diet might explain the presence of ARA in the tissues of animals fed the S-only diet. Eicosapentaenoic acid was also not detected in the S-only diet. The precursors for EPA is ALA, which was not detected any of the dietary treatments, but present in the tissue of all abalone, suggesting that abalone may have attained EFAs from other food sources within the system, such as diatoms where the primary storage material in diatoms is lipid (Dunstan *et al.*, 1994). It is possible that animals fed S-only were consuming diatoms with ALA and converting it to EPA. Furthermore, EDA was absent in the dietary treatments, but was present in the tissues of all abalone, supporting the importance of naturally occurring diatoms in the diet of farmed *H. midae*. The presence of EDA may also suggest that *H. midae* may have the ability to convert this LC-PUFA from its C18 precursor, LA, but this remains to be tested. Although the study was a feeding study, it was conducted on a commercial abalone farm, following stringent farm protocols and therefore physically removing diatoms by scrubbing surfaces would not only result in stress for the animals, but would not be a realistic setting of a farm-scale trial.

Dietary treatment had no influence on the fatty acid profile of abalone tissue in the present study. This means that whether abalone were fed a diet comprising of only one protein source, either fish or plant-based, or a combination of the two, their FA content in the tissues were similar. Although the concentration of PUFAs were higher in the combination diets (fishmeal with plant protein), the proportion of PUFAs as a percentage of total FAs were similar for all four treatments (69 – 73 %). However, n-3 PUFAs were completely absent in the S-only diet and the high proportion of PUFAs were due to the high abundance of LA (68 %). This suggests that excess EFAs, such as LA in the S-diet, hold no additional value. The commercial abalone industry in South Africa rely on a superior, fishmeal and soya-based diet, but although the combination of the two provide more EFAs, these do not appear to be deposited in the tissues. When EFAs were accumulated, they were similar among treatments, suggesting abalone preferentially accumulate EFAs for reproductive purposes independent of the dietary source. This study suggested the possible importance of diatoms as a source of EFAs for farmed *H. midae*. It is important to note that prior to the study, all abalone were fed a fishmeal and soya combination diet, but in the absence of dietary intake specifically from formulated feed, would have been depleted in the tissues. Therefore the only explanation for the presence of EFAs in the tissues of abalone fed S-only were from another nutrient source, such as diatoms. Starved abalone have been reported to utilize protein and carbohydrates instead of fatty acids (Durazo-Beltrán *et al.*, 2004) and since abalone in the present study were not starved and fed to satiation, the presence of EFAs from the previous diet could explain their occurrence. Furthermore, FA analysis was not carried out at day zero before the experimental diets were introduced in this study. Despite this, changes in EFAs between tissues and sex of the abalone were observed in this study.

Differences in tissue EFAs during highest vs lowest gonad volume

Fatty acid composition varied among tissues, suggesting specific roles of different FAs in organisms (Hu *et al.*, 2009). Elevated levels of specific EFAs were found and this was dependent on time of sampling, tissue and sex of the abalone. The digestive gland (DG) and gonads of female and male abalone had markedly elevated levels of EFAs compared to the meat at day 133, coinciding with the period of largest gonad volume relative to the soft tissue of the abalone. The EPA concentrations were similar between DG and gonad, while LA was markedly higher in the DG compared to the gonads in both sexes. Interestingly, ARA was only a contributing FA in the ordination plots in male abalone with high concentrations in the DG. Elevated levels of ARA have been observed in the DG of female Jade Tiger hybrid (*Haliotis laevigata X Haliotis rubra*) abalone in spring, coinciding with peak gonadosomatic index (GSI) values (Mateos *et al.*, 2010). Fatty acids are stored in the form of lipids in the body and reservoirs for these lipids include organs such as the liver, muscle and gonads (Hu *et al.*, 2009). In abalone, the DG is the most important tissue in terms of metabolic participations where lipids are hydrolysed and from there they are transferred to other organs (Soudant *et al.*, 1996), while the foot stores carbohydrates (Mercer *et al.*, 1993). Gametogenesis is an energy and nutrient consuming process and therefore nutrient supply is crucial for normal gamete generation, directly affecting reproduction (Hu *et al.*, 2009). The accumulation of these EFAs in the gonads as they mature may be vital in reproduction (Mercer *et al.*, 1993). The supplementation of ARA in the diets of Atlantic halibut (*Hippoglossus hippoglossus*) and haddock (*Melanogrammus aeglefinus*) has shown improvements in fertilisation, hatching rates and larval quality (Mazorra, 2000; Castell *et al.*, 2003). Changes in lipid content with gonad maturity have been observed in *Haliotis varia* and *H. fulgens* (Hahn, 1989; Najmudeen, 2007). Significant increases in ARA, ALA and EPA have been observed in *H. rubra* during the maturation period, increasing from the immature stage to the ripe and partially spent stage, followed by significant decreases in the spent stage (Litaay *et al.*, 2007). These EFAs may be highly concentrated in the DG and gonads due to their high biological activity in reproductive processes.

In contrast, on day 315 when abalone were least reproductively active, meat samples had higher concentrations of the contributing EFAs compared to the gonad, i.e., ARA, EPA, ALA and LA in females and ARA and LA in males. These differences between meat and gonad were due to the large decrease in EFA concentrations in the gonad at day 315, coinciding with the lowest GBI values. Both the concentration and proportion of EFAs in the meat samples remained similar between both sampling times, since the foot muscle is the carbohydrate store (Mercer *et al*., 1993). Mateos *et al*. (2010) found that the gonad and DG of the Jade Tiger hybrid abalone had higher concentrations of all the main fatty acids throughout the year compared to the muscle tissue. The total lipids of the viscera in *H. discus hannai* and *Haliotis diversicolor* have also been observed to be higher than that of the muscle tissue (Lou *et al*., 2013). These authors also observed the muscle tissue comprised mainly of n-3 (EPA and DPA), while the gonad and DG comprised of n-6 fatty acids, namely LA and ARA (Mateos *et al*., 2010). In addition, Nelson *et al.* (2002) also found that the hepatopancreas/gonad of *H. fulgens* had a higher amount of fatty acid than the foot muscle throughout the year. Nelson *et al.* (2002) found that the seasonal changes of lipid composition in the foot were reflecting that of the macroalgal diets and the temporal variations in the tissue PUFA were attributed to growth and gonadal development. Most lipid in the meat of abalone is present in the cellular membrane as polar lipids, with most being stored in the viscera (Uki *et al*., 1986). Glycogen is the main storage compound in abalone meat (Webber, 1970), but lipids have been shown to accumulate in the meat when fed a diet high in lipids (Dunstan *et al*., 1996). However, all contributing EFAs to the NMDS ordinations showed very little change in the concentrations in the meat samples between the two sampling days, further supporting the basis that changes seen between the DG and gonad were nutrient allocations linked to reproductive development. This suggests that EFAs in the foot muscle are maintained and not influenced by reproductive development.

Although gametogenesis in females and males is very different based on their structure and size, lipids are important for both, with females accumulating lipid reserves for the egg yolk and males incorporating lipids into their sperm plasma membranes (Najmudeen, 2007; Hu *et al*., 2009). At day 133, the essential fatty acids ARA, γ-LA and LA were influential in the separation in female and male gonads, with concentrations being up to five times higher in females than in males. However, at day 315, the only constant contributor to female and male abalone gonads was ARA. Although with overall concentrations much lower than on day 133, females had three times more ARA than males. Grubert *et al*. (2004) found that ARA was higher in the foot of abalone than in the feed, suggesting a preferential accumulation or the synthesis of ARA from C18 precursors. D'Souza and Loneragan (1999) identified ARA as an EFA for penaeid larval development and is considered essential for growth and reproductive quality of sperm and eggs (Bell and Sargent, 2003). In addition, EPA was influential in the gonad ordination, with males having three and a half times more EPA than females. Grubert *et al*. (2004) found that the testis had approximately 10 times more EPA than the ovary in blacklip and greenlip abalone. Sea urchins were able to accumulate ARA in their gonad tissues during maturation (Cook *et al*., 2007). Fatty acids provide structural and energetic material during reproduction (Durazo-Beltrán *et al*., 2003a; Hu *et al*., 2009). At day 133, the concentrations of the contributing EFAs were higher in gonad tissue of females than in the diets, showing the preferential accumulation of these EFAs and their importance in reproduction, while at day 315 concentrations of the important EFAs were lower than those in the diets. There was an overall decrease in the EFAs that contributed to the gonad ordination plots between day 133 and 315 and this was likely linked to the reproductive cycle, with concentrations dropping between approximately four to seventy-five times between the two sampling events. For females, ARA, EPA, γ-LA and LA were important between the two sampling times, while EPA, EDA and ALA were important for males. Grubert *et al*. (2004) found that there was little variation in lipids and fatty acid composition between spent and gravid animals in both *H. rubra* and *H. laevigata*. This was likely because spent animals still retained the residual gonad tissue due to partial spawning and/or incomplete resorption of the gonad tissue. Mateos *et al*. (2010) found that there was no differences in EPA and ARA in the gonad of female Jade Tiger hybrid abalone during periods of highest and lowest GSI values, while DPA and LA were the highest during the lowest GSI values. These differences in EFA concentrations suggest the importance of these specific fatty acids when gonads are the most mature, but inconsistencies between studies suggest that EFAs linked to reproduction may be species specific. Furthermore, the particular stage of maturity, age and diet of the abalone may also attribute to the abundance of these EFAs.

Tissue fatty acid composition

The overall pattern for the percentage composition of fatty acid subtypes was a decrease in saturated fatty acids (SFA) in the DG from day 133 to 315 and an increase in the gonad, while the opposite occurred for the PUFAs, increasing in the DG and decreasing in the gonad between day 133 and 315. This inverse relationship is attributed to the digestive gland functioning as the nutrient storage organ, transferring nutrients to the gonad for reproductive purposes (Soudant *et al*., 1996; Litaay and De Silva, 2003; Najmudeen, 2007). However, there was no differences in the percentage composition of SFA of the meat tissue between the two sampling days. Day 133 of the study was in summer, while day 315 was during the winter months, suggesting the effect of water temperature on fatty acid composition as indicated by Su *et al.*

(2006) and has been observed in blacklip and greenlip abalone (Nelson *et al*., 2002; Su *et al.*, 2006). The sea water temperature range was the highest during summer (11 – 23 °C) and lowest during winter (14 – 18 °C) in the present study and these changes in water temperature may have played a role in the differences in the SFA composition. However, a study that more closely monitors the changes in SFA over time, for example weekly or monthly sampling and in line with changes in water temperature would be required. The highest concentrations of saturated fatty acids in both blacklip and greenlip abalone were observed in summer, however, reproductive development was not measured in these studies (Nelson *et al.*, 2002; Su *et al.*, 2006). Similarly, a study on Jade Tiger hybrid showed peak SFA in the foot, DG and gonad in the summer, coinciding with peak GSI (Mateos *et al*., 2010). The most abundant SFA in all tissues was palmitic acid (C16:0) for both sexes at both sampling times. Palmitic acid has also been found to be the most abundant SFA in other Haliotids (Dunstan *et al*., 1996; Grubert *et al*., 2004; Su *et al*., 2004; Mateos *et al*., 2010; Bilbao *et al*., 2012; Lou *et al*., 2013; Latuihamallo *et al*., 2015).

The monounsaturated fatty acids (MUFA) decreased from summer to winter in all tissues of both sexes in the present study, but has been observed to peak in winter and spring in blacklip and greenlip abalone (Su *et al.*, 2006). In a separate study on the muscle tissue of greenlip abalone, MUFA has been observed to peak in Autumn (Nelson *et al*., 2002). Seasonal variations in the peak total MUFA of female Jade Hybrid abalone tissues have been observed and include the foot (spring), digestive gland (spring and winter) and gonad (winter, summer and spring) (Mateos *et al*., 2010). The most abundant MUFA was oleic acid (C18:1n9c) in all tissues of abalone at both sampling times in the present study and has also been observed in wild and cultured Australian abalone (Su *et al*., 2004). Mateos *et al*. (2010) found that oleic acid was the most abundant MUFA in the DG and gonad in farmed, Jade Tiger hybrid abalone.

These inconsistencies suggest that the abundance of MUFA is species and tissue specific and that season may play a small role in MUFAs in haliotids.

In contrast to the trends we observed for SFAs, the opposite was observed for the percentage composition of PUFAs, with increasing PUFA levels between day 133 and 315 in the DG, while PUFAs decreased in the gonad between the two sampling days, illustrating the transfer of EFAs coinciding with reproduction. Therefore, PUFAs were the highest in the DG and gonads during winter and summer, respectively, coinciding with peak gonad volume during summer. However, percentage composition of PUFAs in the meat tissue remained similar between the two sampling days. Interestingly, the concentration of n-6 PUFAs increased slightly in the foot muscle from summer to winter, while n-3 PUFAs remained similar between the two seasons. High n-6 levels during winter were also observed in the Jade Tiger hybrid, however n-3 concentration also peaked during winter, which did not coincide with GSI (Mateos *et al*., 2010). This suggests that time of sampling in farmed *H. midae* may result in variations in n-3 and n-6 PUFAs although percentage composition is similar. Polyunsaturated fatty acids generally have an inverse relationship with water temperature (Nelson *et al*., 2002), which was not observed in the present study. The temporal variations in the PUFAs in the present study were likely associated with gonad development and not temperature. Inconsistencies between the present study and previous study may have been associated with the availability of EFAs in the respective diets. Polyunsaturated fatty acids have been found to be the most abundant in the foot muscle of greenlip abalone during winter (Nelson *et al*., 2002). Mateos *et al*. (2010) also observed the highest abundance of total PUFAs during winter in the foot and digestive gland of the Jade Tiger hybrid abalone; however, PUFAs were at their highest level during winter, autumn and spring in the gonad tissue, encompassing only a proportion of periods of peak GSI.

The most abundant PUFA in the gonad and meat of abalone in this study was EPA, while the most abundant PUFA in the DG was LA, followed very closely by EPA. These high abundances are likely due to their high abundance in the dietary treatments and their ability to synthesize LC-PUFAs from short-chain precursors. Grubert *et al*. (2004) found that the tissues of blacklip and greenlip abalone had fatty acid profiles that reflected that of their diets, with each tissue having elevated levels of specific EFAs. The foot, testes and ovaries of these two species had elevated levels of ARA, EPA and LA, respectively. In addition, the DG of both males and females had elevated levels of LA (Grubert *et al*., 2004). The high composition of these EFAs were likely due to their high composition in the diets. Dunstan *et al*. (1996) found that the meat of abalone fed an artificial diet had accumulated LA with elevated levels of EDA when compared to abalone that consumed an algal diet throughout their life. Predominant PUFA found in wild and cultured abalone were EPA and DPA, while ARA was also abundant in wild abalone (Su *et al*., 2004). Abalone require EPA in their diet instead of DHA with only small quantities of DHA measured in abalone tissue relative to those present in the diet (Uki *et al*., 1986; Nelson *et al*., 2002; Durazo-Beltrán *et al*., 2003a; Su *et al*., 2004; Hernández *et al*., 2013). Although DHA was not a main contributor to any of the NMDS ordinations, the proportion of DHA was higher than ARA in the DG and gonad at both sampling times. The high content of DHA in the gonad and digestive gland has also been reported in the Jade Tiger abalone (Mateos *et al*., 2010). The lower content of DHA compared to ARA and EPA in the muscle tissues in the present study have been observed in abalone (Nelson *et al*., 2002; Durazo-Beltrán *et al*., 2003a; Su *et al*., 2004; Mateos *et al*., 2010; Hernández *et al*., 2013). When dietary EPA is low, abalone are able to retroconvert DHA to form EPA (Dunstan *et al*., 1996). Mulvaney *et al*. (2015) fed Jade Tiger abalone diets of formulated feed, macro algae and a combination of both and found that the fatty acid subtypes in abalone tissue remained relatively consistent across all dietary treatments. Thus, this suggests that abalone metabolise and accumulate fatty acids from different food sources to a constant level of subtypes, despite macroalgae diets containing much lower SFA and PUFA levels. However, differences were found at a higher resolution of fatty acid profiles between treatments (Mulvaney *et al.*, 2015), as seen in the present study. Both ARA and EPA are precursors for prostaglandins, which have been found to affect reproduction in mollusks, including the abalone, *Haliotis rufescens* (Morse *et al.*, 1997). Due to the decrease in ARA concentration and lower proportion compared to EPA, this suggests that ARA is converted to prostaglandins instead of EPA in *H. midae.* This study was only based on a feeding trial, therefore further research is required.

Conclusion

Understanding the effect of dietary protein sources on the allocation of essential fatty acids in the tissues of abalone during different periods of reproductive development would be beneficial in improving the understanding of abalone nutrition and physiology.

Dietary treatment had no effect on the fatty acid composition of abalone tissues, likely due to the similarity of EFAs in the treatments, availability of diatoms within the experimental systems and perhaps the ability of *H. mid*ae to convert LC-PUFA from C18 precursors.

The remarkable decrease in the concentrations of certain EFAs between the two sampling times suggest the importance of these EFAs in reproductive development. Large differences were seen between the DG and gonads, while the most contributing EFAs that were allocated to the meat and gonad during peak reproductive development were identified. The presence of EDA and ALA in abalone tissue that were not detected in the dietary treatments also indicate their importance in *H. midae* nutrition.

The DG clearly portrayed its role as a nutrient store for fatty acids, while the allocation of these EFAs into the gonad at certain times indicate their essentiality for gonad growth. This was the first study to look at the fatty acid allocation in *H. midae* at two points of the reproductive cycle and contributes to a better understanding of *H. midae* nutrition. In addition, this study paves the way forward for the formulation of broodstock diets as elements that favour reproductive success may be critical for species that reproduce by broadcast spawning (Nelson *et al*., 2002).

CHAPTER 4

A FIRST STUDY OF THE GONAD HISTOLOGY AND SEX STEROID CONCENTRATIONS OF FARMED ABALONE FED DIFFERENT DIETARY PROTEINS

4.1 INTRODUCTION

First reports of vertebrate-type steroids in molluscs occurred in the 1950s (Hagerman *et al.*, 1957), but despite this novel discovery half a century ago, the endocrinology of molluscs is still poorly known (Fernandes *et al.*, 2011). The hormones 17β-oestradiol and testosterone are referred to as sex steroids due to their importance in reproduction and are commonly measured indicators of reproductive behaviour (Zabrzańska *et al.*, 2015). These hormones are pivotal in sexual differentiation, growth, reproduction and sexual maturation in vertebrates (Gooding and LeBlanc, 2004; Lim, 2016). Both 17β-oestradiol and testosterone levels have been measured in the tissue and haemolymph of molluscs and have been found to change, coinciding with reproduction (Carreau & Drosdowsky, 1977; Le Guellec *et al.*, 1987; Bose *et al.*, 1997; Croll and Wang, 2007; Sternberg *et al.*, 2008). In addition, concentrations of these hormones varied between species, sex and tissue type, which allows insight into the biological activity of these hormones (Giusti and Joaquim-Justo, 2013). However, their synthesis and functional role in mollusc reproduction has been controversial (Scott, 2012). One of the key arguments is that molluscs lack the enzyme involved in steroid synthesis (Scott, 2012).

The enzyme 17β-hydroxysteroid dehydrogenase belongs to a large group of multifunctional enzymes (17β-HSDs) that convert 17-keto-steroids into the active 17β-hydroxy- forms in the last steps of sex hormone biosynthesis (Adamski and Jakob, 2001). For mammals, 17β-HSDs are pivotal in steroidogenic pathways, especially in the formation of oestradiol, also known as the most potent natural oestrogen (Zhou *et al.*, 2011). The involvement of 17β-HSDs in the synthesis of testosterone and oestradiol has been identified in aquatic invertebrates such as sea urchins, prawns and oysters (Ghosh and Ray, 1993; Wasson *et al.*, 2000; Le Curieux-Belfond *et al.*, 2001). The 17β-HSDs expressed patterns that were correlated with fluctuations in oestradiol levels in corals (Tarrant *et al.*, 2003). Zhou *et al.* (2011) determined that 17β-HSD-12 plays a key role in steroidogenesis during the reproductive period in abalone, *Haliotis diversicolor supertexta*. These authors found differences in the mRNA expression of 17β-HSD-12 during the pre-spawning, mid-spawning and post-spawning phases, thus suggesting that 17β-HSD plays a potential role in the regulation of sex steroids during reproduction of this species of abalone (Zhou *et al.*, 2011).

Authors who studied steroid hormones typically coupled their measurements with fecundity-related data, such as gonad histological assessments or gonad indices in order to be able to follow the reproductive cycle in line with changes in steroid concentrations (Reis-Henriques and Coimbra, 1990; Gauthier-Clerc *et al.*, 2006; Ketata *et al.*, 2007; Liu *et al.*, 2008; Gust *et al.*, 2011; Smolarz *et al.*, 2018). The gonad tissue forms a large proportion of the visceral mass in abalone and forms a cone-like structure, enveloping the digestive gland, but this physiological feature makes it difficult to accurately separate the two organs (Tutschulte and Connell, 1981). A number of methods have been used to measure the reproductive progress of abalone, including gonad bulk index analysis which calculates the volume of gonad tissue relative to the soft tissue of abalone. However, the most accurate way of measuring maturity stages is with gonad histological evaluations (Litaay and De Silva, 2003), which are typically conducted on female abalone due to the ease in identifying the maturation process compared to males (Gurny and Mundy, 2004). Therefore, monitoring gametogenesis in both females and males will not only contribute to our understanding of gonad development in male abalone, but may be useful in extrapolating information for broodstock studies. Studying the steroid profiles coupled with other methods of reproductive measurement may help understand reproductive phases and gamete development (Ketata *et al.*, 2007). Moreover, oestrogen and androgen profiles have not yet been established in the haemolymph of *Haliotis midae*.

The two phases of oogenesis in molluscs include the generative and vegetative phase (Anderson, 1974). The generative phase is the increase in oogonal cells by mitotic multiplication, while the uptake of nutrients for the maturation of oocytes is the vegetative phase and encompasses vitellogenesis (Pipe, 1987). Therefore, different protein sources may supply abalone with different nutrients, which may affect vitellogenesis, thereby influencing reproductive output. The presence of soya in the formulated diet of farmed *H. midae* influenced reproduction by producing larger gonads as well as changing the rate of oogenesis by consistently producing more mature oocytes, leading to the hypothesis that the phytoestrogens present in soya were responsible for the changes in reproductive activity (Ayres, 2013; Riddin, 2013; Wu *et al.*, 2019). For example, Siberian sturgeon (*Acipenser baeri*) that were fed a soya-based diet had higher plasma vitellogenin levels compared to fish that were fed a fishmeal-based diet (Pelissero *et al.*, 1991). Wu *et al.* (2019) used crystalline isoflavones in fishmeal-only based diets at levels equivalent to those in diets with increasing rates of soya, but found no differences in reproduction with isoflavone inclusion. The lack of effect of crystalline isoflavones on abalone reproduction may have been attributed to a number of reasons; including the length of the study (six months), reproductive status of the animal, the bioavailability of these crystalline compounds or the combination of individual isoflavones that can either act synergistically or antagonistically (Friedman and Brandon, 2001; El-Sayed *et al.*, 2012; Wu *et al.*, 2019). Therefore, the potential hormonal effect of soya in farmed *H. midae* remains unanswered. By using a suitable alternative plant protein to soya with a similar nutritional profile, but without phytoestrogens, namely sunflower (*Helianthus annuus*) meal would be beneficial in elucidating the possible hormonal effects that soya may have on this commercially important species of abalone, *H. midae*.

The aim of this study was to determine the possible role that different dietary protein sources, namely fishmeal, soya and sunflower meal may have on the sex steroid concentrations in farmed *H. midae* and their potential link to gametogenesis. Gonad histological assessments and haemolymph hormones assays of 17β-oestradiol and testosterone in both female and male abalone will be conducted in 45-day intervals over one-year. The aim of this study was achieved by addressing the following objectives:

1) assess gametogenesis in abalone fed single-protein based diets (fishmeal-only and soya-only) and combination diets (fishmeal-soya and fishmeal-sunflower meal);
2) identify the stages of oocyte and spermatocyte development;
3) quantify the oocyte densities of abalone that were fed the respective diets;
4) quantify the frequency of maturity stages of females and males in each dietary treatment;
5) quantify the concentrations of 17β-oestradiol and testosterone in the haemolymph of female and male abalone in each dietary treatment;
6) determine the changes in sex steroids over time; and
7) determine the relationship between the two hormones for each sex within each dietary treatment.

4.2 MATERIALS AND METHODS

Experimental system and animals

The data in this experiment were collected from the same abalone and the same production system that were described in Chapter 2.

Dietary treatments

The same dietary treatments that were introduced in Chapter 2 were used in this experiment (Table 2.2.1). That is, the experimental animals were fed one of four dietary treatments, formulated to contain 30 % crude protein, 6 % lipid and 15.77 - 17.43 MJ kg^{-1} energy. Each treatment included four replicate tanks containing six baskets each. Two diets contained either fishmeal (FM) or soya (S) as the main protein source. A third diet contained a combination of both fishmeal and soya (FM S) and the fourth diet comprised of fishmeal and dehulled sunflower meal (FM SM) as the main protein source (Table 2.2.1, Chapter 2).

Abalone sampling protocol

As described in Chapter 2, sampling occurred at 45-day intervals and data were collected over two days. On both days, two males and two females were randomly collected from each replicate tank and held in labelled mesh bags. These bags were carried in a crate and immediately transported to SPP Canning (Pty) Ltd, Hermanus. Upon arrival, animals were housed in a chilling room at approximately 10 °C. Abalone were removed and placed in numbered 350 ml plastic tubs. On the first day, haemolymph samples were collected (see *Haemolymph collection* below), prior to the abalone being shucked for data that were collected and reported on in Chapters 2 and 3. Whole abalone viscera from animals that were sampled and shucked on day-2 were then stored in 40 ml plastic containers, containing 10 % buffered formalin, prior to histological slide preparation (see *Gonad histological slide preparation* below).

Haemolymph collection

Haemolymph was obtained from the pedal sinus in the middle of the foot with a sterile 25-gauge needle attached to a 3 ml syringe and introduced into 1.5 ml Eppendorf tubes (Hooper *et al*., 2014). A new needle and syringe were used for each sample. Only samples which required less than three insertions of the needle were used. Samples were frozen at -20 °C until further analysis of oestradiol and testosterone was done using enzyme linked immunosorbent assay (ELISA).

Oestradiol ELISA

The haemolymph oestradiol concentrations were analysed using a commercial ELISA kit with a detection range of 0 – 1000 pg ml^{-1} (Diagnostic Automation/Cortez Diagnostics Incorporated, Calabasas, California, USA). Once the haemolymph samples were thawed for approximately one hour and centrifuged (13 x g for 10 min), 25 µl of the haemolymph, standard and control were pipetted in duplicate into separate wells of a pre-coated 96-well microplate used for microtiter reading. One hundred microlitres of the Oestradiol-HRP conjugate reagent was dispensed into each well, followed by 50 µl of rabbit anti-Oestradiol reagent per manufacturer instructions. They were then mixed thoroughly for 30 s and incubated at room temperature (approximately 20 °C) for 90 min. The mixture was then removed by flicking the contents out and the micro-wells, which were then rinsed with distilled water five times, ensuring that the excess water was removed by placing the wells upside down on absorbent paper each time. One hundred microlitres of the TMB reagent were then pipetted into each well, gently mixed for 10 s and then incubated at room temperature for 20 min. The reactions were then stopped by adding 100 μl of stop solution (hydrochloric acid) into each well, followed by gently mixing for 20 s ensuring that all the blue in the wells turned yellow per manufacturer instructions. The absorbance was read at 450 nm with a microtiter well reader (SpectraMax® M2 Microplate Reader, Molecular Devices, San Jose, California, USA) within 15 min.

Testosterone ELISA

The haemolymph testosterone concentrations were analysed using a commercial ELISA kit with a detection range of 0 – 18 000 pg ml^{-1} (Diagnostic Automation/Cortez Diagnostics Incorporated, Calabasas, California, USA). After thawing for approximately one hour and centrifuging at 13 x g for 10 min, 10 μl of the sample, standard and control was dispensed in duplicate into the pre-coated micro-wells as per manufacturer instructions. This was followed by 100 μl of Testosterone-HRP conjugate reagent into each well. After this, 50 μl of rabbit anti-Testosterone reagent were pipetted into each well, thoroughly mixed for 30 s and incubated at 37 °C for 90 min. The mixture was then removed by flicking the contents out and the micro-wells, which were then rinsed with distilled water five times. Excess water was removed by placing the wells upside down on absorbent paper between each rinse. This was followed by 100 μl of TMB reagent which was dispensed into each well, gently mixed for five seconds and incubated at room temperature (approximately 20 °C) for 20 min. The reaction was stopped by adding 100 μl of stop solution (hydrochloric acid) into each well and gently mixed for 30 s, ensuring the blue coloration turned yellow. The absorbance was then read at 450 nm with the same microtiter plate reader within 15 min.

The mean absorbance values for each set of samples, standards and controls were calculated.

A standard curve was constructed by plotting the mean absorbance obtained for each standard against its respective concentration in ng ml^{-1}. The mean absorbance value for each sample was used to determine the corresponding concentration of either oestradiol or testosterone from the standard curve that was derived by calculating a least-squares regression model.

Gonad histological slide preparation

Gonad tissue samples that had been fixed in 10 % buffered formalin for a minimum of two days were sectioned at the tip, middle and base of the conical appendage and placed into ethanol for 24 h. The sections were dehydrated in 70 (1 h), 96 (2 h) and 100 % (4 h) ethanol solutions and cleared in toluene and xylene (4 h). Cassettes were held in wax at 57 °C until they were embedded in paraffin wax at 60 °C. Samples were sectioned with a 4.0 µm microtome and incubated in an oven at 60 °C for 30 min. Sections were cleared and hydrated in toluene for 4 min, rinsed in 100, 96 and 70 % dilution for 3 min each and washed in distilled water for 3 min. The sections were immersed in Harris's haemotoxylin for 10 min and rinsed under running water until all residual haemotoxylin was washed off. The sections were differentiated in acid alcohol for 10 s and rinsed under a running tap for 10 min and then rinsed in 70 % ethanol for 3 min. The sections were counterstained in Eosin for 2.5 min and dehydrated in 96 % ethanol for 6 min and then 3 min in 100 % ethanol. The sections were then cleared in toluene for 4 min, mounted on microscope slides (25 x 75 x 1 mm) and covered with a cover slide.

Slides were viewed under a light microscope (UOP, UB200i compound microscope, serial number: 201101137) at 4, 40 and 100 X magnification. A photograph (UOP, Microscope camera DCM900) of a randomly selected view from the mid-section was taken at 4 X magnification. From the photograph, the oocytes were counted using Fiji software (http://pacific.mpi-cbg.de/). No differences were found in the oocyte numbers between the basal, midpoint and apical zones of farmed *H. midae*, therefore the mid-section was chosen as it comprised mostly of ovarian tissue (Ayres, 2013). The number of oocytes per square millimetre was determined using Equation 12, where the total number of oocytes in the photograph (E_T) was divided by the total area in the photograph (A_T).

$$Number\ of\ oocytes\ per\ mm^2 = \frac{E_T}{A_T} \tag{12}$$

Histological assessment

The development of oocytes was divided into 10 stages, ranging from oogonia to necrotic oocytes, based on the descriptions and illustrations of Georgi and DeMartini (1977), Wood and Buxton (1996), Najmudeen (2008), Roux *et al.* (2013), Ayres (2013), Kim *et al.* (2016) and Wu *et al.* (2019). These authors suggested a combination of sizes and morphology of oogonia, oocytes, nucleus and nucleolus, and their respective staining properties, description of the chorion and jelly coat, and the attachment of oocytes to trabeculae, in order to sort oocytes into up to 10 developmental stages. Their suggestions were followed closely when staging the oocytes in the current study, with only minor modifications, when necessary. The reproductive stages of the ovaries were divided into five stages, from early maturing to degenerating. Stages were assigned based on the predominant oocytes present, size of the lumen and structure of the trabeculae (Georgi and DeMartini, 1977; Capinpin *et al.*, 1998; Kim *et al.*, 2016).

The development of spermatogonia was divided into four stages, based on the descriptions of

Wood and Buxton (1996), Najmudeen (2007), Roux *et al*. (2013) and Velez-Arellano *et al*. (2015). Each stage was based on the structure and size of the cells. The reproductive stage of the testes was divided into five stages, ranging from early maturing to degenerating. Stages were assigned based on the presence of the predominant stages and size of the tubules (Wood and Buxton, 1996; Najmudeen, 2007; Velez-Arellano *et al*., 2015; Kim *et al*., 2016).

Statistical analysis

Oocyte densities

The ripe stage of reproduction was isolated for the analysis as this best represents female abalone fecundity. Analysis of Variance (ANOVA) was performed on the mean oocyte densities between treatments for the ripe stage, followed by Tukey's HSD post hoc test (Tukey, 1960) to determine where significant differences occurred between treatment means.

Sex steroids

A frequency distribution was constructed and due to the large presence of zero and lower concentration scores, a large variability in the means and standard errors was apparent, therefore box and whisker diagrams were constructed in order to have a better measure of central tendency. The five-number summary was explored, which consisted of the median, interquartile, minimum and maximum ranges, thus estimates were less affected by outliers and skewed data (Williamson *et al*., 1989). Data were presented as median, followed by the interquartile range in brackets.

The change in sex steroids as a function of time was also described using regression analysis. Analysis of covariance was used to test the slopes of the regression models that had been estimated for each treatment to model sex steroids as a function of time.

The significance level for all statistical tests was set at $\alpha = 0.05$. Data were presented as means ± standard error, unless stated otherwise. All analyses were performed using Statistica® 13 software package. Frequency distribution were constructed using Microsoft Excel.

4.3 RESULTS

Gonad histology

OOGENESIS

Oocytes began small and round and as they matured they became larger, round and finally polygonal due to the compacting of the mature oocytes in the ovary (Figure 4.3.1). Oocyte stages ranged from oogonia to stage 8 and also included necrotic oocytes (Figure 4.3.1). Stages 1 – 6 were attached to the trabeculae with stages 1 – 3 found predominately in large clusters (Figure 4.3.1a and b). Stage 7, 8 and necrotic oocytes were free within the lumen (Figure 4.3.1c). All developmental stages were identified according to the criteria listed in Table 4.3.1 and with these guidelines, predominant oocytes and the condition of the ovaries were identified and staged according to Table 4.3.2.

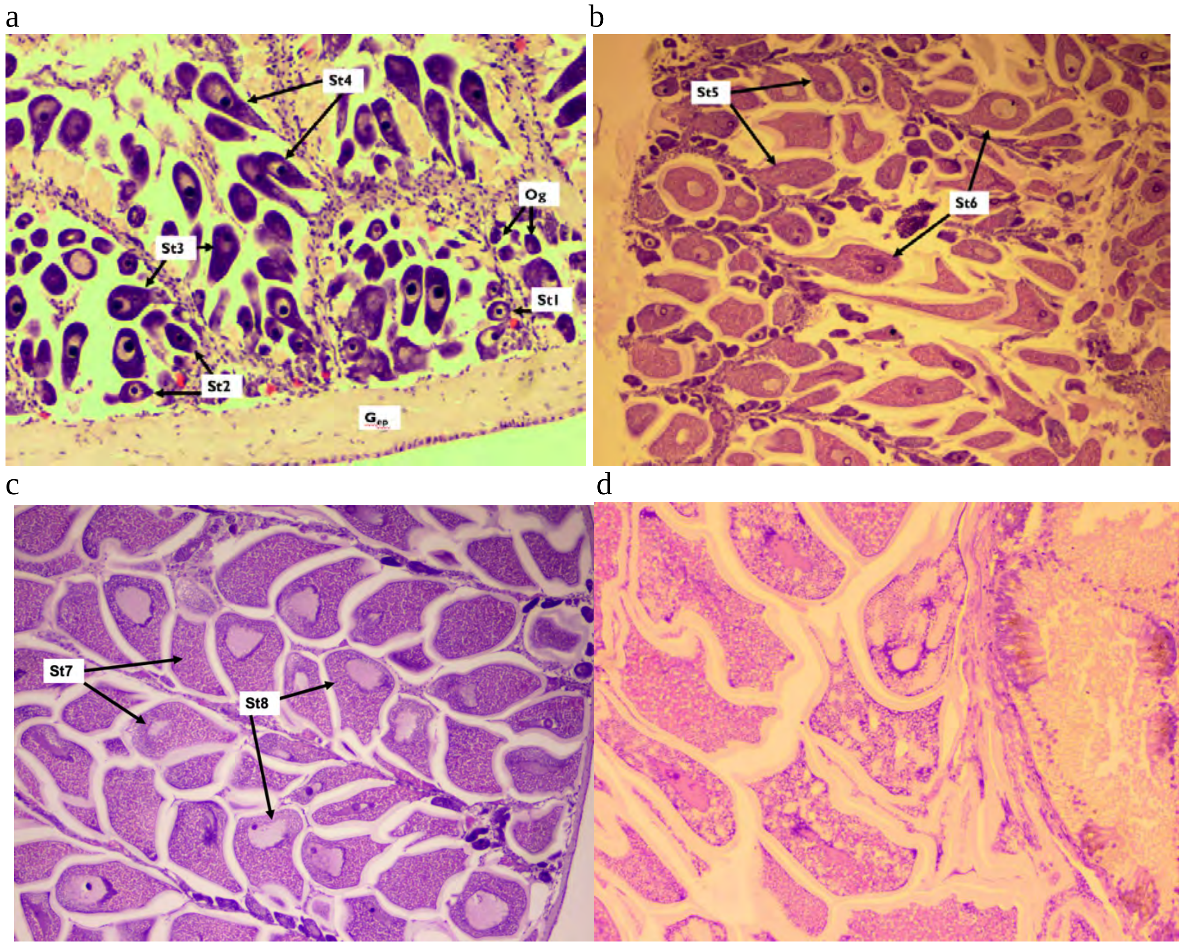
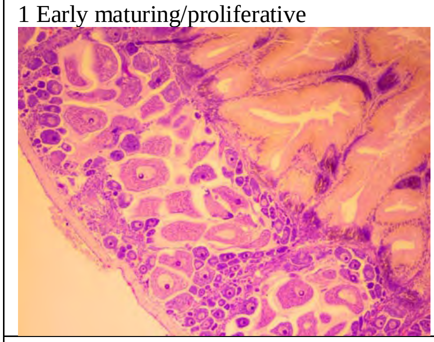
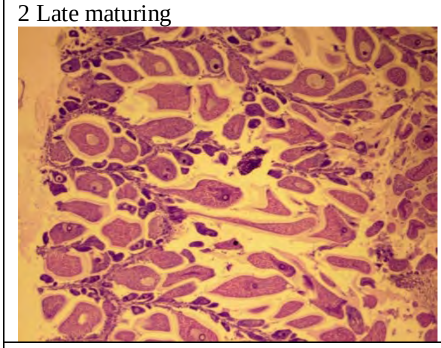
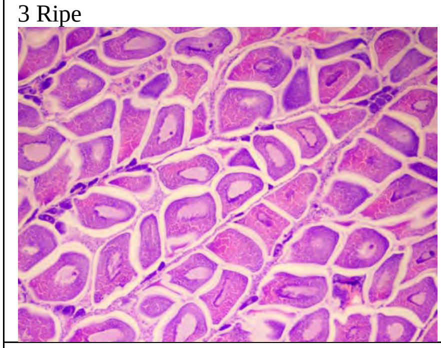
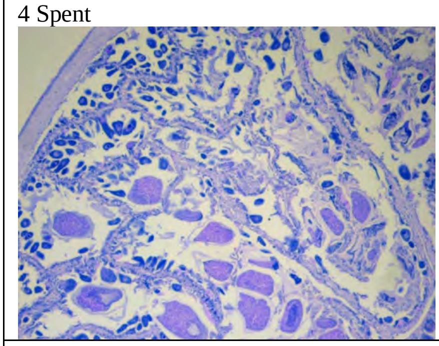
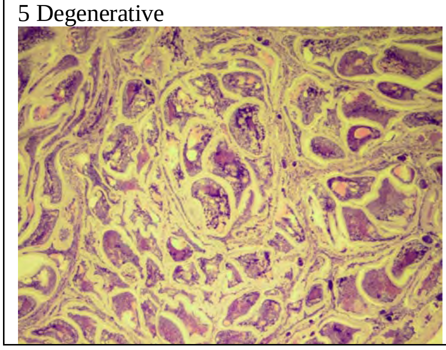


Figure 4.3.1: Transverse section of *Haliotis midae* gonad, depicting: (a) oogonia (O_g) to stage (St) 4 and gonad epithelium layer (G_{ep}); (b) stage 5 and 6; (c) stage 7 and 8; and (d) necrotic oocytes.

Table 4.3.1: Stages of oocyte development in *Haliotis midae*, describing cell structure, nucleus features and distinctive characteristics.

OOGONIA		
Cell structure	*Nucleus*	*Distinctive characteristics*
15 – 20 µm.	Stained dark blue, indicating strong basophilic properties.	Cytoplasm stained dark blue, indicating presence of ribosomes. Oogonia surrounded by flat, squamous follicular cells that lined up against the trabeculae. Separating the gonad into small compartments (Figure 4.3.1a)
STAGE 1 OOCYTE		
Cell structure	*Nucleus*	*Distinctive characteristics*
Oval shaped, 21 – 30 µm in length.	Large and round, approximately 12 µm in diameter. Stained light-blue with a densely packed chromatin network.	Found in clusters, adhering to the trabeculae. Cytoplasm dark blue due to haematoxylin-eosin, indicating strong basophilic properties (Figure 4.3.1a)
STAGE 2 OOCYTE		
Cell structure	*Nucleus*	*Distinctive characteristics*
Scallop-shaped, 31 – 40 µm length.	Large, round, light-blue stained in colour, approximately 15 µm in diameter.	Found in clusters, adhering to the trabeculae. Cytoplasm stained dark-blue, with small lipid droplets. Uncoiling of the chromatic network visible in light-blue stained nucleus (Figure 4.3.1a).
STAGE 3 OOCYTE		
Cell structure	*Nucleus*	*Distinctive characteristics*
Scallop-shaped, 41 – 60 µm.	Large and round, approximately 22 µm in diameter. Light-blue in colour, but slightly lighter than Stage 2, with uncoiling chromatin.	Clustered in groups along the trabeculae, with larger spaces in between oocytes due to the formation of the chorion. Cytoplasm stained dark-blue, with an increase in size and number of lipid droplets (Figure 4.3.1a).
STAGE 4 OOCYTE		
Cell structure	*Nucleus*	*Distinctive characteristics*
Columnar or pear-shaped, 60 - 80 µm.	Round, approximately 25 µm with uncoiling chromatin.	Oocytes attached to the trabeculae at the base of the cell. Dark blue-stained cytoplasm with increase in size and density compared to Stage 3. In addition, chorion became more visible (Figure 4.3.1a).
STAGE 5 OOCYTE		
Cell structure	*Nucleus*	*Distinctive characteristics*
Columnar-shaped, 100 – 180 µm in length.	Oval, approximately 45 µm.	Oocytes began to form a cytoplasmic stalk that was attached to the trabeculae. Cytoplasm stained pink due to presence of yolk platelets that contained many lipids droplets of similar size. The chorion was visible with the additional formation of the jelly coat (Figure 4.3.1b).
STAGE 6 OOCYTE		
Cell structure	*Nucleus*	*Distinctive characteristics*
Tear-drop shaped, 160 – 250 µm in length.	Oval, approximately 60 µm in length. Stained pink with enlarged and lightened round nucleolus of approximately 25 µm in diameter.	Thin cytoplasmic stalk was still attached to the trabeculae. Cytoplasm stained pink due to presence of yolk platelets with variable sized lipid droplets. Each oocyte was encased in a thick jelly coat layer, separating cells into loose clusters (Figure 4.3.1b).
STAGE 7 OOCYTE		
Cell structure	*Nucleus*	*Distinctive characteristics*
Polygonal, 200 – 250 µm in length.	Oval and pink, approximately 80 µm in length.	Cytoplasmic stalk detached from the trabeculae and cells were situated feely within the lumen, surrounded by the chorion and a thick jelly coat. Cytoplasm stained pink with numerous white lipid droplets (Figure 4.3.1c).
STAGE 8 OOCYTE		
Cell structure	*Nucleus*	*Distinctive characteristics*
Polygonal, 250 – 300 µm in diameter.	Oval, approximately 90 µm in diameter.	Cells were free within the lumen. The chorion and jelly coat became thinner as cells became more compressed. Cytoplasm was stained pink and contained clusters of lipid droplets (Figure 4.3.1c).
NECROTIC OOCYTES		
Cell structure	*Nucleus*	*Distinctive characteristics*
Cells larger than 150 µm.	Broken down, shrunk or disappeared.	Numerous small vacuoles in the cytoplasm. Cell membrane disappeared with ruptured chorion (Figure 4.3.1d).

Table 4.3.2: Descriptions of ovary maturity stages in female *Haliotis midae*.

MATURITY STAGE	DESCRIPTION
1 Early maturing/proliferative 	Small lumen, with mostly oogonia and previtellogenic oocytes (stage 1 and stage 2). Oogonia and oocytes form clusters on trabeculae wall.
2 Late maturing 	Vitellogenesis is initiated at this stage. Large lumen with stage 5 and stage 6 as the most abundant oocytes that are still attached to the trabeculae.
3 Ripe 	Large lumen with mainly stage 7 and stage 8 oocytes. Oocyte tightly packed, becoming distorted and difficult to measure. Very few oogonia and previtellogenic oocytes present and mostly towards the peripheral wall of the gonad.
4 Spent 	Trabeculae collapsed. Slight gametogenic activity with few mature oocytes, but some areas may still be densely packed. Oocytes mostly necrotic. Immature oocytes may be present. Areas devoid of cells where oocytes were expelled.
5 Degenerative 	Remnant eggs from previous spawning degenerating, breaking down and becoming reabsorbed. Lumen still full of oocytes and may be tightly packed, resulting in irregular shape of oocytes. Majority of the mature oocytes are necrotic. Immature oocytes may still be present. Trabeculae collapsed.

SPERMATOGENESIS

Spermatogonia began large, round and lightly stained and changed to small, elongated and darkly stained cells (Figure 4.3.2). The shape of the cells changed at each stage of maturity, becoming increasingly darker and moving out into the lumen (Figure 4.3.2). All five developmental stages were identified according to the criteria listed in Table 4.3.3 and with these characteristics, predominant cells and the state of the gonads were identified and staged according to Table 4.3.4.

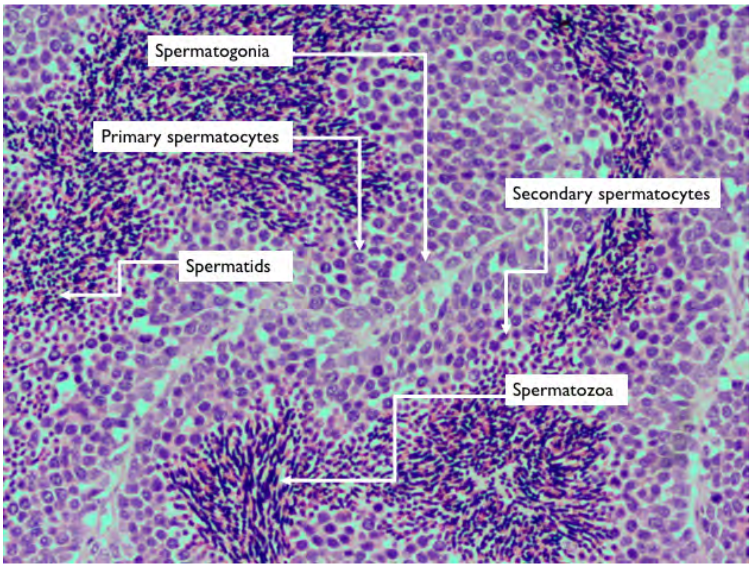
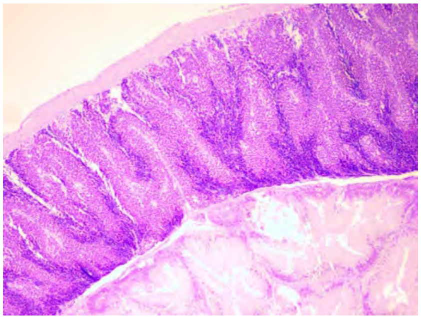
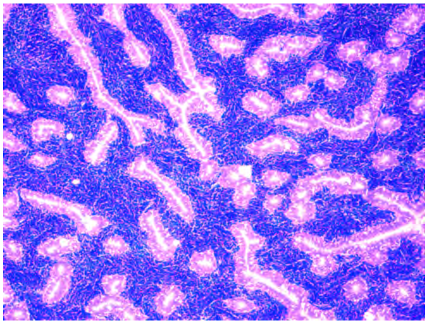
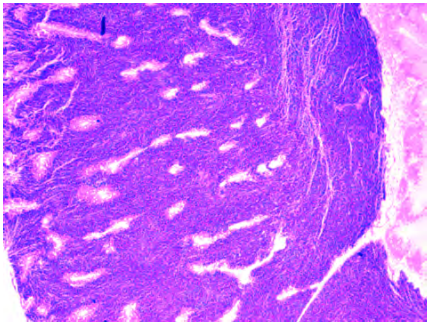
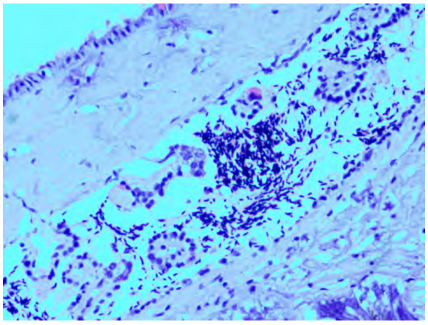


Figure 4.3.2: Cross section through the gonad of male *Haliotis midae* depicting the five stages of sperm development.

Table 4.3.3: Stages of sperm development in *Haliotis midae* describing cell structures and distinctive characteristics.

SPERMATOGONIA	
Cell structure Oval, 5 – 8 µm in length.	*Distinctive characteristics* Clear nucleus, spots of light blue. Cells are attached to trabeculae, forming clusters that are spread out into the lumen (Figure 4.3.2).
PRIMARY SPERMATOCYTES	
Cell structure Round, 3 – 5 µm in diameter.	*Distinctive characteristics* Dark stained nucleolus, darker shade of purple compared to spermatogonia. Cells moving further out from the trabeculae (Figure 4.3.2).
SECONDARY SPERMATOCYTES	
Cell structure Oval, 2 – 5 µm in length.	*Distinctive characteristics* Increasingly darker than primary spermatocytes, cells form clusters along primary spermatocytes (Figure 4.3.2).
SPERMATIDS	
Cell structure Round, 1 – 2 µm in diameter.	*Distinctive characteristics* Stained darker than secondary spermatocytes. Free from trabeculae (Figure 4.3.2).
SPERMATOZOA	
Cell structure Elongated, 3 – 4 µm in length.	*Distinctive characteristics* Dark, blue stained cells and free within the lumen (Figure 4.3.2).

Table 4.3.4: Descriptions of gonad maturity stages of male *Haliotis midae*.

MATURITY STAGE	DESCRIPTION
1 Early maturation 	Mainly spermatogonia and spermatocytes. Multiple wide and narrow tubules positioned transversely from gonad epithelial layer towards digestive gland with dense clusters of mainly spermatogonia and spermatocytes. Stained lightly.
2 Late maturing 	Mainly spermatids and spermatozoa. Numerous clusters of lightly stained secondary spermatocytes loose within lumen, surrounded by dense spermatids and spermatozoa, making image appear darker than the early maturing stage.
3 Ripe 	Large lumen with dense spermatozoa and spermatids. Small clusters of secondary spermatocytes loose within the lumen. Trabeculae present, with few immature cells attached.
4 Spent 	Gonad size reduced greatly. Areas devoid of any cells where spermatozoa had been expelled. Slight gametogenic activity with spermatozoa loosely spread in the lumen and few, small clusters of spermatocytes.

Gametogenesis

All stages of gonad maturity were inconsistently present in female and male abalone throughout the study. However, the degenerative and ripe stages were present in the females of all treatments at each sampling time, while the late maturing stage was consistently present in all male abalone throughout the study (Figure 4.3.3). Female abalone gonads appeared to be in the stage of maximum ripeness at day 91 and day 224, while males were in maximum ripeness between days 91 and 273. Although spent ovaries were first identified at day 133, it is suggested that not all animals spawned in a single event. Low proportion of spent gonads were seen unevenly distributed throughout the study, with one to two of the eight animals sampled per sex for each treatment at each time showing signs of spent gonads at the respective sampling times (Figure 4.3.3).

At least half of the ovaries of abalone at day zero were in the degenerative stage, followed by a decrease at day 91 and then an increase, peaking at day 133 in females from all treatments (Figure 4.3.3). Degenerative ovaries remained similarly high at day 182, followed by a large drop in the proportion of ovaries in this stage, which remained relatively low until day 315.

Early maturing and late maturing ovaries were more prevalent in abalone that were fed the single-protein diets as well as those fed FM SM. Female abalone that were fed FM S had relatively more ripe ovaries over the one-year study, while male abalone that were fed FM SM had relatively more ripe and spent testes throughout the study (Figure 4.3.3).

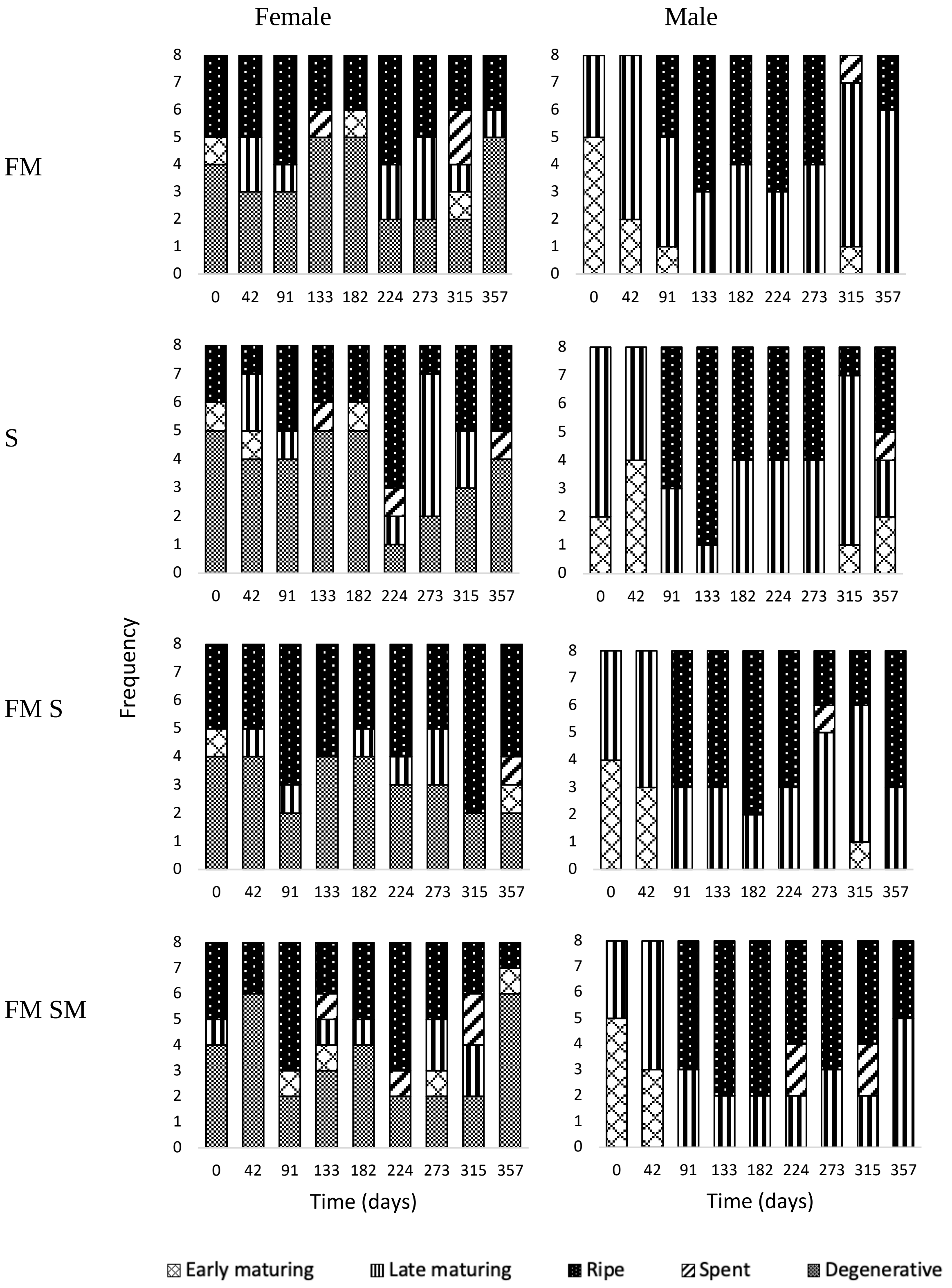


Figure 4.3.3: Frequency of gonad maturity stages in female and male *Haliotis midae* fed a fishmeal-only based diet, soya-only based diet (S), a combination of fishmeal and soya (FM S) and a combination of fishmeal and sunflower meal (FM SM) diet over a year.

Oocyte density

There were no significant differences in the number of oocytes per mm^2 in the ripe stage between treatments (ANOVA, $F_{(3, 12)} = 0.78$, $p = 0.53$) with an average of 72.68 ± 1.94 oocytes mm^{-2}.

Sex steroids

Frequency distribution

The frequency distribution of oestradiol and testosterone in the haemolymph of both females and males from all dietary treatments were not normally distributed and skewed towards samples with an estimated concentration of zero pg ml^{-1} (Figure 4.3.4).

Approximately 30 % of the females that were fed the single protein diets had non-detectable oestradiol levels when measured in the haemolymph over the one-year period, while females fed FM S and FM SM had higher frequencies of non-detectable oestradiol concentrations in the haemolymph at 44 and 42 %, respectively (Figure 4.3.4a). Oestradiol concentrations ranging from non-detectable to 0.45 pg ml^{-1} were the most measured values in the haemolymph of females from all treatments (50 – 61 %), except those fed the FM SM (33 %) diet (Figure 4.3.4a). More than 40 % of males in the S, FM S and FM SM treatments had non-detectable oestradiol levels in the haemolymph over the one-year period, while 33 % of males fed FM had non-detectable levels of oestradiol in the haemolymph (Figure 4.3.4b). In addition, between 40 and 50 % of males fed FM, S and FM SM measured between non-detectable and 0.45 pg ml^{-1} of oestradiol in the haemolymph over the year, while 36 % of males fed FM S measured between this range (Figure 4.3.4b).

Fifty percent of females fed FM and FM SM had non-detectable levels of testosterone in the haemolymph over the one-year period, while those fed FM S had a higher frequency of non-detectable levels at 67 %, contrasted with those fed S at 47 % (Figure 4.3.4b). The second most measured range of testosterone in the haemolymph was between non-detectable and 550 pg ml^{-1} for females in all treatments (11 – 25 %), except those fed FM, with 14 % of animals measuring 801 – 850 pg ml^{-1} in the haemolymph over the year (Figure 4.3.4b). Male abalone fed FM (56 %) and FM S (50 %) had the highest frequencies of non-detectable testosterone levels in the haemolymph, while abalone fed FM SM and S had similar frequencies of non-detectable concentrations in the haemolymph at 39 and 36 %, respectively over the one-year (Figure 4.3.4b). The range between non-detectable and 550 pg ml^{-1} of testosterone in the haemolymph was the second most recorded range in male abalone that were fed FM S (22 %), FM SM (36 %) and S (25 %), while those fed FM had similar concentrations in the haemolymph compared to the females, mostly occurring in the range 801 – 850 pg ml^{-1} at 14 % (Figure 4.3.4b).

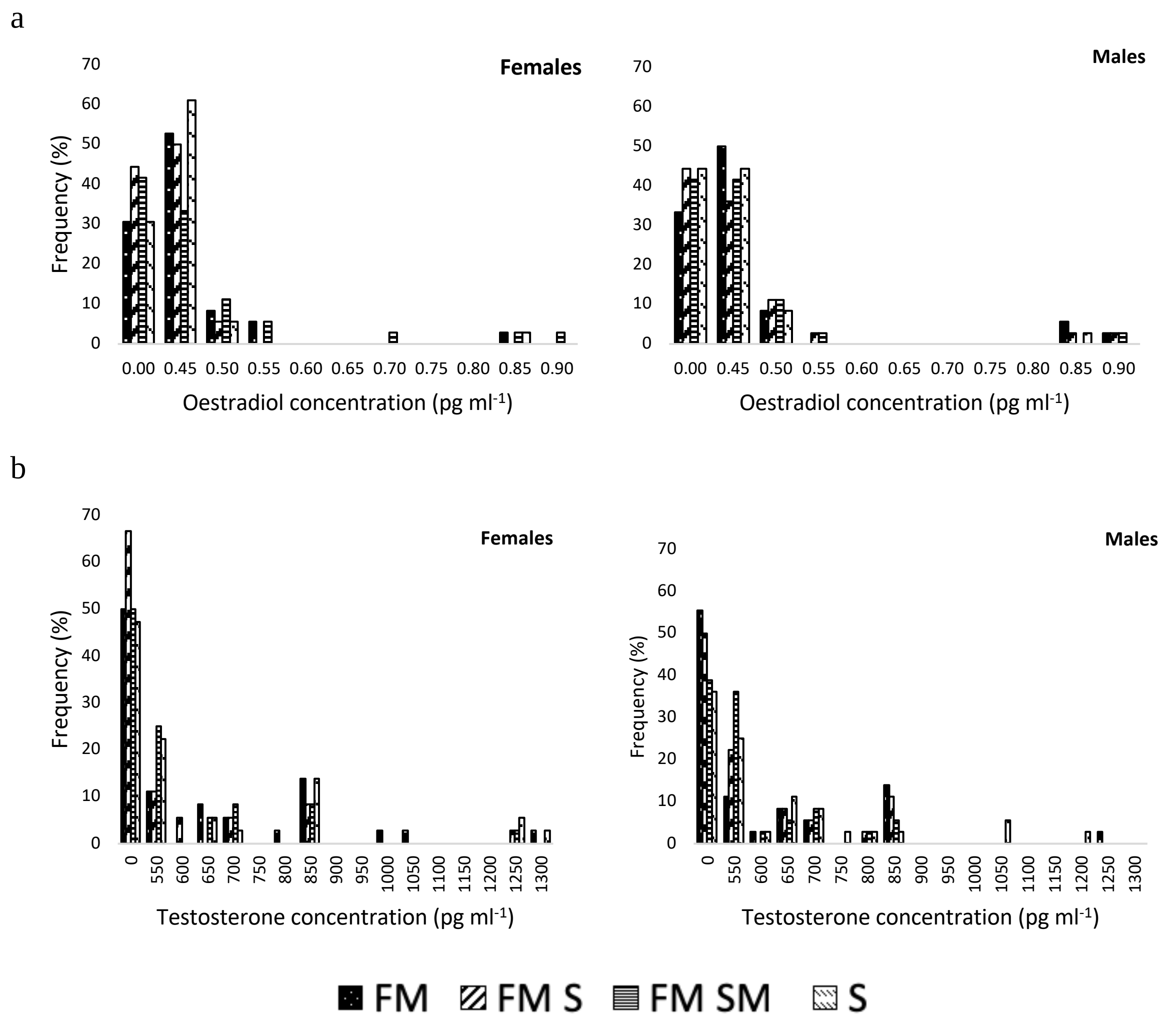


Figure 4.3.4: Frequency distribution (%) of (a) oestradiol (pg ml^{-1}); and (b) testosterone (pg ml^{-1}) concentrations in female (n = 288) and male (n = 288) *Haliotis midae* fed a fishmeal-only based diet (FM), soya-only based diet (S), a combination of fishmeal and soya (FM S) and a combination of fishmeal and sunflower meal (FM SM) diet over a one-year period.

Box and whisker plots

Oestradiol

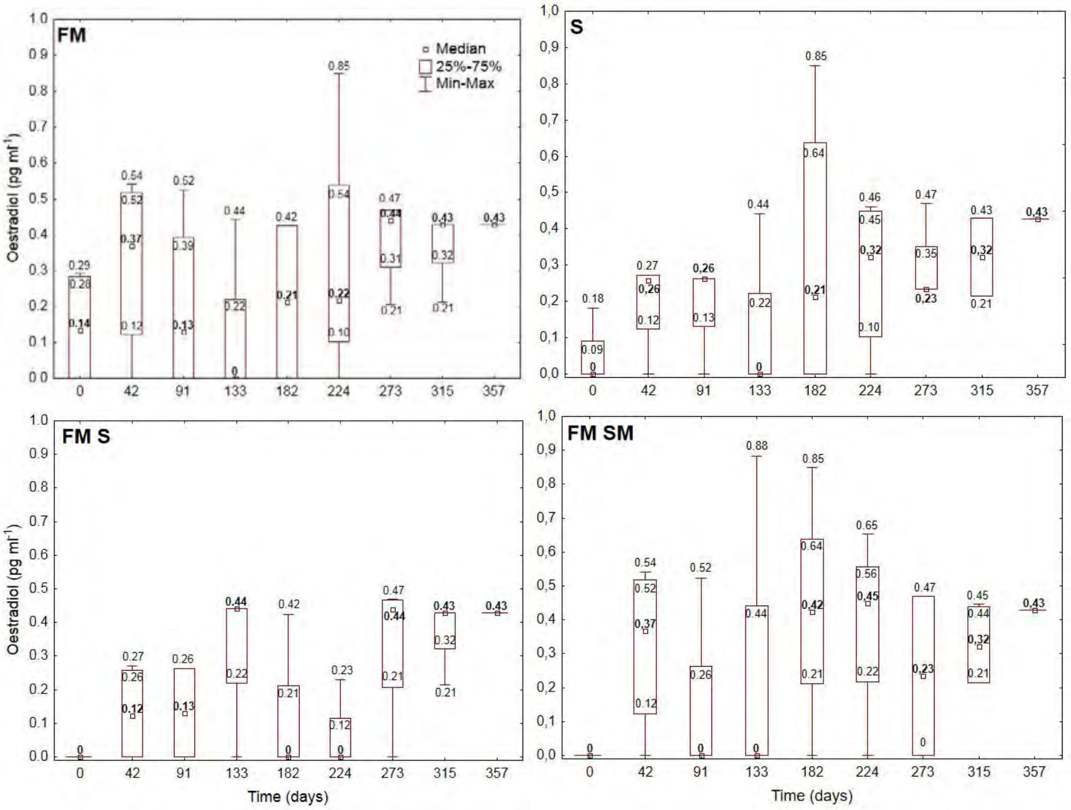
Females

Oestradiol concentrations in female abalone showed distinctive peaks in median values and periods of very low oestradiol concentrations (Figure 4.3.5a). Peaks were seen at day 42 and towards the end of the study for females fed the single-protein based diets, while those fed FM S had peaks at day 133 and towards the end of the study. Females fed FM SM peaked at day 42 and at the middle and the end of the study (Figure 4.3.5a). Dips in median oestradiol concentration were seen at day 133 for abalone that were fed the single-protein based diets and the FM SM diet, while those that were fed FM S showed dips at day 182 and 224 (Figure 4.3.5a). Highest median concentrations ranged between 0.43 and 0.45 pg ml^{-1} between treatments. The highest lower quartile was 0.43 pg ml^{-1} for females in all treatments, while the highest upper quartile ranged between 0.47 and 0.64 pg ml^{-1} (Figure 4.3.5a). Non-detectable concentrations were present in abalone from all treatments, while maximum values of 0.85 pg ml^{-1} were found in females fed the single-protein based diets. In addition, females fed FM SM reached a maximum concentration of 0.88 pg ml^{-1}, while those fed FM S had a maximum concentration of 0.47 pg ml^{-1} over the one-year period (Figure 4.3.5a).

Males

Oestradiol concentrations in the haemolymph of male abalone also showed distinctive peaks and periods of very low oestradiol concentrations (Figure 4.3.5b). Very large interquartile ranges were present, particularly at day 133 and 182 where the highest maximum concentrations were present (Figure 4.3.5b). Peak oestradiol concentrations were found at day 42 for males fed FM and FM SM, while those fed FM only peaked again at day 224, those fed FM SM peaked again at day 133 and 273, ending with similar median oestradiol concentrations at the end of the study (Figure 4.3.5b). Male abalone fed S and FM S had very low median oestradiol concentrations at the first four and three sampling times, respectively, followed by gradual increases reaching median oestradiol concentrations similar to abalone fed FM-only and FM SM by the end of the study (Figure 4.3.5b). The highest median concentration was slightly higher compared to females, ranging from 0.43 – 0.47 pg ml^{-1} (Figure 4.3.5b). Similar to females, the highest lower quartile 0.43 – 0.44 pg ml^{-1}, while the highest upper quartile ranged between 0.64 and 0.66 pg ml^{-1} (Figure 4.3.5b). Non-detectable levels were present in abalone from all treatments, while maximum concentrations were similar to females, ranging from 0.85 – 0.88 pg ml^{-1} (Figure 4.3.5b).

a Females



b Males

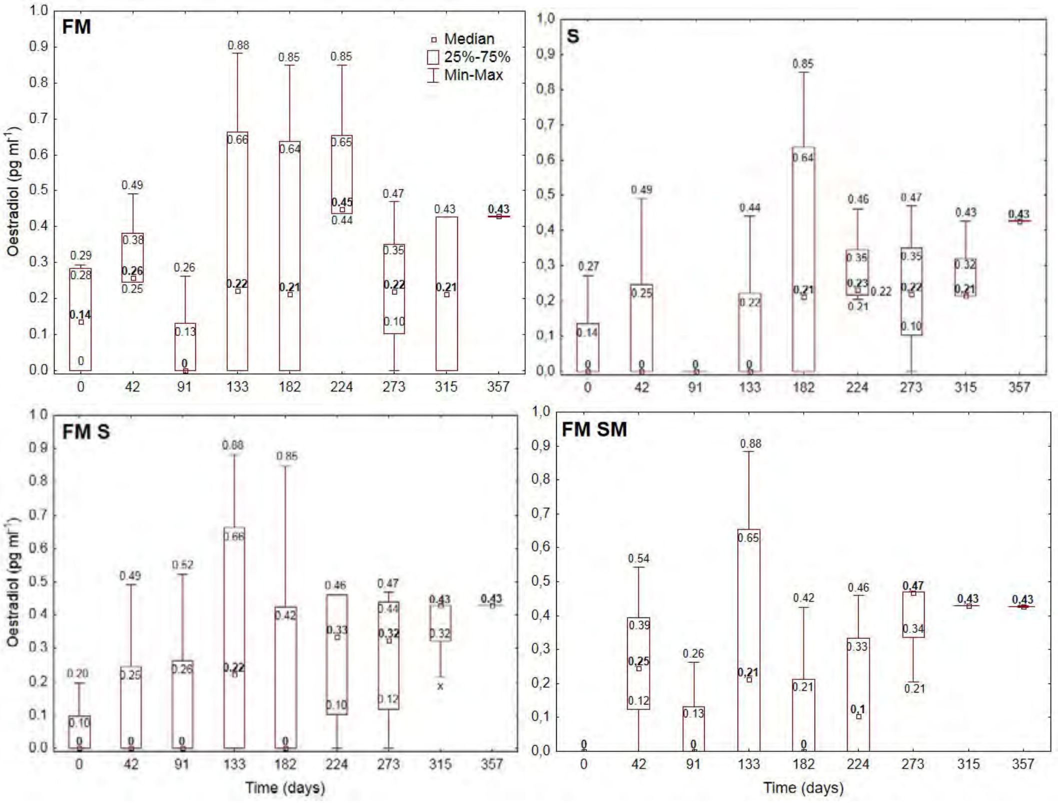


Figure 4.3.5: Box and whisker plots of oestradiol concentrations (pg ml^{-1}) in the haemolymph of female and male *Haliotis midae* fed fishmeal (FM), soya (S), fishmeal and soya (FM S) and fishmeal and sunflower meal (FM SM) based diets over one year.

Testosterone

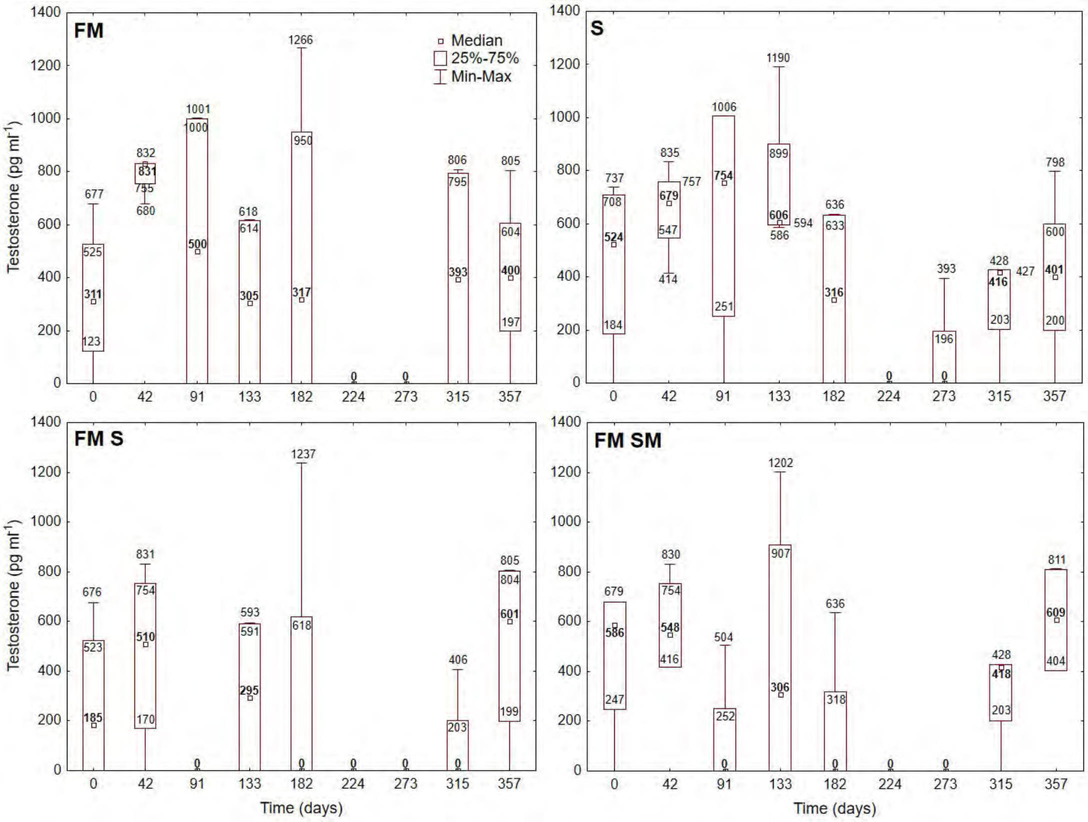
Females

Testosterone concentrations in female abalone showed peaks in median values as well as periods of approximately zero testosterone concentrations in the haemolymph (Figure 4.3.6a). The first peak in median testosterone concentration was seen at day 42 for abalone from most treatments, but not in those that were fed FM SM (Figure 4.3.6a). A second peak in median testosterone concentration occurred at day 133 for animals in most treatments, but not those fed FM, followed by the last peak at the end of the study, seen in animals from all treatments (Figure 4.3.6a). Dips in median testosterone concentration were seen at day 91 and day 224 to 273 for animals from all treatments (Figure 4.3.6a). However, very large interquartile ranges could be seen throughout the study, particularly from day 91 to 182 (Figure 4.3.6a). The highest median concentration amongst treatments ranged between 600 and 904 pg ml^{-1} with those fed FM S having the lowest and S with the highest median (Figure 4.3.6a). The highest lower quartile ranged between 199.34 and 755 pg ml^{-1}, while the highest upper quartile ranged 804 to 1222 pg ml^{-1} (Figure 4.3.6a). Non-detectable concentrations were present in abalone from all treatments, while the highest maximum values amongst treatments ranged from 1202 to 1266 pg ml^{-1} with abalone that were fed FM SM being at the lower end of that range, while abalone fed FM had the highest testosterone concentration over the one-year period (Figure 4.3.6a). Testosterone in the haemolymph was non-detectable at day 224 and 273 in females from all treatments, except for females that were fed S at day 224 Figure 4.3.6a).

Males

Testosterone concentrations in male abalone showed distinctive peaks in median values as well as periods of very low testosterone levels in the haemolymph over the one-year experimental period (Figure 4.3.6b). Median concentrations first peaked at day 42 for abalone in most treatments, but not in those abalone fed S as it continued increasing, peaking at day 91 Figure 4.3.6b). Abalone fed S and FM S had two peaks in median testosterone concentration, the last one being at the end of the study, while abalone fed FM and FM SM had a second peak at day 133 and day 224, respectively, with the last peak occurring at the end of the study (Figure 4.3.6b). Abalone fed FM and FM SM had two dips in oestradiol median concentrations, at day 91 and 133, respectively, with the third dip lasting over three and two sampling times, respectively. Abalone fed S and FM S had one dip in median oestradiol concentrations, around the middle of the study and after the second half of the study, respectively, with the last dip lasting two and four sampling times, respectively (Figure 4.3.6b). The highest median concentration amongst treatments was lowest for abalone fed FM S (718 pg ml^{-1}), while the highest median for the other three treatments ranged between 754 to 756 pg ml^{-1} (Figure 4.3.6b). The highest lower quartile between treatments ranged between 340 to 594 pg ml^{-1}, while the highest upper quartile ranged from 805 – 1006 pg ml^{-1}. Non-detectable levels occurred in abalone from all treatments, while the highest maximum value between treatments ranged between 830 to 1207 pg ml^{-1}, with animals that were fed single protein-based diets exceeding 1000 pg ml^{-1}, while those that were fed combination diets never had values higher than 837 pg ml^{-1} (Figure 4.3.6b).

a Females



b Males

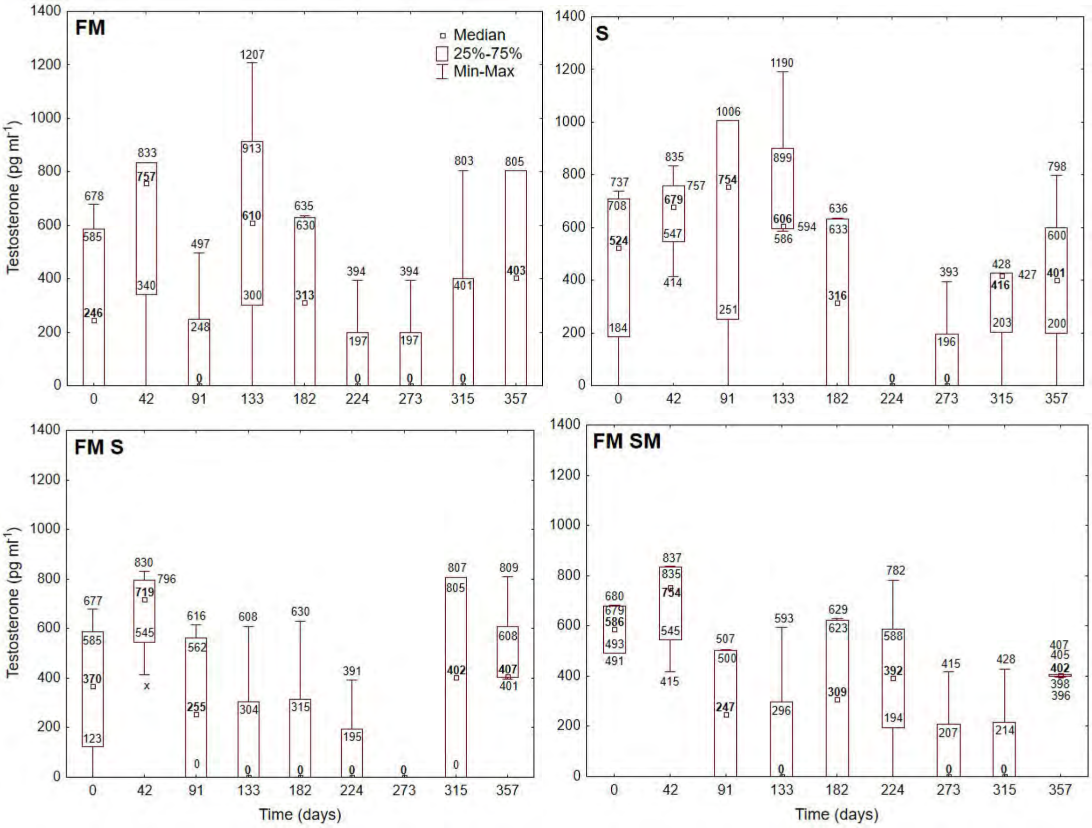


Figure 4.3.6: Box and whisker plots of testosterone concentrations (pg ml^{-1}) in the haemolymph of female and male *Haliotis midae* fed fishmeal (FM), soya (S), fishmeal and soya (FM S) and fishmeal and sunflower meal (FM SM) based diets over one year.

Changes in sex steroids over time

There was a significant positive relationship between the oestradiol concentration in the haemolymph of female abalone from all treatments and time over the one-year period (see exact p-values in Figure 4.3.7). Similarly, there was a significant positive relationship between haemolymph oestradiol concentration of male abalone from most treatments (see exact p-values in Figure 4.3.7), but not for male abalone that were fed the FM diet and time ($p = 0.17$; Figure 4.3.7). Analysis of covariance (ANCOVA) showed significant results for the slopes between treatments for oestradiol concentrations in the haemolymph of both females (ANCOVA $F_{(4,136)} = 7.80$, $p < 0.0001$) and males (ANCOVA $F_{(4, 136)} = 7.27$, $p < 0.0001$). Females that were fed the FM S diet had a slope that was at least 10 times steeper than abalone from the other three treatments, which had similar slopes, while male abalone that were fed the combination diets and S-only based diets had similar slopes (Figure 4.3.7).

There were no significant relationship between testosterone concentrations and time in the haemolymph of female abalone from all treatments (See exact p-values in Figure 4.3.7). Similarly, there was no significant relationship between testosterone levels in the haemolymph of male abalone that were fed FM and FM S diets over time ($p = 0.36$ and $p = 0.49$, respectively; Figure 4.3.7); however, there was a significant negative relationship between the testosterone concentration in the haemolymph and time for male abalone that were fed S and FM SM diets ($p = 0.02$ for both; Figure 4.3.7). The slopes for testosterone levels in the haemolymph of males over time were significantly different between treatments (ANCOVA $F_{(4, 136)} = 2.87$, $p = 0.03$), but not in female abalone (ANCOVA $F_{(4, 136)} = 0.84$, $p = 0.50$). Male abalone that were fed FM SM and S-only had similar slopes for haemolymph testosterone, while abalone that were fed FM S and FM-only had no slope (Figure 4.3.7). Therefore, over time the oestradiol levels found in female and male abalone haemolymph differed depending on dietary treatment, while testosterone concentrations in the haemolymph were only influenced by dietary treatments in male abalone.

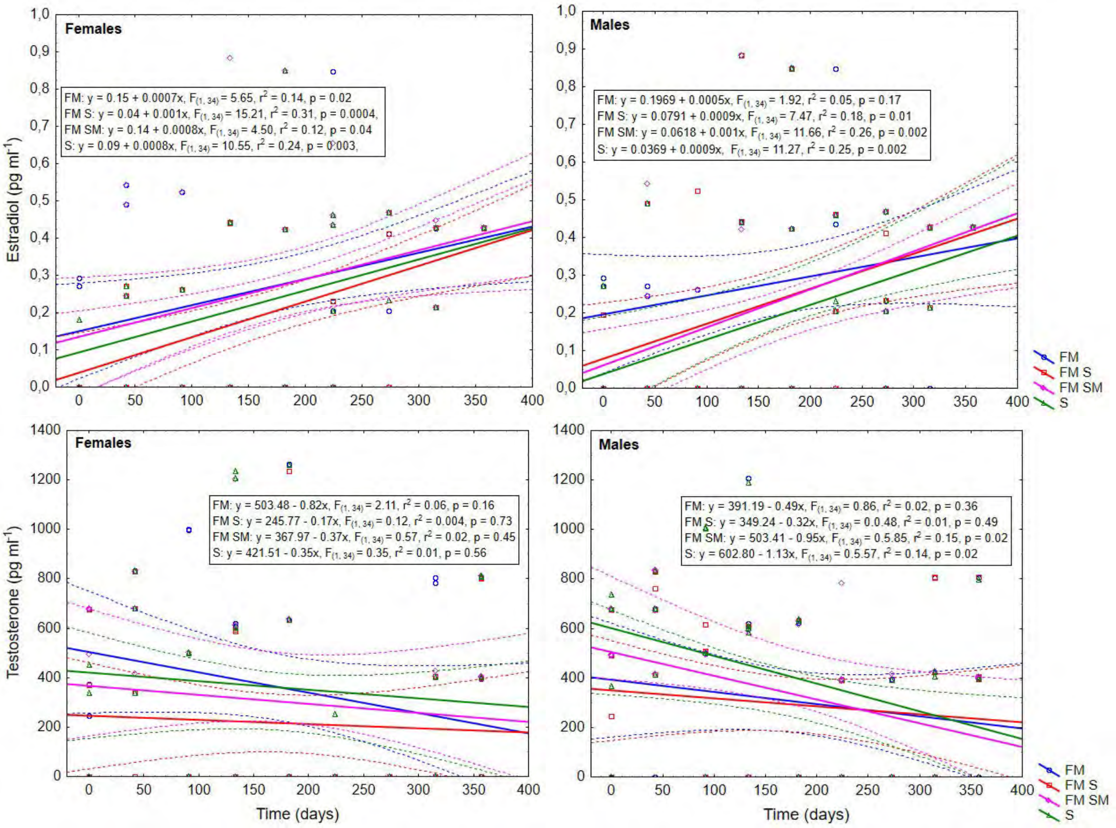


Figure 4.3.7: The change in oestradiol (pg ml^{-1}) and testosterone (pg ml^{-1}) concentrations in female and male *Haliotis midae* fed fishmeal-only (FM), soya-only (S), fishmeal and sunflower meal (FM SM) and fishmeal and soya (FM S) based diets. Dotted lines represent 95 % confidence bands.

Relationship between oestradiol and testosterone

There was no significant relationship between oestradiol and testosterone concentrations in the haemolymph of both female and male abalone within in each treatment ($p > 0.05$; Table 4.3.5). Therefore, one sex steroid hormone had no influence on the other in animals from all treatments.

Table 4.3.5: Regression analysis of oestradiol concentration as a function of testosterone concentration in the haemolymph of female and male *Haliotis midae* fed fishmeal (FM), soya (S), combination of fishmeal and soya (FM S) and fishmeal and sunflower meal (FM SM) based diets.

Abalone sex	Treatment	F	r^2	p
Female	FM	0.65	0.02	0.43
	FM S	3.64	0.10	0.06
	FM SM	0.96	0.03	0.34
	S	0.90	0.03	0.35
Male	FM	2.37	0.07	0.13
	FM S	1.30	0.04	0.26
	FM SM	0.03	0.001	0.87
	S	0.08	0.002	0.78

4.4 DISCUSSION

The assessment of gonad histology, coupled with steroid hormone assays is an informative way of understanding the variations in the reproductive cycle of farmed abalone, *Haliotis midae*. Although still scant, knowledge on molluscan endocrinology has increased over the last decade due to their significance in aquatic ecosystems worldwide as well as the use of molluscs in aquaculture (Guisti and Joaquim-Justo, 2013) and their importance as bioindicators (Ketata *et al.,* 2008). The steroid hormones 17β-oestradiol and testosterone have been measured in the tissue and haemolymph of molluscs, including gastropods, bivalves and cephalopods with patterns coinciding with reproduction, inferring that they play a role in reproduction (D'Aniello *et al*., 1996; Gooding and LeBlanc, 2004; Croll and Wang, 2007; Zabrzańska *et al*., 2015). The key enzyme involved in steroidogenesis was present in *Haliotis diversicolor supertexta*, which plays critical role during the steroid mediated reproductive process by converting estrone to oestradiol (Zhou *et al*., 2011). The abalone 17 β-HSD-12 enzyme was also differentially expressed during different stages of reproduction (Zhou *et al*., 2011), suggesting that sex steroids could potentially play a role in abalone reproduction.

Gonad histological assessments is one of the most accurate methods of assessing the reproductive status of an animal (Litaay and De Silva, 2003), and when coupled with steroid hormone assays, this research may contribute to the understanding of the abalone reproductive cycle and the possible role that steroid hormones plays in gametogenesis. Furthermore, the influence of dietary protein sources on the sex steroid hormones in abalone has not been assessed. The method of gonad histology and sex steroid hormone assays had been conducted in studies on molluscs (Reis-Henriques and Coimbra, 1990; Gauthier-Clerc *et al*., 2006; Ketata *et al*., 2007; Liu *et al.*, 2008), but farmed abalone have not been studied using a combination of these methods.

The aim of this study was to determine the effects of dietary protein sources on the reproductive physiology of farmed *H. midae*, coupled with sex steroid concentrations over one year, allowing us to investigate the effect of diet on the possible role of sex steroids in the reproduction of farmed *H. midae.* This was achieved through the identification and quantification of oocyte and spermatocyte development. The sex steroid hormones 17β-oestradiol and testosterone were measured in the haemolymph of both female and male abalone fed single-protein based diets (fishmeal-only and soya-only) as well as combination diets (fishmeal-soya and fishmeal-sunflower meal). The changes in haemolymph sex steroids over time as well as the relationship between the two hormones were assessed.

Gametogenesis

Haliotis midae is a temperate species of abalone, found along the coast of southern Africa. Temperate species usually have distinct spawning seasons throughout the year (Capinpin *et al.*, 1998; Setyono, 2006). Many abalone species do not have a resting phase in the reproductive cycle, with gametogenesis initiated immediately after each spawning event (Webber, 1970;

Wood and Buxton, 1996). Gametogenesis in abalone is a continuous process with gonads containing several stages of development at any given time, therefore the assignment of a single reproductive stage is typically based on the prevalence of cell types observed in the gonad (Capinpin *et al*., 1998). The spawning season of *H. midae* has been found to vary, with breeding peaking in late autumn and early winter (April – July) and smaller spawning peaks in October and January in wild abalone (Wood and Buxton, 1996), while farmed *H. midae* have exhibited spawning peaks in the summer months (Ayres, 2013; Riddin, 2013).

Haliotis midae used in the present study showed asynchronous spawning patterns as seen in both wild and farmed *H. midae* (Wood and Buxton, 1996; Litaay and De Silva, 2003; Ayres, 2013). Maximum ripeness in females was at day 91 and 224, during spring and autumn, respectively, while for males the maximum gonadal ripeness occurred from day 91 up to day 273, encompassing the spring, summer and autumn months of October to April. Haliotid species have complex spawning cycles with the occurrence of multiple spawning events where not all individuals spawn in a single spawning event, resulting in a decrease in the frequency of spawning events (Gurny and Mundy, 2004). Male clams (*Ruditapes decussatu*s) continue to discharge spermatozoids despite the complete release of gametes in females (Capinpin and Corre, 1996). Similarly, this was seen in the uneven distribution of spent ovaries and testes in the present study. Farmed *Haliotis asinina* exhibited irregular periodic spawning throughout the year, with prolonged periods of spawning in male abalone compared to females (Sobhon *et al*., 1999). This pattern was reflected by the prolonged period of maximum ripeness in males compared to females. Spawning in the present study may have been sporadic among individuals or in small groups, as was also seen in wild abalone and farmed abalone (Wood and Buxton, 1996; Sobhon *et al*., 1999).

Relatively more females fed FM S had consistently more ovaries that were in the ripe stage over the one-year trial compared to abalone fed the single protein diets and FM SM, where early maturing and late maturing ovaries were more prevalent. The replacement of soya with sunflower meal did not appear to have similar effects on the frequency distribution of ripe ovaries, but it resulted in more abalone with testes that consistently showed signs of ripeness and relatively more gonads that were spent throughout the one-year study. The ovaries of farmed *H. midae* fed a combination diet of fishmeal and soya contained predominately stage eight oocytes compared to females that were fed the FM-only based diet, where ovaries contained predominately Stage-7 oocytes (Ayres, 2013). Wu *et al.* (2019) showed that an increase in dietary soya inclusion resulted in an increase in the percentage of Stage-7 oocytes in farmed *H. midae* (Wu *et al.*, 2019). These authors did not conduct gonad histological assessments on male abalone, as the maturation process is easier to monitor in females and is commonly the preferred choice for reproductive studies (Gurny and Mundy, 2004). It has been hypothesized that the differences seen due to the soya inclusion were as a result of the uptake and production of nutrients (Ayres, 2013; Wu *et al.*, 2019). Prolonged periods of ripening and spawning may be due to variations in temperature and availability of nutrients as suggested for clams (Capinpin and Corre, 1996). Temperature was consistent between treatments throughout the study, which suggests that the combination of fishmeal and soya were more favourable in maintaining ripe ovaries throughout the year in females, while the inclusion of sunflower meal may have been more effective in producing more abalone with ripe testes throughout the year. A dose-dependent study on the effects of sunflower meal on spermatogenesis would be required to further understand the effects of this alternate protein source on reproduction in male, farmed *H. midae.* Although dietary treatment had no influence on the fatty acid profiles of abalone gonad tissue (Chapter 3), a possible reason for the two combination diets maintaining relatively more ripe ovaries and testes may have been linked to essential amino acids or the bioavailability of nutrients from both fishmeal and a plant protein source that was more favourable for females and males.

Necrotic oocytes and consequently degenerative ovaries were found throughout the study. Necrosis is the breakdown and reabsorption of all unspawned genetic material immediately after spawning. This activity has been found to occur in the ovaries of *H. midae* even during the early maturing phase and during vitellogenesis (Wood and Buxton, 1996). At least half of the ovaries in all the treatments at day 133 and 182 were in the degenerative stage, followed by a decrease in degenerative ovaries at day 224, coupled with the presence of spent ovaries, which suggests that a spawning event had occurred. This can further be corroborated by the gradual decrease in gonad bulk indices from day 133 onwards, indicating a spawning event (Chapter 2). However, necrotic sperm was not visible under light microscopy. Wood and Buxton (1996) observed necrotic sperm after apparent spawning, using light microscopy, however, a description or images of the necrotic sperm was not provided in the study.

Oocyte densities

The number of oocytes in the ripe stage was similar for abalone in all treatments. Farmed *H. midae* fed a fishmeal-only based diet and a combination of fishmeal and soya showed similar oocyte densities (Ayres, 2013), as reflected in the present study. The inclusion of soya at graded levels also did not influence oocyte densities (Wu *et al.*, 2019). In addition, the replacement of soya with sunflower meal, as well as the feeding of a soya-only based diet did not have an effect on oocyte densities in abalone with ripe gonads. The ripe phase of oogenesis falls under the growth phase, where vitellogenesis takes place and requires the uptake of nutrients, as opposed to the proliferative phase, during which oogonal cells increase by mitotic multiplication (Anderson, 1974). Therefore, this suggests that all diets were sufficient in providing nutrients to produce mature oocytes. However, abalone that were fed FM S required significantly less feed to meet the nutritional requirements as indicated by the lower FCR values (Chapter 2), both during and outside the peak reproductive periods.

Sex steroids

The role that sex steroids play in the reproduction of abalone has not been studied and understanding the steroid profiles is useful in understanding the possible link they have with gametogenesis. The assessment of gonad maturation that is linked to differences caused by different dietary protein sources in farmed abalone was central. Both oestradiol and testosterone were measured in female and male abalone haemolymph in all treatments in the present study. However, non-detectable levels of oestradiol were recorded between 30 to 44 % of the haemolymph samples in females and males throughout the year, while testosterone had higher frequencies of non-detectable concentrations, ranging 47 – 67 % and 36 – 56 % in female and male abalone, respectively. Due to the large frequency of non-detectable values, the descriptive five-number summary was used, thus allowing the comparison of median values to concentrations provided in the literature as this method is less affected by outliers in data sets that are not normally distributed. Linear regression analysis was used and although the r^2 values were low, despite significant relationships, a better understanding of the data set over the one-year period was drawn, given the limitations presented with the large fluctuations and variability in sex steroid concentrations throughout the study.

Few studies have looked at steroid hormone concentrations in the haemolymph instead of concentrations in the gonadal tissue. As seen in vertebrates, Bose *et al*. (1997) suggested that steroids synthesized at various sites are released into the haemolymph and are positively correlated to the sexual phase of the animal. However, concentrations in the haemolymph have been reported to be lower than in the gonad and digestive gland tissue in molluscs (D'Aniello *et al*., 1996; Croll and Wang, 2007). This may be the reason for the high frequencies of undetectable levels of both hormones in females and males throughout the study.

The median testosterone levels in the haemolymph of *H. midae* fell within the range of mean concentrations in the haemolymph of other gastropods, while oestradiol concentrations in this species were much lower than those depicted in other molluscs. The adult garden snails (*Helix aspersa*) measured 300 pg ml^{-1} of testosterone in the haemolymph, much lower than the testosterone in the male adult giant African land snail (*Achatina fulica*) which measured approximately 690 pg ml^{-1}, while females had the lowest concentration of approximately 33 pg ml^{-1} (Le Guellec *et al*., 1987; Bose *et al*., 1997). However, the sex of the adult garden snails was not differentiated prior to analysis so samples may have been taken from females and males (Le Guellec *et al*., 1987). Hermaphroditic African land snail had around 615 pg ml^{-1} of testosterone and 6340 pg ml^{-1} of oestradiol, while values in females were approximately 2000 pg ml^{-1} of oestradiol, with males having non-detectable concentrations in the haemolymph (Bose *et al*., 1997). Adult male *Octopus vulgaris* had 500 pg ml^{-1} of testosterone and 80 pg ml^{-1} of oestradiol in the haemolymph (D'Aniello *et al*., 1996). Thus, comparing concentrations between molluscs is difficult as the ranges of sex steroids in molluscs is variable.

In this study, females and males displayed similarities and dissimilarities in median oestradiol and testosterone concentrations in the haemolymph, as well as fluctuations throughout the one-year experiment. Similarities in oestradiol concentrations between females and males have been reported in other molluscs, such as the mud snail (*Ilyanassa obsolete*), clam (*Mytilus edulis*) and scallop (*Patinopecten yessoensis*) (Osada *et al*., 2004; Gauthier-Clerc *et al*., 2006;

Sternberg *et al*., 2008), while studies have reported an abundance of estrogens in females and androgens in males (De Longcamp *et al*., 1974; Ketata *et al*., 2007; Smolarz *et al*., 2018). In contrast, the opposite has been reported in the blue mussel (*Mytilus edulis trossulus*) where females had higher testosterone concentrations and males contained higher oestradiol concentrations (Zabrzańska *et al*., 2015). Therefore, hormone concentrations varied between species and sex with no particular pattern of sex related differences amongst them (Giusti and Joaquim-Justo, 2013).

There was no relationship between oestradiol and testosterone concentrations in the haemolymph of animals within in each treatment, indicating that both sex steroids were not increasing or decreasing concurrently in the animals. The lack of relationship between oestradiol and testosterone concentrations suggest the possible role that aromatase might play in converting androgens to estrogens in abalone. However, although aromatase activity has been reported in a number of molluscs, including gastropods and bivalves (Matsumoto *et al*., 1997; Morcillo and Porte, 1999; Le Curieux-Belfond *et al*., 2001; Lavado *et al*., 2006), the activity is low compared to vertebrates and its effects are possibly not related to aromatization (Scott, 2018). The Taiwan abalone (*Haliotis diversicolor supertexta*) was exposed to tributyltin, a well-known endocrine disrupting compound and the haemolymph protein expression profile of the animals was assessed (Zhou *et al*., 2010). An aromatase-like protein was identified, which was down-regulated upon tributyltin exposure and it was suggested that this was likely caused by inhibition of aromatase activity, which could cause dysregulations in hormones (Zhou *et al*., 2010). Female giant abalone (*Haliotis madaka*) exposed to tributyltin and triphenyltin were masculinized (Horiguchi *et al*., 2005). Imposex in gastropods has been increasingly documented and the inhibition of aromatase activity by endocrine disrupting compounds that leads to increasing androgen levels has been suggested to be the cause (Oberdörster and McClellan-Green, 2002; Horiguchi *et al*., 2005). However, it was discovered that the effects of tributyltin were exerted via the retinoic acid receptor pathway (Horiguchi *et al*., 2008). To date, the evidence that suggests molluscs can synthesize oestradiol is considerably low (Scott, 2018), therefore, any conversion of oestradiol from testosterone remains to be evaluated in abalone.

Sex steroids and reproduction

There was a significant relationship between haemolymph oestradiol concentration and time for females in all treatments, while no relationship was seen in haemolymph testosterone levels. This indicates that despite the constant fluctuations and large variability in oestradiol levels, oestradiol levels were being accumulated over time. The rate of increase in haemolymph oestradiol concentration was higher in females that were fed FM S compared to females from the other three treatments, but concentrations in females all increased to the point of similar oestradiol levels at the end of the study. When corroborated with the gonad histological data, this may suggest that the higher rate of change in haemolymph oestradiol concentration in animals that were fed FM S were able to produce more ripe gonads throughout the study. However, although the rate of change in females fed FM S was faster, haemolymph oestradiol concentrations never exceeded 0.47 pg ml^{-1}, along with less variability over the year, while animals from the other three treatments exceeded this concentration throughout the study, with maximum concentrations exceeding 0.80 pg ml^{-1}. This may suggest that females that consumed the FM S diet maintained oestradiol concentrations in the haemolymph, which may have assisted in maintaining relatively more ripe ovaries throughout the study. Females that were fed the single-protein based diets and FM SM combination may have been accumulating more esterified steroids than those that were fed FM S and since the assay determined total oestradiol concentrations, this could not be differentiated and although the concentrations were higher, the sex steroids were not available for metabolism. Janer *et al.* (2005) found a dose-dependent effect of esterified oestradiol concentrations in the mussel (*Mytilus galloprovincialis*) when exposed to increasing oestradiol concentrations, while free oestradiol were only elevated when exposed to high levels. The eastern mud snail (*Ilyanassa obsolete*) maintained a constant free testosterone concentration, while the remaining testosterone were esterified (Gooding and LeBlanc, 2004). Female abalone appear to preferentially accumulate oestradiol and not testosterone over the one-year period and the reason for this remains unclear. A study that exposes abalone to increasing rates of dietary steroids and measuring both free and esterified steroids would have to be conducted to determine the ability for *H. midae* to maintain and accumulate free and esterified sex steroids, respectively. It is possible that the diets themselves contained sex steroids since the diets were comprised of fishmeal and may have been derived from reproductively mature teleost fishes with high levels of androgens and estrogens (Sower and Iwamoto, 1985). It is also possible that the gonads of these fishes used in the production of fishmeal were incorporated into the diets.

Peaks in oestradiol and testosterone levels were seen in females, but these were not consistent with the known spawning peaks and periods of maximum ripeness. Oestradiol and testosterone concentrations have been found to peak during the reproductive season in the female New Zealand mud snail (*Ilyanassa obsolete*) (Sternberg *et al*., 2008; Gust *et al*., 2011), while Ketata *et al*. (2007) found that the oestradiol concentrations of female clams (*Ruditapes decussatus*) increased at the beginning of vitellogenesis and testosterone concentrations increased at the end of gametogenesis. The oestradiol concentration in the gonad of the cockle (*Fulvia mutica*) and clam (*Mya arenaria*) peaked during the beginning of gametogenesis and during a spawning event (Gauthier-Clerc *et al*., 2006; Liu *et al.,* 2008). Therefore, inconsistencies have been found in molluscs and the patterns in the present study do not appear to relay any of those seen in other molluscs. Furthermore, changes in sex steroid hormone levels have been found to better correlate with water temperature as opposed to gamete development in the blue mussel (*Mytilus trossulus*) (Smolarz *et al*., 2018), which in this study is unlikely since water temperature was similar in all treatments throughout the one-year period, but spikes and troughs in both steroid hormones were variable for both sexes in all treatments. It is not likely that oestradiol and testosterone concentrations play a role regulating oogenesis since there was no consistent pattern in sex steroids during this period. Furthermore, the overall increase in oestradiol concentrations in the haemolymph of females from all treatments over time, despite an evident spawning peak in the middle of the study as indicated by GBI values (Chapter 2), suggests that oestradiol does not play a role in regulating oogenesis and indicates the ability of abalone to accumulate oestradiol over time.

The oestradiol concentrations in the haemolymph of male abalone had a significant positive relationship with time, but only when a plant protein source was present in the diet. This suggests that this may have been diet related. Male abalone have the ability to accumulate oestradiol, particularly when soya and sunflower meal were present in the diet. This accumulation, however, was not observed in testosterone levels since male abalone that were fed S and FM SM resulted in a significant negative relationship between haemolymph testosterone concentration and time. The presence of phytoestrogens in soya and its subsequent estrogenic effects have been well documented in fish (Pelissero *et al*., 1991; El-Sayed *et al*., 2012), but studies on molluscs are lacking. Phytoestrogens are considered antinutrients and are not found in sunflower oil cake, which may have been the reason why the inclusion of sunflower oilcake at varying inclusion rates had no estrogenic effects on male African catfish (*Clarias gariepinus*) with similar, low (< 1 ng ml^{-1}) plasma oestradiol levels over a period of four months (Nyina-Wamwiza *et al.*, 2012). A similar study looked at the effect of diet on the growth, gonad development and sex steroid hormones in *Oreochromis* sp., however, the protein sources used were fishmeal and cottonseed meal and that study focused on the presence of gossypol, an antifertility agent, which is not found in sunflower meal either (Rinchard *et al.*, 2002). The oestrogenic content in sunflower meal as well as the possible estrogenic effects that sunflower meal may have on farmed abalone remains to be tested.

Male abalone from all treatments exhibited maximum ripeness from day 91 to day 273, with fluctuating median values for both oestradiol and testosterone concentrations during this period. The maximum testosterone concentration for each treatment was recorded between days 91 and 273, but non-detectable levels were also recorded at numerous sampling times for animals in all treatments. Abalone fed FM SM had consistently more ripe and spent testes compared to abalone in the other treatments; however testosterone concentrations never exceeded 837 pg ml^{-1}, while those fed FM and S reached maximum concentrations of 1207 and 1190 pg ml^{-1}, respectively. Like the females, it could be possible that males that were fed FM, S and FM S were differentially accumulating testosterone esters and had higher rates of hydrolysis, since testosterone did not appear to accumulate over time. Mature freshwater snail (*Biomphalaria alexandrina*) had a higher concentration of testosterone in the haemolymph than immature or spent snails (Omran, 2012), while testosterone levels were highest in the gonad tissue of the male mud snail (*Ilyanassa obsolete*) during the reproductive season **(**Sternberg *et al.*, 2008). In the male clam, *Mya arenaria*, testosterone concentrations also peaked during spawning (Gauthier-Clerc *et al.*, 2006), while testosterone levels in the gonads of clam, *Ruditapes decussatus*, increased at the end of gametogenesis in males (Ketata *et al.*, 2007). The increase and decrease in oestradiol and testosterone concentrations, respectively suggest that oestradiol instead of testosterone is more prevalent in the haemolymph of male abalone, but fluctuations in testosterone concentrations did not follow particular patterns that coincided with peak spawning periods or periods of maximum ripeness. During the periods of maximum ripeness in males, at least 50 % of the sampling times, non-detectable median concentrations were recorded, while oestradiol concentration fluctuated inconsistently between treatments.

Conclusion

The role played by sex steroids in mollusc reproduction and endocrinology is still unclear (Capinpin and Corre, 1996; Giusti and Joaquim-Justo, 2013); however, the use of gonad histological assessments combined with sex steroid assays has allowed the exploration of this possibility in farmed abalone. Gametogenesis in abalone fed single-protein based diets and combination diets were assessed through the identification and quantification of oocyte and spermatocyte development. The sex steroids, oestradiol and testosterone were quantified in the haemolymph of both females and males in all dietary treatments and exhibited changes over time, with no relationship between the two steroids in animals from each treatment.

Dietary protein sources had an effect on the frequency distribution of maturity stages, where females that were fed FM S produced more ripe gonads and males that were fed FM SM contained more testes that showed signs of ripeness over the one-year study. However, abalone from all treatments exhibited periods of overlapping maximum ripeness in females and males, with males having prolonged periods of relatively more ripe testes. Spawning periods indicated by decreasing GBI values (Chapter 2) were corroborated by the relatively high proportion of degenerative and spent ovaries. The identification of necrotic sperm requires further research as these could not be identified in the present study.

Based on this study, it is suggested that the changes in sex steroid levels that are potentially linked to gametogenesis varied and that these patterns are not as clear as usually seen in fish, where those fluctuations are linked with specific endocrinological effects (Scott, 2018).

Dietary protein influenced the sex steroid concentrations in females and males, exhibiting fluctuations throughout the one-year study, but with no distinct pattern linked to gametogenesis. Female abalone appear to have the ability to accumulate oestradiol in the haemolymph, while only the presence of a plant-protein source resulted in oestradiol accumulation in males, however, their subsequent effects on gametogenesis remain unclear. Although the inclusion of soya and sunflower meal were more favourable in terms of gonad ripeness in females and males, respectively, the inconsistent fluctuations in both sex steroids in abalone showed no distinct patterns linked to gametogenesis.

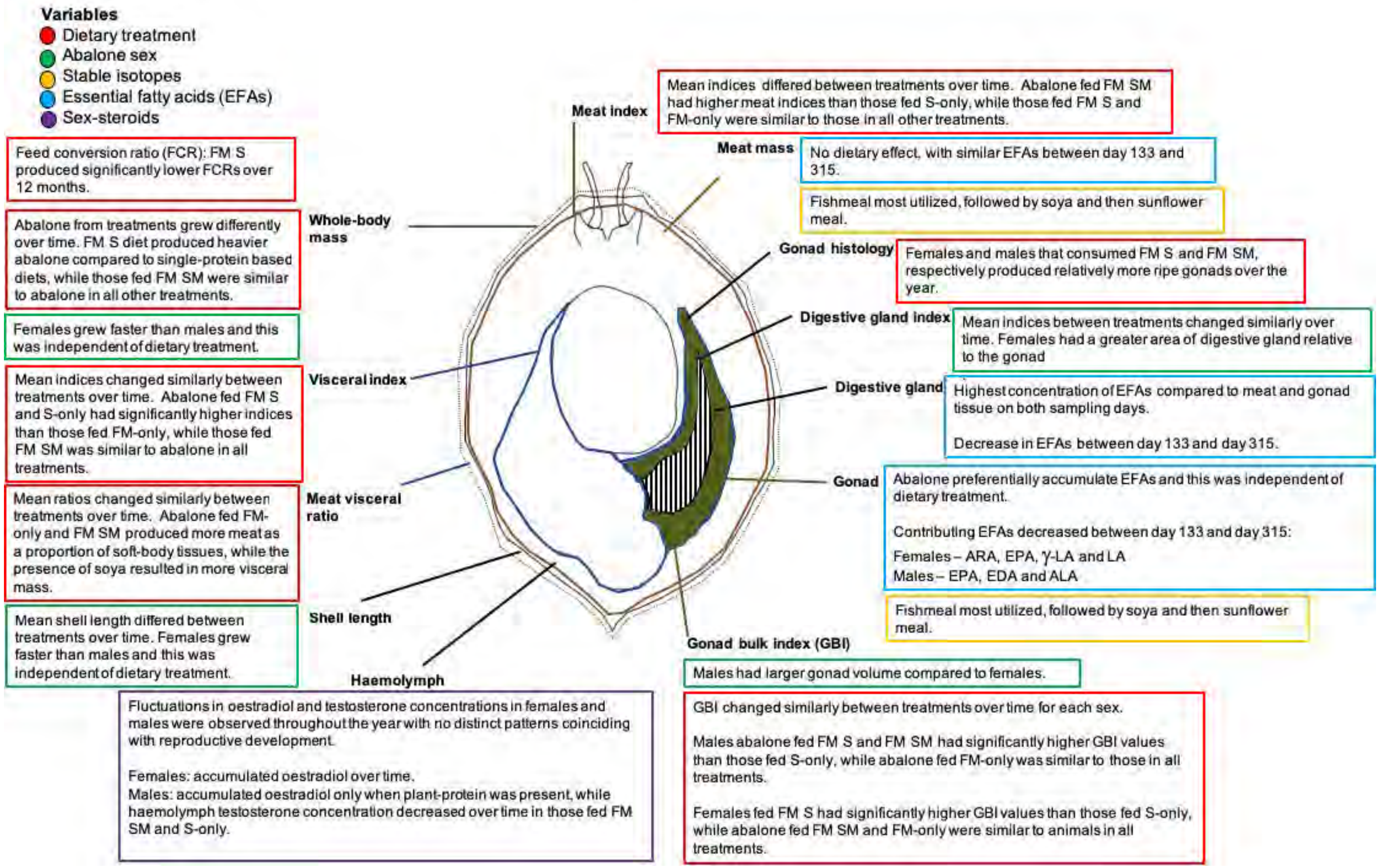
CHAPTER 5

CONCLUSION

The aim of this research was to use a combination of techniques to contribute to the understanding of the relationship between growth, reproductive and nutritional physiology of farmed *Haliotis midae*. The effects of the dietary protein source on the growth and reproduction of *H. midae* were explored in Chapters 2 – 4. These experiments were designed to examine the allocation of protein and nutritional components to somatic and reproductive tissue. The research included a first attempt to investigate the role that sex steroid hormones plays during gametogenesis. The methods used in this investigation included the following:

1) stable isotope analysis was used to determine the relative contribution of fishmeal, soya and sunflower meal to the growth of somatic and reproductive tissue;
2) fatty acid analysis made it possible to ascertain the allocation of essential fatty acids (EFA) from different protein sources in somatic and reproductive tissue during periods of peak reproductive investment and during a quiescent phase; and
3) gonad histological assessments on both females and males was tested coupled with haemolymph steroid assays in order to stage reproductive development and to determine the potential role that estradiol and testosterone may have in gametogenesis in this species, and to see if these related to dietary protein source.

This trial was conducted over one-year and on a full commercial scale that included a total of 780 kg of abalone stocked into 16 abalone raceways on an operational abalone farm. The length and scale of this study was designed so as to cover an entire reproductive cycle and to allow for measurable changes in weight gain to occur under full commercial conditions. Based on



Feed conversion ratio (FCR): FM S produced significantly lower FCRs over 12 months.

Abalone from treatments grew differently over time. FM S diet produced heavier abalone compared to single-protein based diets, while those fed FM SM were similar to abalone in all other treatments.

Females grew faster than males and this was independent of dietary treatment.

Mean indices changed similarly between treatments over time. Abalone fed FM S and S-only had significantly higher indices than those fed FM-only, while those fed FM SM was similar to abalone in all treatments.

Mean ratios changed similarly between treatments over time. Abalone fed FM-only and FM SM produced more meat as a proportion of soft-body tissues, while the presence of soya resulted in more visceral mass.

Mean shell length differed between treatments over time. Females grew faster than males and this was independent of dietary treatment.

Mean indices differed between treatments over time. Abalone fed FM SM had higher meat indices than those fed S-only, while those fed FM S and FM-only were similar to those in all other treatments.

No dietary effect, with similar EFAs between day 133 and 315.

Fishmeal most utilized, followed by soya and then sunflower meal.

Females and males that consumed FM S and FM SM, respectively produced relatively more ripe gonads over the year.

Mean indices between treatments changed similarly over time. Females had a greater area of digestive gland relative to the gonad

Highest concentration of EFAs compared to meat and gonad tissue on both sampling days.

Decrease in EFAs between day 133 and day 315.

Abalone preferentially accumulate EFAs and this was independent of dietary treatment.

Contributing EFAs decreased between day 133 and day 315:

Females – ARA, EPA, γ-LA and LA

Males – EPA, EDA and ALA

Fishmeal most utilized, followed by soya and then sunflower meal.

Males had larger gonad volume compared to females.

Fluctuations in oestradiol and testosterone concentrations in females and males were observed throughout the year with no distinct patterns coinciding with reproductive development.

Females: accumulated oestradiol over time.

Males: accumulated oestradiol only when plant-protein was present, while haemolymph testosterone concentration decreased over time in those fed FM SM and S-only.

GBI changed similarly between treatments over time for each sex.

Males abalone fed FM S and FM SM had significantly higher GBI values than those fed S-only, while abalone fed FM-only was similar to those in all treatments.

Females fed FM S had significantly higher GBI values than those fed S-only, while abalone fed FM SM and FM-only were similar to animals in all treatments.

Figure 5.1: Summary of data chapters 2 – 4, depicting the results of dietary treatment, abalone sex, stable isotope analysis, essential fatty acids and haemolymph sex steroid hormones on the different components of *Haliotis midae* growth and reproductive output. Dietary treatments include fishmeal-only (FM), soya-only (S), fishmeal-soya (FM S) and fishmeal-sunflower meal (FM SM) based diets. Day 133 and 315 represented the periods of highest and lowest gonad volume, respectively.

the combination of results from all studies conducted for this thesis, Figure 5.1 presents the variables that were monitored over the year and their influence on abalone growth, reproduction and nutritional physiology. These insights into abalone physiology contribute to our understanding of the physiology of this commercially important species and they may also be useful to farm managers. The key findings from the three data chapters have been summarized in Figure 5.1, highlighting the conclusions and the limitations involved in these studies. The novel contribution of this research towards abalone nutritional physiology, the implications of these findings as well as potential considerations for future studies will be addressed.

Novel contribution to our understanding of abalone growth and reproduction and its relationships with dietary protein

The natural diet of haliotids comprises of micro and macroalgae, however, these gastropods successfully utilise fishmeal and land-based plant protein sources for growth as denoted by the large number of abalone nutrition studies that have been conducted over the past two decades (Britz, 1996a, 1996b; Iba, 2008; Bansemer *et al.*, 2016; Mau and Jha, 2018). Although *H. midae* are better able to utilise the protein component from macroalgae for growth compared to formulated feed (Kemp, 2018), the constant supply and efficient utilisation of these compound diets dictate its importance in the abalone aquaculture industry (Bautista-Teruel *et al.*, 2003). Feeding abalone the compound diets and measuring growth and reproductive outputs translated the effect of the diet as a whole into the individual components (somatic tissue and reproductive tissue). However, stable isotopes were used as a tool to trace the allocation of the individual protein ingredients into the respective tissues over time in sexually mature abalone, which has not been explored before.

The allocation of fishmeal to somatic and reproductive tissue was the greatest and increased during increased reproductive investment, suggesting the importance of fishmeal in the metabolism of both tissue types. Kemp (2018) fed juvenile abalone (approximately 7.0 g abalone^{-1}) a mixed diet of formulated feed and macroalgae and found that macroalgae contributed more to tissue growth and that fishmeal was poorly utilised compared to soya. This discrepancy is likely due to the size of the abalone, since sexually mature, reproductively active animals are likely to have energy requirements that differ from those of rapidly growing younger animals (Barkai and Griffiths, 1988). This research emphasises the necessity for laboratory studies in obtaining data required for the use of stable isotopes and mixing models in abalone. In terms of abalone physiology, these findings suggests that the ability for *H. midae* to utilise fishmeal and soya is dependent on the size of the animals. Only one other study on *H. midae* used stable isotope analysis as a tool in estimating dietary contributions (Kemp, 2018), so that this experimentation was able for the first time to contribute to our understanding of the importance of abalone body mass as a potential factor that influences nutrient allocation within the body. These findings illustrate the importance of conducting nutritional studies on mature, grow-out animals and not only juvenile abalone.

Sunflower meal has been used successfully as a soya replacement in the diets in a variety of cultured animals (Sanz *et al*., 1994; Carellos *et al*., 2005; de Morais Oliviera *et al*., 2016; Hossain and Chakraborty, 2017), but has for the first time been used in farmed *H. midae* in combination with various analyses; namely fatty acid analysis, gonad histological assessments and haemolymph sex steroid assays. The inclusion of sunflower meal in a fishmeal diet produced similar growth outputs to those that were fed a fishmeal-soya diet. The only significant difference observed when soya was replaced with sunflower meal was in the meat visceral ratio, where the presence of soya resulted in more visceral tissue as a proportion of soft tissue mass. Although fishmeal produces more muscle tissue growth, the partial replacement of fishmeal with sunflower meal appears to be favourable in the allocation of nutrients into meat instead of visceral mass. Since the EFAs in the meat and gonad tissue of abalone were not influenced by dietary protein source, the higher ratio of meat tissue was likely due to the essential amino acids from the two fishmeal and plant-protein diet combinations.

Diatoms are hypothesised to be an important additional food source for grow-out animals due to: (1) the presence of eicosadienoic acid in all the tissues, despite the absence in all the dietary treatments; and (2) the presence of eicosapentaenoic acid in the tissues of abalone fed the soya-only based diet without its precursor, α-linolenic acid in the diet. Abalone used in the present study were housed outdoors and exposed to natural light and seawater and they had full access to diatoms that are present in commercial abalone production systems. The lack of a dietary effect on the fatty acid composition of the tissues may have been attributed to the preferential accumulation of the required EFAs acquired from these diatoms and this suggests the importance of diatoms in the diet of sexually mature, animals in their grow-out phase, not only in the hatchery of abalone culture facilities. Abalone use their radula to graze on their feed and may potentially pick up diatoms from the grazing motion and this may contribute to the dietary nutrients (Nel *et al.*, 2018). These diatoms may also contain essential amino acids, affecting both growth and reproductive development.

The change in the abundance of EFAs in the gonad tissue between peak spawning and the supposed period of the lowest reproductive investment, suggests that particular EFAs are important for females (ARA, EPA, γ-LA and LA) and males (EPA, EDA and ALA). Abalone in the present study appeared to accumulate the necessary fatty acids from their respective diets, and they possibly converted essential fatty acids from their precursors and consumed an additional source of essential nutrients, such as diatoms.

Differences in gonad development were observed between dietary treatments, illustrated by higher gonad bulk index (GBI) values in abalone fed diets that included both fishmeal and a plant-protein source and this was corroborated by gonad histological assessment. Females and males that consumed the FM S and FM SM diets, respectively had more gonads that were in the ripe stages of development. However, sex steroid hormones in the haemolymph of abalone were not reflective of the differences in reproduction as a result of dietary protein source. The patterns of hormone levels typically recorded in fish were not observed in the present study, suggesting the lack of hormonal effect of the phytoestrogens in soya as previously hypothesised for abalone (Ayres, 2013; Riddin, 2013). For example, vitellogenesis (oocyte development) is generally considered to be linked to increased levels of oestradiol, and once vitellogenesis is complete, a decrease in the production of oestradiol and testosterone occurs (Levavi-Sivan *et al.*, 2006). A reason for this inconsistency may be because the ovaries and testes contain oocytes and sperm cells at all stages of development. Both oestradiol and testosterone were present in female and male abalone over the one-year study, with similar variations of both steroids measured periodically in both sexes. This prolonged interchangeable nature of steroid hormones in females and males has not been studied in this species, so that the data produced in this study has made a first contribution to the endocrinology in this species and allow for some comparative assessments. For example, oestradiol levels in fish are produced at much higher levels compared to males and androgen production in males remains high throughout sexual maturation (Devlin and Nagahama, 2002). As has been found in fish (Scott and Ellis, 2007), abalone may also have the ability to accumulate these hormones from the water via diffusion through the gills, or possibly through ingestion as the dietary treatments may have contained these steroid hormones, as suggested for land-living invertebrates (Scott, 2018).

Suggestions for future research

Abalone in the present study appeared to accumulate the necessary fatty acids from their respective diets, and they possibly converted essential fatty acids from their precursors and consumed an additional source of essential nutrients. Therefore, using a metabolomic approach in understanding abalone nutrition contributes to our understanding of the importance of artificial diets for haliotids. The metabolic responses of abalone to diets of various compositions such as varying protein sources, lipid sources, dietary energy and carbohydrates will assist in understanding previously unstudied physiological processes in abalone so as to assist farm managers in choosing an optimal diet that allows for fast growth, reduced reproductive activity of grow-out stock and a body composition that improves the marketability of the abalone.

Haliotis midae have been shown to preferentially accumulate EFAs in the present study for reproductive development and potentially convert EFAs from its prerequisites. However, the differences in growth that were observed were likely linked to the supply of essential amino acids. Particular EFAs were prominent between peak reproduction and the periods of least reproductive investment in females and males and the potential effect of these EFAs and possibly essential amino acids from different dietary sources on the quality of sperm and oocytes should be tested in future studies. The identification and allocation of individual protein sources to somatic and gonadal tissue was achieved through the use of stable isotope analysis, but using compound-specific stable isotope analysis would be advantageous in tracing individual amino acids and fatty acids from their dietary source and should be considered in future studies. Such results may be useful for broodstock management of South African abalone. Furthermore, the use of stable isotope analysis and mixing models could also be applied to the naturally occurring diatoms in grow-tanks, thereby discerning the relative contribution of diatoms to both somatic and gonadal tissue growth in mature abalone.

The sex of the abalone contributed to differences in the growth of abalone and this was independent of dietary treatment. This suggests the potential influence of pheromones secreted by either females or males, which may have an effect on the concentration of sex steroid hormones. Female *Haliotis asinina* have been shown to exhibit behavioural changes when exposed to sexually mature male abalone, with the gonad being the source of this attractant (Nhan *et al.*, 2010); however, growth and reproductive output were not measured in this study. The effects of these attractants may consequently have an effect on the growth of abalone. However, Ayres (2013) sex-sorted animals, with tanks containing only females, only males and a mixed-sex treatment and found no differences in growth and reproduction. A laboratory study may be necessary to control the presence of alternative or possibly previously undetected compounds that may influence growth and reproduction in haliotids.

The results from this research suggest that the assessment of the importance of diet on the endocrinology of farmed abalone using sex steroid hormones is a very challenging option as the results showed large fluctuations in hormone levels as well as accumulation of the hormones. It is hypothesised that more rigorous sampling, such as daily sampling instead of using 45-day sampling may lead to a better understanding of abalone endocrinology. However, because of the high proportion of non-detectable hormone levels throughout the study and although enzyme linked immunoassay kits are a useful alternative to radioimmunoassay (RIA), the use of RIA might be necessary for validation in future studies. However, due to the need to use radioactive material and involve specialised equipment, this may not be feasible in trials run on a full commercial-scale. In future studies it may be useful to determine the levels of sex steroids in both the dietary treatments as well as the incoming sea water to obtain a better estimate of the concentrations in the available sources. In addition, the potential of the presence of endocrine-disrupting compounds in diatoms could be studied. This research direction is suggested here based on the fact that diatoms may have played a role as a source of essential fatty acids, that may influence both somatic growth and reproductive activity. Thus, although the research on the endocrinology in this species could not explain all aspects of the role of steroids for gametogenesis in this species, the findings can be used as a foundation for future studies of abalone physiology. For example, hormone assays in combination with the quantification of fatty acids in somatic and reproductive tissues could be used to study the physiology of abalone that graze on diatoms as the only dietary source compared to animals receiving an artificial diet.

Implications to the abalone aquaculture industry

The ideal condition on a commercial abalone facility would be for abalone to invest their metabolisable energy into somatic growth instead of using nutrients for reproduction. The essential nutrients required for both these anabolic processes are provided through formulated pellets as well as the diatoms that naturally form on the tank surfaces (Nel *et al*., 2018). Fishmeal was the most utilised for both somatic and reproductive tissue over the one-year, followed by soya and lastly sunflower meal. The combinations i.e., fishmeal-soya and fishmeal-sunflower meal had an influence on the allocation of nutrients to tissues of the two sources within each dietary treatment. Despite the very low utilisation of sunflower meal by abalone in the somatic and reproductive tissue, the inclusion of sunflower meal resulted in more muscle tissue as a proportion of soft tissue mass. On the other hand, soya inclusion produced a higher visceral mass, while both combinations maintained similar growth rates and whole-body mass values. The visceral mass comprises of the gonad, digestive gland, respiratory and circulatory organs and during processing, it is separated from the foot muscle and discarded, with only the foot muscle being processed for market, unless the abalone are used for live export (Venter *et al.*, 2016). This would appear to be beneficial for the abalone industry; however, the inclusion of sunflower meal resulted in a higher FCR compared to soya and also required a higher fishmeal inclusion rate to maintain the required protein content. However, if increased reproductive performance was required, for example in broodstock, gonad histological assessments suggest that a FM S diet could be fed to female abalone, while a FM SM diet would be beneficial for male abalone.

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