

The effects of dietary soya and crystalline phytoestrogens  
on the growth, gonad development and histology of  
farmed abalone, *Haliotis midae*



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## ABSTRACT

The inclusion of soya as a dietary protein source in the formulated feed, Abfeed® S34 (Marifeed Pty (Ltd), Hermanus) for farmed abalone, *Haliotis midae* has resulted in larger gonads during reproductive seasons compared to the gonads of abalone fed kelp or diets that included fishmeal as the only main protein source. The aim of this study was to determine if the isoflavones present in the soya were responsible for this increase in gonad size and the subsequent effects on farmed abalone growth.

Animals weighing between 40-50 g were fed one of seven isonitrogenous and isoenergetic diets containing either 0, 25, 50 or 100 % of the soya component of the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) from September 2013 to March 2014. An additional three diets were formulated to include crystalline isoflavone (ISO). These diets were identical to the 0 % soya diet (i.e. the fishmeal only diet - FM), only ISO was included at the same rate that ISO occurred in the three soya diets. Data were analysed using a multiple forward stepwise regression analysis (MSR) to test the effects of ISO concentration, soya concentration, time, sex, time by concentration interaction and sex by concentration interaction on growth and gonad development and to identify those variables that most contributed to the model.

The inclusion of crystalline ISO failed to promote larger gonads and had no effect on abalone growth, while growth and gonad development was dose dependent on soya inclusion rates with sex and time contributing to the models. Mean monthly weight gain in males correlated with increasing soya concentrations (c) (MSR,  $y = 3.24 + 0.002c$ ,  $r^2 = 0.23$ ,  $p = 0.03$ ), ranging from  $3.11 \pm 0.55$  g abalone<sup>-1</sup> month<sup>-1</sup> to  $4.43 \pm 0.46$  g abalone<sup>-1</sup> month<sup>-1</sup>, while both male and female monthly length gain was not influenced by soya concentration with an overall mean of  $1.62 \pm$

0.05 mm abalone<sup>-1</sup> month<sup>-1</sup> (MSR,  $p = 0.05$  and  $p = 0.81$ , respectively). By December, the whole body mass, meat mass and visceral mass in both males and females decreased with increasing soya levels. However, by February, female whole body mass, meat mass and visceral mass positively correlated with soya levels. At the end of the study, male abalone fed FM with soya equivalent to the commercial feed had the highest whole body mass ( $69.00 \pm 2.48$  g abalone<sup>-1</sup>), meat mass ( $41.80 \pm 1.12$  g abalone<sup>-1</sup>), visceral mass ( $9.00 \pm 2.47$  g abalone<sup>-1</sup>) and gonad bulk index ( $42.70 \pm 9.82$  g abalone<sup>-1</sup>), while females were not influenced by soya concentrations with an overall whole body mass of  $63.46 \pm 0.79$  g abalone<sup>-1</sup>. Weight loss was observed in all treatments between February and March, probably due to a spawning event. The moisture content in the meat was not influenced by treatment, however, visceral water loss (%) was effected by both ISO and soya concentration with time and sex contributing to the model. The visceral water loss of females fed graded levels of soya decreased as a function of soya from December to March, and from December to February for males, whereas females fed ISO-enriched diets decreased as a function of ISO concentration ( $c$ ) at the end of the study from  $74.98 \pm 0.88$  to  $73.10 \pm 0.75$  % (MSR,  $y = 74.97 - 0.0025c$ ,  $r^2 = 0.20$ ,  $p = 0.048$ ).

The inclusion of crystalline ISO had no significant effect on oogenesis in female farmed *Haliotis midae*, while the distribution of the predominant oocyte stage, stage 7 (second last stage prior to spawning) was dose-dependent in abalone fed increasing soya concentration ( $c$ ) (MSR,  $y = 33.38 + 0.03c$ ,  $r^2 = 0.32$ ,  $F_{(1, 18)} = 8.52$ ,  $p = 0.01$ ). The increase in stage 7 oocytes in abalone fed FM with soya did not reduce the number of oocytes ( $44.96 \pm 3.01$  oocytes mm<sup>-2</sup>) present within the lumen, while the number of oocytes ( $o$ ) in abalone fed the FM-only based diets decreased with increasing abundance of stage 7 oocytes (MSR,  $y = 58.28 - 0.48o$ ,  $r^2 = 0.38$ ,  $F_{(1, 18)} = 12.51$ ,  $p = 0.002$ ), possibly due to the increase in size of the oocytes with thicker jelly coats.

This study provided evidence that crystalline isoflavone had no influence on abalone gonad development over five months, while soya had a dose-dependent effect on growth, gonad mass and oogenesis in farmed *Haliotis midae*. Formulated abalone feed could be manipulated at certain times of the year to obtain maximum growth. These implications and further studies were discussed.

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## TABLE OF CONTENTS

ABSTRACT.....	i
ACKNOWLEDGEMENTS.....	iv
LIST OF TABLES.....	vii
LIST OF FIGURES.....	ix
LIST OF ABBREVIATIONS.....	xvii
<b>CHAPTER 1</b>	
GENERAL INTRODUCTION.....	1
AIMS AND OBJECTIVES.....	3
<b>CHAPTER 2: EFFECT OF PHYTOESTROGENS ON GROWTH AND GONAD DEVELOPMENT</b>	
INTRODUCTION.....	4
MATERIALS AND METHODS.....	10
RESULTS.....	22
DISCUSSION.....	65
<b>CHAPTER 3: EFFECT OF PHYTOESTROGENS ON OOGENESIS</b>	
INTRODUCTION.....	77
MATERIALS AND METHODS.....	79
RESULTS.....	82
DISCUSSION.....	91
<b>CHAPTER 4</b>	
GENERAL DISCUSSION.....	100
<b>REFERENCES.....</b>	<b>105</b>

## LIST OF TABLES

**Table 1.1:** Formulation and composition of dietary treatments, including a fishmeal (FM) only based diet and FM with graded levels of isoflavone (ISO) equivalent to the ISO present in soya (S) at 25, 50 and 100 % of the S in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

**Table 1.2:** Variables used in multiple forward stepwise regression analysis.

**Table 2.1:** Proximate analysis of dietary treatments, including a fishmeal (FM) only based diet and FM with graded levels of isoflavone (ISO) equivalent to the ISO present in soya (S) at 25, 50 and 100 % of the S in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

**Table 2.2:** Isoflavone (ISO) concentrations ( $\text{mg kg}^{-1}$ ) in a fishmeal (FM) only based diet and FM with increasing rates of ISO equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed (Pty) Ltd, Hermanus).

**Table 2.3:** Biomass loss per tank between days 136 and 180 of animals fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavone (ISO) equivalent to diets with 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

**Table 2.4:** Growth of abalone fed a fishmeal (FM) only based diet and increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days. The inclusion of the following variables indicates a significant contribution to the model. c = concentration, s = sex, sc = sex by concentration, t = time, tc = time by concentration, log = log transformed data. A dash reflects a lack of contribution of all variables to the model. Data was analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

**Table 2.5:** Gonad development of abalone fed fishmeal only and increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya in the commercial feed (Abfeed ® S34, Marifeed Pty (Ltd), Hermanus) over 180 days. The inclusion of the following variables indicates a significant contribution to the model over time. c = concentration, s = sex, sc = sex and concentration interaction, t = time, tc = time and concentration interaction, log = log transformed data. Data was analysed with a multiple forward stepwise regression analysis with an error level of 5 %.

**Table 2.6:** Feed conversion ratio of animals fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed ® S34, Marifeed Pty (Ltd), Hermanus) from November to February. Means with different superscripts within each row and column indicate significant differences (RM-ANOVA,  $p \leq 0.05$ ).

**Table 2.7:** Mean ( $\pm$  standard deviation) water temperature, oxygen (O<sub>2</sub>), pH and total ammonia nitrogen (TAN) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (RM-ANOVA,  $p > 0.05$ ).

## LIST OF FIGURES

**Figure 1.1:** Graphical representation of dietary treatments, including a fishmeal (FM) only based diet and FM with soya included at the same level as the soya in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) and at 50 and 25 % of that level, and diets with isoflavone (ISO) equivalent to the ISO present in the 100, 50 and 25 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

**Figure 1.2:** (A) Photograph illustrating the measurement of the conical appendage ( $L_{ca}$ ) in a male abalone; and (B) a photograph of the cross section through the midpoint of the conical appendage where linear dimension of the digestive gland (a and b) and viscera (x and y) are illustrated. The same method was applied in measuring female gonads.

**Figure 2.1:** Mean monthly weight gain ( $\text{g abalone}^{-1} \text{ month}^{-1}$ ) and length gain ( $\text{mm abalone}^{-1} \text{ month}^{-1}$ ) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 or 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) equivalent to 12, 236, 452 and 760  $\text{mg kg}^{-1}$  of diet. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

**Figure 2.2:** Frequency of tanks in each treatment with biomass loss between days 130 and 180 of animals fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

**Figure 2.3:** Growth of abalone fed a fishmeal (FM) only based diet and increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial

feed (Abfeed®S34, Marifeed Pty (Ltd, Hermanus) over 180 days. The inclusion of the following variables indicates a significant contribution to the model. c = concentration, s = sex, sc = sex by concentration, t = time, tc = time by concentration, log = log transformed data. A dash reflects a lack of contribution of all variables to the model. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

**Figure 2.4:** The mean whole mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.5:** The average meat mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

**Figure 2.6:** The average meat mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.7:** The mean meat index (%) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180

days between September and March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

**Figure 2.8:** The average shell length of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days from September to March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

**Figure 2.9:** The average shell length of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.10:** The average condition factor of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.11:** The average condition factor of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.12:** The average meat water loss of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 %

of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.13:** The mean visceral water loss of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.14:** The mean visceral water loss of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.15:** The increase in mean visceral mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days from September to March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

**Figure 2.16:** The mean visceral mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.17:** The change in mean visceral index (%) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus)

over 180 days from September to March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.18:** The mean visceral index (%) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.19:** The change in mean gonad bulk index (GBI) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days from September to March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.20:** The mean gonad bulk index (GBI) of abalone fed a fishmeal (FM) only based and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya(S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.21:** The change in mean digestive gland (DG) index (%) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days from September to March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 2.22:** The mean digestive gland (DG) index (%) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25,

50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Figure 3.1:** Transverse sections of the mid-section of the conical appendage of a female *Haliotis midae*. (A) Oogonia (Og) stained dark blue; (B) stage 1 oocytes (St1) attached to the trabeculae (Tr) containing squamous follicular cells (Fc); (C) scallop-shaped stage 2 oocytes (St2) in clusters; (D) larger stage 3 oocytes (St3) with a light blue-stained nucleus (N) and a dark nucleolus (Nu); (E) columnar stage 4 oocyte (St4) adhering to the trabeculae alongside the digestive gland (DG); and (F) large columnar stage 5 oocytes (St5) with pink cytoplasm (Cy) containing numerous lipid droplets (v) a prominent jelly coat (JC) and chorion (C).

**Figure 3.2:** Transverse sections of the mid-section of the conical appendage in a female *Haliotis midae* depicting mature oocyte development. (A) Teardrop-shaped stage 6 oocyte (St6) with long cytoplasmic stalk (CS) and cytoplasm containing numerous lipid droplets (v); (B) stage 7 oocytes (St7) surrounded by a thick chorion (C) and jelly coat (JC); (C) stage 8 oocyte (St8) alongside the digestive gland (DG) surrounded by a thin jelly coat and thin chorion; and (D) necrotic (Ne) oocyte with a large vacuole (V), collapsed chorion (C) and disappearance of the nucleus (N). In all stages except in necrotic oocytes the nucleolus (Nu) was stained light-blue.

**Figure 3.3:** Transverse sections of the mid-section of the conical appendage in female *Haliotis midae*, depicting oocytes of animals fed different diets. (A) Animal fed a fishmeal (FM) only based diet with clusters of early maturity stage oocytes (Oc) and areas of the lumen that were devoid of oocytes (DeO); (B) animal fed the diet containing 100 % soya inclusion level, illustrating necrotic oocytes and a thick jelly (JC); (C) animal fed the FM diet with the inclusion of 100 % isoflavones, illustrating necrotic oocytes (Ne) and areas devoid of oocytes (DeO) with

a thick jelly coat (JC) surrounding all oocytes; and **(D)** animal fed the FM diet with 25 % soya inclusion level, illustrating a cluster of immature oocytes along the gonad epithelium (GEp) and the presence of necrotic oocytes (Ne).

**Figure 3.4:** Average distribution of oocyte development in female abalone, *Haliotis midae* fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either no soya, 25, 50 and 100 % of the soya (S) in the commercial feed, equivalent to 12, 236, 452 and 760 mg kg<sup>-1</sup> of diet. N = necrotic; St = stage; Og = oogonia.

**Figure 3.5:** Mean percentage of stage 7 oocytes of females fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of soya (S) in the commercial feed, equivalent to 12, 236, 452 and 760 mg kg<sup>-1</sup> (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

## LIST OF ABBREVIATIONS

A <sub>b</sub>	total number of abalone
A <sub>T</sub>	total area in photograph
C	chorion
c	concentration
CF	condition factor
CS	cytoplasmic stalk
Cy	cytoplasm
d	days
DeO	devoid of oocytes
DG	digestive gland
EDC	endocrine disrupting compound
ER	oestrogen receptor
E <sub>T</sub>	total number of oocytes
EVG	effective gonad volume
F	female
Fc	follicular cells
FCR	feed conversion ratio
FM	fishmeal
FM ISO 25	fishmeal with the addition of isoflavones equivalent to the isoflavone concentration in 25 % of the soya component in the commercial feed
FM ISO 50	fishmeal with the addition of isoflavones equivalent to the isoflavone concentration in 50 % of the soya component in the commercial feed
FM ISO 100	fishmeal with the addition of isoflavones equivalent to the isoflavone concentration in 100 % of the soya component in the commercial feed
FM S 25	fishmeal with the addition of 25 % of the soya in the commercial feed

FM S 50	fishmeal with the addition of 50 % of the soya in the commercial feed
FM S 100	fishmeal with the addition of 100 % of the soya in the commercial feed
GBI	gonad bulk index
GEp	gonad epithelium
h	hours
hER	human oestrogen receptor
ISO	isoflavone
JC	jelly coat
L	length
L <sub>f</sub>	final length
log	log transformed data
L <sub>t</sub>	initial length
M	male
M <sub>D</sub>	total dry mass of foot muscle
M <sub>DS</sub>	dry mass of meat sample
M <sub>f</sub>	final wet whole mass
M <sub>m</sub>	wet meat mass
M <sub>t</sub>	initial wet whole mass
M <sub>T</sub>	total wet meat mass
M <sub>WS</sub>	wet mass of meat samples
MSR	multiple forward stepwise regression analysis
N	nucleus
Ne	necrotic oocyte
Nu	nucleolus
o	number of oocytes mm <sup>-2</sup>

O <sub>c</sub>	oocyte clusters
O <sub>g</sub>	oogonia
O <sub>s</sub>	oocyte developmental stage
O <sub>2</sub>	oxygen
PVC	polyvinylchloride
RM-ANOVA	repeated measures analysis of variance
S	soya
Sc	sex by concentration interaction
St	stage
t	time
TAN	total ammonia nitrogen
tc	time by concentration interaction
Tr	trabeculae
v	lipid droplets
V	large vacuole
V <sub>D</sub>	dry visceral mass
V <sub>m</sub>	visceral wet mass
W	whole mass
W <sub>m</sub>	wet whole mass
W <sub>S</sub>	shucked mass
W <sub>t</sub>	current weight of abalone
W <sub>t+1</sub>	predicted weight of abalone

## CHAPTER 1

### GENERAL INTRODUCTION

*Haliotis midae* is an intensively farmed species of abalone, with products that are of high value due to the overexploitation of natural stocks and the increasing demand in Asia (Hauck and Gallardo-Fernandez, 2013). However, abalone is a slow growing animal and because of high capital investment and running costs, small improvements in growth can be beneficial (Hauck and Sweijd, 1999). Studies on growth of abalone are readily available (Britz, 1996; Capinpin and Corre, 1996; Guzmán and Viana, 1998; Kim *et al.*, 1998; Shipton and Britz, 2001; Bautista-Teruel *et al.*, 2003; Dlaza *et al.*, 2008; Ismail *et al.*, 2009; Green *et al.*, 2011; Ayres, 2013; Riddin, 2013; O'Mahoney *et al.*, 2014), but an understanding of gonad development is less readily available.

The growth rate of farmed abalone has been accelerated with the use of formulated feed, particularly feeds fortified with plant protein (Dlaza *et al.*, 2008) such as soya (Ayres, 2013). However, the inclusion of soya as a dietary protein source in the formulated feed, Abfeed®S34 (Marifeed Pty (Ltd), Hermanus) for farmed abalone, *H. midae* has resulted in larger gonads during reproductive seasons compared to gonads of abalone fed kelp or diets that included fishmeal only as the main protein source (Ayres, 2013; Riddin, 2013). This change in the investment of energy into gonad growth rather than somatic growth is not beneficial in abalone farming when abalone are processed and canned after the viscera (digestive gland and gonad) have been discarded. It has been suggested that some phytoestrogens present in soya could be responsible for an increase in gonad mass of farmed, South African abalone, *H. midae* (Ayres, 2013; Riddin, 2013).

Soya bean meal is the main source of plant protein in animal diets worldwide, due to its good essential amino acid profile, constant supply and lower cost compared to other plant protein sources (Rumsey *et al.*, 1994; Refstie *et al.*, 2000; Drew *et al.*, 2007; El-Sayed *et al.*, 2012). However, soya contains many endogenous antinutritional factors, including phytoestrogens, protease inhibitors, phytic acid and antivitamin (Burrells *et al.*, 1999; Francis *et al.*, 2001; El-Sayed *et al.*, 2012). These plant-derived phytoestrogens include genistin, glycitin, daidzin, genistein, glycitein and daidzein, with genistin and daidzin making up the majority of the total glucoside concentrations (El-Sayed *et al.*, 2012). Due to the structural similarity of isoflavones (ISO) to oestrogen, they are able to mimic and/or have anti-oestrogenic effects (Patisaul and Jefferson, 2010). The effect of dietary isoflavones is species specific and it also varies depending on the reproductive status of the animal (El-Sayed *et al.*, 2012). Oestrogenic activity of isoflavones has been reported in Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*) and the African catfish (*Clarias gariepinus*) (Bennetau-Pelissero *et al.*, 2001; Yilmaz *et al.*, 2009; El-Sayed *et al.*, 2012). The effect of ISO present in soya on the sexual differentiation in the Nile tilapia was successfully verified by the inclusion of pure, crystalline genistein and daidzein in a formulated feed (El-Sayed *et al.*, 2012).

The combinations of both plant and animal protein exhibit better growth compared to using fish meal, soya and other plant proteins as the sole protein source (Shipton and Britz, 2001; Guzmán and Vianna, 1998; Kim *et al.*, 1998; Lee, 1998; Bautista-Teruel *et al.*, 2003; Ayres, 2013).

However, the inclusion of soya resulted in a relative increase in gonad mass (Tung and Alfaro, 2012; Ayres 2013; Riddin, 2013) with an additional effect on oogenesis (Ayres, 2013). Thus, the importance of diet on reproduction needs to be considered when formulating a diet (Nyameasem and Borketey-La, 2014).

The aim of the current study was to determine whether the phytoestrogens present in soya are responsible for larger gonads and this was achieved by addressing the following objectives:

1. correlate the growth and gonad development of abalone fed diets that contain graded levels of soya to those fed diets that contain crystalline ISO at equivalent inclusion rates;  
and
2. compare oocyte histology in abalone fed either crystalline ISO or soya.

## CHAPTER 2

# THE EFFECT OF PHYTOESTROGENS ON GROWTH AND GONAD DEVELOPMENT

### INTRODUCTION

Phytoestrogens are considered endocrine disrupting compounds (EDC), because of their ability to alter endocrine functions (Jobling *et al.*, 2003; Sternberg *et al.*, 2008; Patisaul and Jefferson, 2010). This chapter outlines the importance of phytoestrogens for molluskan endocrinology and tests how growth and gonad development was influenced by these compounds in farmed abalone, *H. midae* that were fed diets that included soya or isoflavones added to a fishmeal-based diet.

Isoflavones are a class of phytoestrogens found in the soybean plant, *Glycine max*. They are among the most widely researched group of phytoestrogens (Patisaul and Jefferson, 2010). Isoflavones bind to human oestrogen receptors (hER), specifically hER- $\alpha$  and ER- $\beta$ . Although the binding affinity to hER- $\alpha$  and hER- $\beta$  is lower than to 17 $\beta$ -estradiol, they can compete with 17 $\beta$ -estradiol for binding to the ERs (Ørgaard and Jensen, 2008). Little is known about molluskan endocrinology when compared to that of vertebrates (Bannister *et al.*, 2007). To date, no studies have been published on the effects of phytoestrogens on mollusks; however, xenoestrogenic studies have been conducted (Oehlmann *et al.*, 2000; Siah *et al.*, 2003; Sternberg *et al.*, 2008; Zhang *et al.*, 2012). Xenoestrogens are synthetic EDCs that mainly result from sewage plants, industrial pollution and pesticides. They include chemicals such as tributyltin, bisphenol-A, octylphenol and ethinylestradiol (Sternberg *et al.*, 2008; Zhang *et al.*, 2012). Soft shell clams, *Mya arenaria* exposed to tributyltin exhibited a delay in sexual maturation, a lower

gonado-somatic index and low progesterone levels (Siah *et al.*, 2003). The freshwater snail, *Marisa cornuarietis* and the marine prosobranch, *Nucella lapillus*, exhibited enlargement of accessory pallial sex glands when exposed to bisphenol-A and octylphenol, while *N. lapillus* males exhibited reduced penis length and reduced prostate glands (Oehlmann *et al.*, 2000). These studies suggest that the changes in reproduction due to EDC exposure are indicative of a disruption of the reproductive endocrinology of mollusks.

Vertebrate-type sex steroids ( $17\beta$ -estradiol and testosterone) have been identified in molluskan tissue and haemolymph and have been reported to fluctuate with time of year, consistent with reproductive events (De Longcamp *et al.*, 1974; Reis-Henriques *et al.*, 1990; Matsumoto *et al.*, 1997; Di Cosmo *et al.*, 2001; Sternberg *et al.*, 2008). However, there has been little evidence that they are able to produce these steroids *de novo* (Scott, 2012). The *in vitro* release of vertebrate-type steroids has been reported in the giant African land snail, *Achatina fulica* and in the common octopus, *Octopus vulgaris* (Bose *et al.*, 1997; Kanda *et al.*, 2006). In addition, mollusks can absorb these steroids from the environment and store them in the form of fatty acid esters (Gooding and LeBlanc, 2001; Peck *et al.* 2007). Although the ER gene has been cloned in mollusks, studies showed that oestrogen signalling in mollusks was independent of ERs (Keay *et al.*, 2006; Bannister *et al.*, 2007). Studies and reviews conducted on vertebrate sex steroids in mollusks between 1970 and 2012 were critically evaluated and it was concluded that there was no substantial evidence of vertebrate steroid synthesis in mollusks, nor do mollusks have functional steroid receptors (Scott, 2012; Scott, 2013). With the evidence provided, one might hypothesise that crystalline isoflavones added to feed will not have an effect on abalone gonad development and subsequent growth, if the isoflavones cannot attach to the ER. However, their

evaluation did not include abalone and since xenoestrogens have been found to influence gonad development in molluscs, there remain many unanswered questions in this field.

Numerous studies on the effect of phytoestrogens on fish reproduction used only daidzein and genistein (Ko *et al.*, 1999; Bennetau–Pelissero *et al.*, 2001; Ng *et al.*, 2006; El-Sayed *et al.*, 2012), as they have been shown to produce the highest oestrogenic activity for hER- $\alpha$  and  $\beta$  (Miyahara *et al.*, 2003). However, different combinations may result in variable outcomes as individual isoflavones may differ in biological potency, and certain combinations may act synergistically or antagonistically (Friedman and Brandon, 2001). Thus, the effects of crystalline isoflavones on abalone growth and gonad development may be due to a number of reasons that need to be investigated.

Measuring abalone gonad mass is difficult as the gonad is located between the outer epithelial layer of the conical appendage and the digestive gland (Gurny and Mundy, 2004). To measure the gonad mass relative to the whole abalone mass would be unfeasible as it is difficult to separate the gonad from the digestive gland (Tutschulte and Connell, 1981). Therefore the gonad bulk index (GBI) was formulated to include the 3-dimensionality of the gonad. The effective gonad volume was divided by the shucked (removal of the shell) body mass to determine the gonad volume relative to the soft tissue of the abalone (Tutschulte and Connell, 1981).

However, to make calculations, a cross section of the conical appendage is required. Four sections of the ovary were taken and GBI decreased with increasing distance from the tip of the conical appendage (Hooker and Creese, 1995). Therefore, the cross section of the conical appendage must be measured from the same location for all abalone sampled within a study (Gurny and Mundy, 2004). A drop in mean GBI indicates a spawning event (Tutschulte and Connell, 1981; Wood and Buxton, 1996), however, results should be interpreted with care in the

presence of necrotic oocytes as the gonad volume may be reduced by oocyte degeneration and reabsorption (Lleonart, 1992). The GBI of farmed *H. midae* peaked during the summer months of Oct 2012 and Jan 2013, followed by a decline (Ayres, 2013). Male abalone had higher GBI values than female abalone (Ayres, 2013). In addition, abalone fed a diet that contained soya as one of the protein sources had significantly higher GBI values than abalone fed a fishmeal-based or kelp diet (Riddin, 2013; Ayres, 2013).

The visceral index and meat mass index measures the mass of viscera and meat as a proportion of the whole mass of the abalone. These measurements give an indication of the allocation of nutrients, as the meat index decreased during the periods of increased GBI, suggesting that meat growth was compromised during gonad growth (Ayres, 2013). However, reproductive investment did not reduce abalone whole mass gain, as abalone continued to increase in weight, possibly due to the increase in gonad mass (Riddin, 2012). A reduction in daily growth rate in the Philippine abalone, *Haliotis asinina* was due to energy being channelled into gonad development (Capinpin and Corre, 1996). Therefore, market value or product quality of abalone that are sold live are not affected by the allocation of energy investment into gonad growth as the viscera form part of the product (Ayres, 2013). However, abalone that are processed and canned are losing mass due to the discarded viscera.

The digestive gland (DG) index is a measure of the percentage of the DG in relation to the gonad tissue. The DG decreases in size as gonad tissue mass increases during reproductive development as nutrients are drawn from the DG (Carefoot *et al.*, 1998; Litaay and De Silva, 2003). The DG index of abalone fed a fishmeal-only based diet was significantly higher compared to abalone fed a diet that included both FM and soya, indicating a lower proportion of gonad tissue in relation to

DG size (Ayres, 2013). Therefore, the inclusion of soya resulted in higher investment into gonad tissue.

Glycogen is the major storage form of energy in abalone and muscle glycogen content can be correlated to water loss following handling and transport (Laas and Vosloo, 2010). For every gram of glycogen stored, three to four grams of water are required (Kreitzman *et al.*, 1992). As glycogen levels decreased, lipid levels increased (Webber, 1970) and the moisture content in the viscera decreased with increasing lipid level (Liyana-Pathirana *et al.*, 2002; Riddin, 2012).

Lipids are an important component of the gonad (O'Mahoney *et al.*, 2014) and are the nutritive storage product of the eggs (Webber, 1970). Lipids were accumulated in the gonad during gonad development (Laas and Vosloo, 2010), while glycogen content decreased (Webber, 1970).

Abalone that were fed a FM-only based diet and a combination of FM and soya diet exhibited similar water loss in both meat and viscera (Ayres, 2013). However, water loss increased after spawning and decreased during spawning (Ayres, 2013). Therefore, water loss may give an indication of the seasonal variation in lipid accumulation for spawning events.

To date, no published research has evaluated the effect of phytoestrogens on abalone growth and reproduction. The aim of this study was to determine the growth and gonad development of farmed abalone, *H. midae* fed a fishmeal-only based (FM) diet and FM with increasing concentrations of isoflavones (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya in the commercial feed, Abfeed@S34. The aim was achieved by addressing the following objectives:

- 1) produce a FM diet and FM with 0, 25, 50 and 100 % of the soya present in Abfeed@S34;

- 2) produce a FM diet with the addition of crystalline ISO that reflect the concentrations of natural, plant derived isoflavones in the soya diets;
- 3) quantify the growth morphometric data (whole mass, meat mass, shell mass, shell length) and gonad development (visceral mass, GBI and DG index) of abalone fed these dietary treatments;
- 4) determine whether water loss was influenced by the inclusion of ISO or soya;
- 5) determine the correlation between growth and gonad mass development and ISO and soya concentration; and
- 6) compare the correlation between growth and gonad development of ISO-fed animals to soya-fed animals.

## MATERIALS AND METHODS

### *Experimental system*

The experiment was conducted at Aquafarm Development (Pty) Ltd, Hermanus, South Africa (34°26'04.35"S; 19°13'12.51"E) for 180 days from September 2013 to March 2014. Animals were kept in thirty-five 85 L (660 x 430 x 360 mm) plastic containers. Within each tank was an oyster mesh basket (550 x 350 x 380 mm) that contained a vertical rack with six black high impact polyethylene sheets (530 x 190 x 2 mm), separated with 15 mm polyvinylchloride (PVC) pipes. An asbestos feeder plate (490 x 100 x 2 mm) with a surface area of 0.94 m<sup>2</sup> was placed horizontally on the racks, six centimetres below the water surface. Aeration was supplied via 20-mm PVC piping that was raised 50 mm from the bottom of the tank below the oyster mesh basket. Holes were drilled in the pipes that ran along the lengths and one width of the tank. Seawater was pumped from the ocean and was filtered through a micro screen drum filter with a pore diameter of 90 µm and gravity-fed via the farm's header tank to each experimental tank at 20 L h<sup>-1</sup>, thus forming a flow-through system. Tanks were drained, scrubbed and the remaining waste was siphoned off once a week. One tank in the system did not contain abalone to allow for shifting baskets when cleaning.

### *Experimental animals and acclimation*

Hatchery-reared abalone, *H. midae* (40 – 50 g abalone<sup>-1</sup>, 53 – 59 mm abalone<sup>-1</sup>, 26 months old, n = 2526), spawned from several females were used in the study. The abalone were raised in the farm hatchery and subsequently subject to farm management procedure prior to the experiment. During this time, the abalone were fed a commercial abalone feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus). They were placed into the experimental tanks three weeks prior to the

experiment and allowed to acclimate. They were fed the same commercial feed during acclimation. Each tank was stocked with 29 males and 29 females.

#### *Dietary treatments and experimental design*

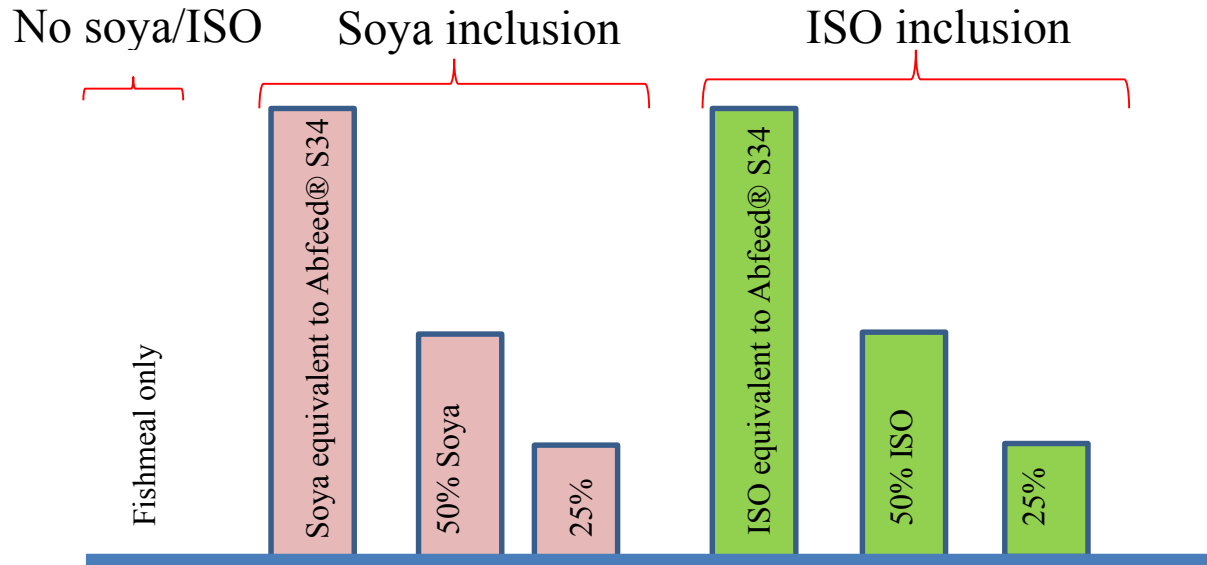
Animals from a treatment were fed one of seven isonitrogenous and isoenergetic diets (Table 1.1). The diets were formulated to contain 34 % crude protein, 5 % lipid and 17.7 MJ/kg energy. There were five replicates for each of seven treatments, randomly allocated to the system. One treatment was a fishmeal (FM) diet with no inclusion of soya and isoflavones (ISO). Three dietary treatments contained soya at graded levels. Soya (S) 100 contained the same amount of soya currently used in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus), while FM S 50 and FM S 25 contained FM with 50 % and 25 % of soya, respectively. Three FM diets contained graded levels of ISO. The treatment ISO 100 contained a concentration equivalent to that of isoflavones in the FM S 100 diet. The isoflavone concentrations in ISO 50 and ISO 25 were equivalent to the concentrations of each ISO found in FM S 50 and FM S 25, respectively (Table 1.1 and Figure 1.1).

**Table 1.1:** Formulation of dietary treatments, including a fishmeal (FM) only based diet and FM with graded levels of isoflavone (ISO) equivalent to the ISO present in soya (S) at 25, 50 and 100 % of the S in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

Ingredient	FM S 100	FM S 50	FM S 25	FM ISO 100	FM ISO 50	FM ISO 25	FM
Fishmeal	325.0	390.4	423.1	455.9	455.9	455.9	455.9
Soya	190.3	95.1	47.6	0.0	0.0	0.0	0.0
Grain starch*	466.7	481.2	488.5	495.8	495.8	495.8	495.8
Vitamin mix*	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Marine fish oil	9.0	5.9	4.3	2.8	2.8	2.8	2.8
Non-nutritive filler**	8.0	26.3	35.4	43.7	44.1	44.3	44.6
Isoflavones:							
Daidzin	0.0000	0.0000	0.0000	0.3580	0.1790	0.0895	0.0000
Glycitin	0.0000	0.0000	0.0000	0.0479	0.0239	0.0119	0.0000
Genistin	0.0000	0.0000	0.0000	0.4556	0.2278	0.1139	0.0000
Daidzein	0.0000	0.0000	0.0000	0.0113	0.0056	0.0028	0.0000
Glycitein	0.0000	0.0000	0.0000	0.0029	0.0014	0.0007	0.0000
Genistein	0.0000	0.0000	0.0000	0.0124	0.0062	0.0031	0.0000
<b>Total</b>	<b>1000.0</b>	<b>1000.0</b>	<b>1000.0</b>	<b>1000.0</b>	<b>1000.0</b>	<b>1000.0</b>	<b>1000.0</b>
Protein (%)	34.00	34.00	34.00	34.00	34.00	34.00	34.00
Lipid (%)	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Gross energy (MJ/kg)	17.70	17.70	17.70	17.70	17.70	17.70	17.70

\* Proprietary information (Marifeed Pty (Ltd), Hermanus)

\*\* Diatomaceous earth



**Figure 1.1:** Graphical representation of dietary treatments, including a fishmeal (FM) only based diet and FM with soya included at the same level as the soya in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) and at 50 and 25 % of that level, and diets with isoflavone (ISO) equivalent to the ISO present in the 100, 50 and 25 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

Abalone were fed a known amount of feed daily between 16h30 and 17h00. This volume of feed was determined for a 45-day sampling period, and was determined using farm feeding methods based on shell length, and a farm average monthly shell length gain of 1.9 mm month<sup>-1</sup> abalone<sup>-1</sup> (Equation 1) to calculate weight (Equation 2) (Yearsley, Aquafarm Development (Pty) Ltd, pers. comm. 2013). The total feed required was the product of the total number of abalone (Ab) in each tank, weight gain, where  $W_{t+1}$  was the predicted weight and  $W_t$  was the current weight and the on-farm feed conversion ratio (FCR) of 1.2 g weight gain<sup>-1</sup> (Yearsley, Aquafarm Development (Pty) Ltd, pers. comm. 2013). The total feed required was then divided by the number of days (d) between sampling times to determine the feed required per day (Equations 3).

$$\text{Length (mm abalone}^{-1} \text{ month}^{-1}) = L + 1.9 \quad (1)$$

$$\text{Weight (g abalone}^{-1}\text{)} = 0.1531 \left(\frac{L}{10}\right)^{3.0899} \quad (2)$$

$$\text{Feed required per day (g)} = \frac{Ab(W_{t+1} - W_t)1.2}{d} \quad (3)$$

### *Leaching trial*

Leaching trials were carried out to determine the ISO concentration in commercial feed containing soya after exposure to seawater. Thirty grams of Abfeed® S34 were placed in a small oyster mesh basket and then placed in seawater with no abalone for eight hours and then removed without the additional rinsing of fresh water and dried at 38 °C for 72 h. This is the average time that feed remains in the water before being eaten (Jones, Rhodes University, pers. comm. 2013). In addition, ISO concentrations were also tested in feed samples prior to leaching. Composite samples of a number of unleached and leached feed samples were used for ISO analysis.

Isoflavone concentrations were determined at a commercial laboratory (Covance Laboratories Ltd, Wisconsin, USA) using official methods of the AOAC (2000). The samples were extracted using a solution of hydrochloric acid and reagent alcohol heated on a steam bath or hot plate. The extract was brought to volume, diluted and centrifuged. An aliquot of the supernatant was placed onto a C 18 solid-phase extraction column. The undesirable components of the matrix were rinsed off with 20 % methanol and isoflavones were eluted with 80 % methanol. The samples were analysed using high-performance liquid chromatography with ultraviolet spectrophotometric detection and compared against known standards (AOAC, 2000).

In addition, the solid leaching rate of each diet was calculated as the dry weight loss during the eight hour period in seawater.

### *Feed formulation*

Crystalline isoflavone (Lc Laboratories, Massachusetts, United States of America), including daidzin, genistin, glycitin, daidzein, genistein and glycitein were dissolved in dimethylsulphoxide at quantities provided by the supplier, prior to being added into the feed mixture (Table 1.1). Formulated feed was subjected to leaching trials to determine leaching rates of the crystalline isoflavones.

### *Data collection*

All abalone were purged for 48 h prior to sampling after which individual weight was recorded (0.01 g) using an electronic balance (Kern PLS 4200-2F, serial number: WIC1200486) and shell length was measured (0.01 mm) using vernier callipers.

Three male and three female abalone were randomly selected from each replicate tank at the start and again at 45 d intervals for the duration of the trial. Abalone were placed in labelled cotton mesh bags and transferred to a chilling room at about 10 °C. Animals were shucked (removal of the shell) and the viscera (digestive gland and gonad) were separated from the foot. Wet whole mass, shucked mass, meat mass and visceral mass were weighed and shell length was measured. These animals were used to determine the percentage of water loss in meat and viscera. The following day, an additional three males and three females per replicate were randomly selected and subjected to the same sampling protocol, however, the viscera were used for gonad bulk index (GBI) calculations. At the end of study, four males and four females per replicate were randomly selected with one female per replicate used for histological analysis (Chapter 3).

Mean monthly weight gain ( $\text{g abalone}^{-1} \text{ month}^{-1}$ ) and mean monthly length gain ( $\text{mm abalone}^{-1} \text{ month}^{-1}$ ) were determined at the end of the 6-month trial (Equations 5 and 6; respectively), where the initial wet whole mass ( $M_t$ ) was subtracted from the final wet whole mass ( $M_f$ ), divided by the duration of the study (180 days) and multiplied by 30 days to obtain the mean monthly weight gain. Mean monthly length gain was obtained by subtracting the initial length ( $L_t$ ) from the final length ( $L_f$ ), divided by the durations of the study (180 days) and multiplied by 30 days.

$$\text{Weight gain (g abalone}^{-1} \text{ month}^{-1}) = \left( \frac{M_f - M_t}{180} \right) \times 30 \quad (5)$$

$$\text{Length gain (mm abalone}^{-1} \text{ month}^{-1}) = \left( \frac{L_f - L_t}{180} \right) \times 30 \quad (6)$$

Meat mass index, which is the meat mass as a percentage of whole mass was determined at each sampling period (Equation 7), where  $M_m$  was the wet meat mass ( $\text{g abalone}^{-1}$ ) and  $W_m$  was the wet whole mass ( $\text{g abalone}^{-1}$ ). In addition, visceral mass as a percentage of whole mass was determined (Equation 8), where  $V_m$  was the visceral wet mass ( $\text{g abalone}^{-1}$ ) and  $W_m$  was the wet whole mass ( $\text{g abalone}^{-1}$ ). Condition factor was determined (Equation 9; Britz, 1996), where  $W$  was the whole mass ( $\text{g abalone}^{-1}$ ) and  $L$  was total length (mm).

$$\text{Meat mass index (\%)} = \left( \frac{M_m}{W_m} \right) \times 100 \quad (7)$$

$$\text{Visceral index (\%)} = \left( \frac{V_m}{W_m} \right) \times 100 \quad (8)$$

$$CF = \left( \frac{W}{L^{2.99}} \right) \times 5575 \quad (9)$$

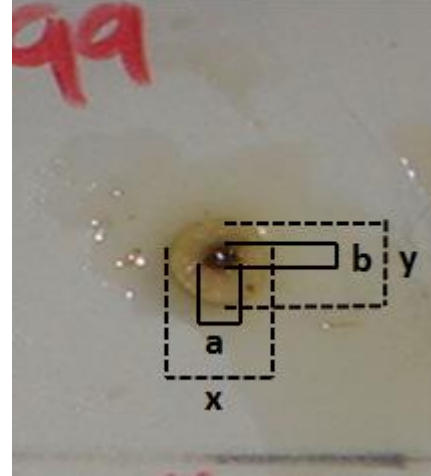
The viscera of the three males and three females from each replicate tank were weighed and each placed into a 40 ml plastic bottle containing Davidson's fixative (20 % formalin, 10 % glycerol,

10 % acetic acid, 30 % absolute ethanol and 30 % salt water) for a minimum of one month. These samples were used to determine the gonad bulk index (GBI; Tutschulte and Connell, 1981). 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. The length of the conical appendage (mm) was determined using SIGMASCAN® PRO 5 (Systat Software, San Jose, CA, USA; Figure 1.2A). The viscera were sectioned through the middle of the conical appendage and linear measurements were taken of the digestive gland (mm) and the gonad (mm) (Figure 1.2B) to calculate the effective gonad volume (EGV; Equation 10), where  $L_{ca}$  was the length of the conical appendage,  $x$  and  $y$  were the linear dimensions of the digestive gland, and  $a$  and  $b$  were the linear dimensions of the gonad. The GBI was then determined by dividing the EGV by shucked mass ( $W_s$ ) (Equation 11; Tutschulte and Connell, 1981). The linear dimensions of the digestive gland and gonad were used to calculate the digestive gland (DG) index (%) (Equation 12), which was the area of the digestive gland relative to the gonad area as a percentage (Tutschulte and Connell, 1981).

A



B



**Figure 1.2:** (A) Photograph illustrating the measurement of the conical appendage ( $L_{ca}$ ) in a male abalone; and (B) photograph of the cross section through the midpoint of the conical appendage where linear dimension of the digestive gland (a and b) and viscera (x and y) are illustrated. The same method was applied in measuring female gonads.

$$EGV (mm^3) = \frac{L_{ca} \pi}{96} \left[ 8(x + y)^2 \frac{(x+y+a+b)^3}{x+y} \right] \quad (10)$$

$$GBI (mm^3 g^{-1}) = \frac{EGV}{W_s} \quad (11)$$

$$DG \text{ index } (\%) = \frac{(x \times y)}{(a \times b)} \times 100 \quad (12)$$

#### *Water loss*

The meat and viscera were weighed, placed in a foil container and placed in a drying oven at 50 °C for 96 h. The foot muscle was too large to dry and was therefore cut into quarters and only half of the foot muscle was weighed and dried. The total dry mass ( $M_D$ ) of the foot was calculated (Equation 13) prior to the determination of meat water loss (Equation 14), where  $M_T$  was the total wet meat mass ( $g \text{ abalone}^{-1}$ ),  $M_{WS}$  was wet mass of the meat samples ( $g \text{ abalone}^{-1}$ )

and  $M_{DS}$  was the dry mass of the meat samples (g abalone<sup>-1</sup>). The dried gonad sample was used to calculate visceral water loss (%) (Equation 15), where  $V_M$  was the wet visceral mass (g abalone<sup>-1</sup>) and  $V_D$  was the dry visceral mass (g abalone<sup>-1</sup>).

$$M_D = M_T - \left[ M_T \times \left( \frac{M_{WS} - M_{DS}}{M_{WS}} \right) \times 100 \right] \quad (13)$$

$$\text{Meat water loss (\%)} = \left( \frac{M_T - M_D}{M_T} \right) \times 100 \quad (14)$$

$$\text{Visceral water loss (\%)} = \frac{V_M - V_D}{V_M} \times 100 \quad (15)$$

Uneaten feed was removed every morning and placed into plastic bags (Ziploc®, S.C. Johnson and Son, Wisconsin), labelled with the tank number and sampling session (e.g. day 0 – 45) and frozen at -20 °C. Frozen feed samples were dried at the end of every sampling interval and subtracted from the quantity of feed fed to abalone in each tank over the sampling period to calculate feed conversion ratio (FCR; Equation 16). Dried feed samples were adjusted for dry matter loss during leaching. The initial moisture content of the diets were not taken into account.

$$FCR = \frac{\text{Dry feed consumed (g)}}{\text{Wet mass gained (g)}} \quad (16)$$

### *Water quality*

Water quality variables including water temperature, dissolved oxygen and pH were measured once a week with a meter (YSI Inc. Pro Plus Multi-parameter Meter, Yellow Springs, Ohio, USA). Mean pH values were obtained by log-transforming the data, averaging the logged data, and using the anti-log of the average value. The total ammonia nitrogen (TAN) concentrations were determined using the Solórzano method (1969). Acid-washed glassware was used to collect

50 ml water samples from each replicate tank. The water samples were kept dark for one hour after reagents were added. Water samples were placed in a spectrophotometer (Prim light, Secomam, 30319, Ales, France) for absorbance readings at a wavelength of 640 nm. Linear regression standard curves were derived from known concentrations of ammonium chloride and used to determine TAN values.

### *Statistical analysis*

The study followed a 2 x 3 x 5 design with diet (FM and soya-based) and three ISO-concentrations as independent effects and five independent and randomly assigned replicates per treatment combination. An FM diet without ISO was included as a control. To avoid bias from pseudoreplication, values measured from abalone in the same tank were averaged prior to analysis. In addition, there were repeated measures per replicate at 45-day intervals. As ISO concentration data were of the continuous data type, due to experimental conditions, their values differed slightly between diets of similar ISO level. In addition, the design was not balanced as the FM-only based diet did not have an equivalent diet compared to the three soya diets at graded levels with their equivalent crystalline ISO diets. It was thus not possible to analyse the data using a multifactor repeated measures analysis of variance, which is commonly used for categorical independent data, but to apply multiple forward stepwise regression analysis (MSR) in order to determine the contribution of several independent variables (Table 1.2) towards a model to estimate dependent variables. Interaction terms between independent variables were included by adding the product of these variables into the model and testing, whether it was a significant contributor. This would allow testing the null hypothesis of independent data or their combinations not having an effect on the dependent variables.

All data were tested for homogeneity of variance (Levene, 1960) and for the normal distribution of the residuals (Shapiro and Wilk, 1965). If the data did not meet the assumptions they were log transformed before analysis. Data from the two sampling days were pooled (e.g. 15 males from first sampling day with the addition of 20 males from the second sampling day to make 35 males and females per treatment at each sampling interval). If sex did not significantly contribute to the model then data were pooled for males and females. If sex significantly contributed to the model, then males (red line) and females (blue line) were analysed with sex as an independent variable, and plotted separately.

A multivariate repeated measures analysis of variance (RM-ANOVA) was used for feed conversion ratio data (log transformed) and water quality parameter data at an error level of 5 % ( $p \leq 0.05$ ). If log transformed data did not meet the assumptions of homogeneity of variance, a repeated measures ANOVA was still used to compare treatments means over time (Moser and Stevens, 1992). Tukey's HSD post-hoc test (Tukey, 1960) was used to determine where significant differences occurred between treatment means.

Results in tables are mean  $\pm$  standard error unless indicated otherwise. All analyses were performed using Statistica 12® software package.

**Table 1.2:** Variables used in multiple forward stepwise regression analysis.

Variables	Used in model
Sex	s
ISO Concentration (mg kg <sup>-1</sup> )	c
Time (days)	t
Sex x concentration (product)	sc
Time x concentration (product)	tc

Linear regression analysis was used to determine the correlation between percentage leaching and isoflavone concentrations in unleached diets. In addition, linear regression analysis was used to determine the correlations between isoflavone concentrations in unleached and leached diets.

## RESULTS

### *Diet proximate analysis*

All dietary treatments contained similar protein, lipid, moisture and ash content (Table 2.1).

Protein ranged from 34.1 to 34.4 %, while lipid content ranged from 2.6 to 2.9 %. The percentage moisture of diets ranged from 12.2 to 12.8 %, while ash content ranged from 11.1 to 13.9 % (Table 2.1).

**Table 2.1:** Proximate analysis of dietary treatments, including a fishmeal (FM) only based diet and FM with graded levels of isoflavone (ISO) equivalent to the ISO present in soya (S) at 25, 50 and 100 % of the S in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

Dietary treatment	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)
FM	34.3	2.9	12.2	13.9
FM S 100	34.1	2.7	12.8	11.1
FM S 50	34.2	2.6	12.6	12.6
FM S 25	34.3	2.7	12.5	12.9
FM ISO 100	34.3	2.8	12.2	12.9
FM ISO 50	34.3	2.8	12.2	12.9
FM ISO 25	34.4	2.6	12.1	13.2

### *Phytoestrogens*

The FM-only based diet contained the least concentration of isoflavones with only daidzin being detectable prior to leaching, but after eight hours in seawater only trace amounts of daidzin were present (Table 2.2). The FM diet with the inclusion of 100 % isoflavone contained the highest concentration of isoflavones before and after leaching with 792 and 504 mg kg<sup>-1</sup> respectively, followed by its equivalent soya diets with 760 and 424 mg kg<sup>-1</sup> (Table 2.2). The inclusion of 25 % soya and its equivalent ISO diet contained a similar concentration of isoflavone before leaching (236 mg kg<sup>-1</sup>), but the inclusion of soya diet contained more isoflavone post leaching (Table 2.2). Overall, the isoflavone concentrations in the soya diets and FM with the addition of crystalline ISO were similar.

The percentage of isoflavone leaching in all dietary treatments was concentration-independent (MSR,  $p = 0.16$ ). Thus, the changes in isoflavone concentrations in each diet after eight hours in seawater were similar in all diets. The isoflavone concentrations ( $c$ ) in the unleached and leached diets were significantly correlated (MSR,  $y = -49.28 + 0.67c$ ,  $r^2 = 0.98$ ,  $F_{(1,4)} = 169.44$ ,  $p = 0.0002$ ).

**Table 2.2:** Isoflavone (ISO) concentrations (mg kg<sup>-1</sup>) in a fishmeal (FM) only based diet and FM with increasing rates of ISO equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed (Pty) Ltd, Hermanus).

	Treatments						
	FM	FM S 100	FM ISO 100	FM S 50	FM ISO 50	FM S 25	FM ISO 25
<b>Unleached</b>							
Total glucosides	11.70	760.00	792.00	452.00	398.00	236.00	236.08
<b>Isoflavones:</b>							
Daidzin	11.70	334.00	308.00	195.00	165.00	100.00	79.80
Glycitin	<0.50	50.60	52.60	33.60	28.80	20.80	22.00
Genistin	<0.50	376.00	398.00	224.00	204.00	114.00	58.20
Daidzein	<0.50	<0.50	11.06	<0.50	<0.50	<0.500	27.60
Glycitein	<0.50	<0.50	<0.50	<0.50	<0.50	<0.500	<0.50
Genistein	<0.50	<0.50	10.50	<0.50	<0.50	<0.500	18.50
<b>Leached</b>							
Total glucosides	<0.50	424.00	504.00	260.00	232.00	124.00	78.80
<b>Isoflavones</b>							
Daidzin	<0.50	150.00	169.00	97.80	82.80	48.40	40.00
Glycitin	<0.50	21.00	26.60	15.70	15.70	<0.50	<0.50
Genistin	<0.50	236.00	262.00	145.00	133.00	75.20	38.80
Daidzein	<0.50	<0.50	16.60	<0.50	<0.50	<0.50	<0.50
Glycitein	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
Genistein	<0.50	10.40	12.30	<0.50	<0.50	<0.50	<0.50

### *Weight and length gain*

Mean monthly weight gain of animals fed the fishmeal (FM) diet and FM with graded levels of ISO was not influenced by the inclusion level of ISO with an overall mean of  $3.52 \pm 0.10$  g abalone<sup>-1</sup> month<sup>-1</sup> (MSR,  $p = 0.87$ ; Figure 2.1A). Sex by concentration (sc) significantly contributed to the mean monthly weight gain of abalone fed soya diets (MSR,  $y = 3.25 + 0.0008sc$ ,  $r^2 = 0.19$ ,  $F_{(1, 38)} = 10.28$ ,  $p = 0.003$ ), as only the mean monthly weight gain of males fed increasing levels of soya increased with an increase in dietary soya concentration (c) (MSR,  $y = 3.24 + 0.002c$ ,  $r^2 = 0.23$ ,  $p = 0.03$ ; Figure 2.1B), while female monthly weight gain was

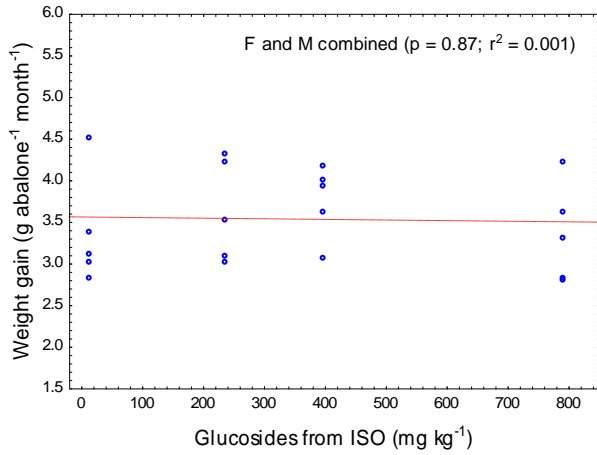
similar to that of the animals fed the FM diet with an overall mean of  $3.54 \pm 0.15$  g abalone<sup>-1</sup> month<sup>-1</sup> (MSR,  $p = 0.23$ ; Figure 2.1B).

Mean monthly length gain was not influenced by the addition of ISO, however females had a higher average length gain ( $1.67 \pm 0.06$  mm abalone<sup>-1</sup> month<sup>-1</sup>) than males ( $1.59 \pm 0.08$  mm abalone<sup>-1</sup> month<sup>-1</sup>; Figure 2.1C). The product of sex and concentration (sc) significantly contributed to the mean monthly length gain of abalone fed diets with the inclusion of soya (MSR,  $y = 1.55 + 0.00022sc$ ,  $r^2 = 0.12$ ,  $F_{(1, 38)} = 6.19$ ,  $p = 0.02$ ; Figure 2.1D), where males had a higher mean monthly length gain ( $1.71 \pm 0.07$  mm abalone<sup>-1</sup> month<sup>-1</sup>) than females ( $1.62 \pm 0.15$  mm abalone<sup>-1</sup> month<sup>-1</sup>). The mean monthly length gain was not influenced by increasing soya levels in both males and females (MSR,  $p = 0.05$  and  $p = 0.81$ , respectively; Figure 2.1D).

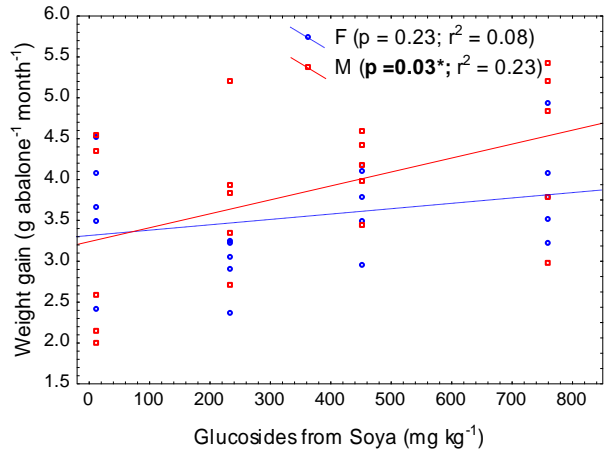
### *Weight loss*

In at least one tank (replicate) of every treatment abalone lost biomass between day 136 and the end of the study. The two tanks that had the highest decrease in biomass between day 136 and 180 were from the FM treatment (36.96 and 73.16 g tank<sup>-1</sup>, respectively; Table 2.3) with 2.8 and 7.6 % loss of biomass, followed by the FM diet with the inclusion of 100 % ISO (32.05 g tank<sup>-1</sup>; Table 2.3) with a biomass loss of 3.4 %. The two lowest decreases in weight loss were found in the FM and 100 % S treatments (8.67 and 9.03 g tank<sup>-1</sup>; Table 2.3) with 0.90 and 0.79 % biomass loss. The number of tanks with decreasing biomass increased as dietary soya increased (Figure 2.2), while FM with the inclusion of 25 % soya, 25 % ISO and 50 % ISO had a similar weight loss in only one replicate tank (Figure 2.2). Fishmeal and FM with the inclusion of 100 % ISO had similar weight loss in two tanks (Figure 2.2).

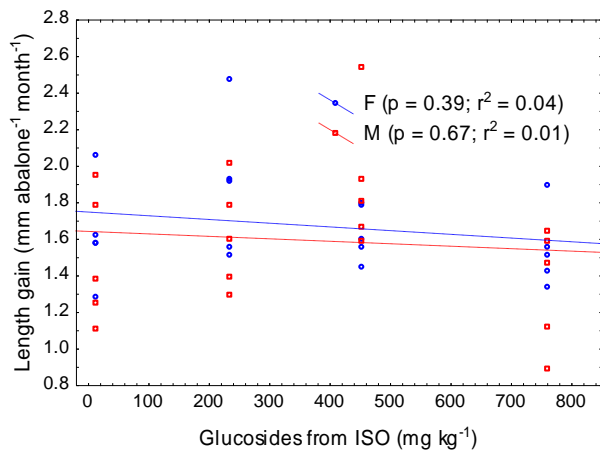
A – Fishmeal + crystalline isoflavone



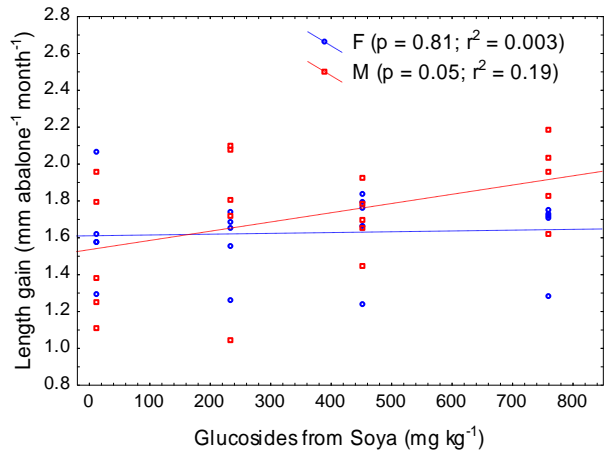
B – Fishmeal + graded levels of soya



C – Fishmeal + crystalline isoflavone



D – Fishmeal + graded levels of soya



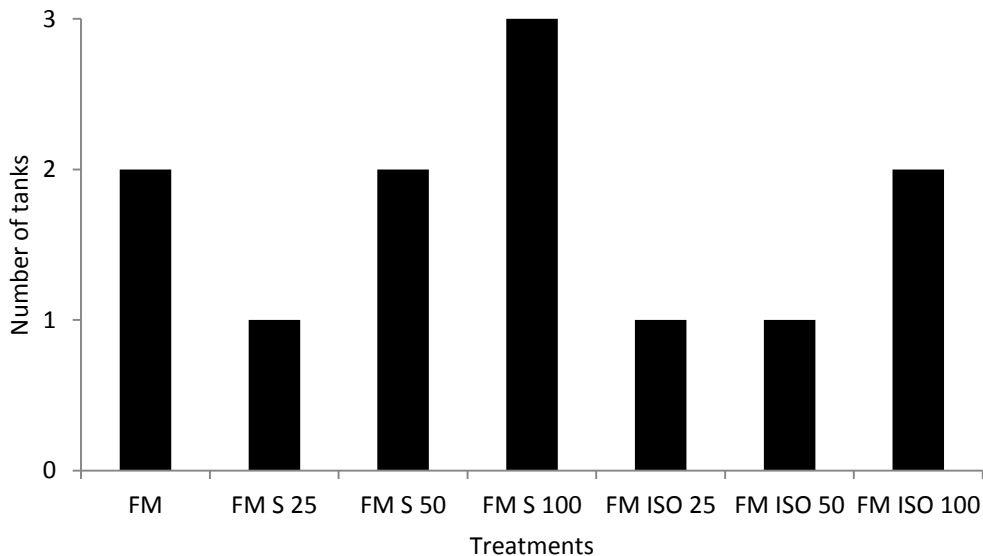
**Figure 2.1:** Mean monthly weight gain (g abalone<sup>-1</sup> month<sup>-1</sup>) and length gain (mm abalone<sup>-1</sup> month<sup>-1</sup>) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 or 100 % of the soya (S) in the commercial feed (Abfeed ® S34, Marifeed Pty (Ltd), Hermanus) equivalent to 12, 236, 452 and 760 mg kg<sup>-1</sup> of diet. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

(B) Male:  $y = 3.24 + 0.002x$

FM      FM ISO 25      FM ISO 50      FM ISO 100

**Table 2.3:** Biomass loss per tank between days 136 and 180 of animals fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavone (ISO) equivalent to diets with either no soya, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus). A dash indicates that the treatment did not experience a loss in biomass.

Treatment	Replicate	Biomass loss (g tank <sup>-1</sup> )	Biomass loss (%)
FM	1	36.96	3.77
	2	73.16	7.56
	3	-	-
	4	-	-
	5	-	-
FM S 100	1	21.18	2.00
	2	08.67	0.90
	3	09.03	0.79
	4	-	-
	5	-	-
FM S 50	1	24.93	2.37
	2	24.95	2.54
	3	-	-
	4	-	-
	5	-	-
FM S 25	1	12.30	1.30
	2	-	-
	3	-	-
	4	-	-
	5	-	-
FM ISO 100	1	27.42	2.70
	2	32.05	3.44
	3	-	-
	4	-	-
	5	-	-
FM ISO 50	1	12.38	1.26
	2	-	-
	3	-	-
	4	-	-
	5	-	-
FM ISO 25	1	27.91	2.81
	2	-	-
	3	-	-
	4	-	-
	5	-	-



**Figure 2.2:** Frequency of tanks in each treatment with biomass loss between days 130 and 180 of animals fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus). Note that there were a total of five tanks in each treatment.

### *Abalone whole mass*

The whole mass of abalone fed FM with crystalline ISO increased over time ( $t$ ) with no effect of sex and ISO concentration (MSR,  $y = 43.33 + 0.13t$ ,  $r^2 = 0.90$ ,  $F_{(1, 98)} = 930.09$ ,  $p < 0.001$ ; Table 2.4). Whole abalone body mass increased from  $43.29 \pm 0.35$  g abalone<sup>-1</sup> at the start of the trial increasing to an overall average of  $64.46 \pm 0.72$  g abalone<sup>-1</sup> over 180 days, with similar averages for days 136 and 180 (Figure 2.3A).

Whole body mass of abalone fed soya increased over time ( $t$ ), with an additional influence of time by concentration interaction ( $tc$ ), sex ( $s$ ) and sex by concentration interaction ( $sc$ ) (MSR,  $y = 43.51 + 0.12t + 0.00004tc + 1.40s - 1.07\log(sc)$ ,  $r^2 = 0.79$ ,  $F_{(1, 38)} = 4.34$ ,  $p = 0.04$ ; Table 2.4), where female and male whole body mass increased differently over time as they were affected

differently by soya concentration from day 136 to the end of the study (Figure 2.3B and Figure 2.3C, respectively). The initial weight of abalone fed FM and FM with graded levels of soya was  $42.81 \pm 0.46$  g abalone<sup>-1</sup>, with similar whole body mass at the end of the study for females fed the FM-only based diet ( $64.94 \pm 1.45$  g abalone<sup>-1</sup>) and females fed FM with 100 % soya ( $64.89 \pm 1.15$  g abalone<sup>-1</sup>). Males fed the FM-only based diet had the lowest whole body mass at the end of the study ( $62.94 \pm 3.27$  g abalone<sup>-1</sup>), contrasted with the highest whole body mass of males fed FM with 100 % soya inclusion rate ( $69.01 \pm 2.48$  g abalone<sup>-1</sup>).

The inclusion of ISO in FM diets had no effect on the average whole mass of animals over the period of the experiment. However, on day 136, males had a significantly higher average whole mass ( $64.08 \pm 0.69$  g abalone<sup>-1</sup>) than females ( $61.92 \pm 0.77$  g abalone<sup>-1</sup>; MSR,  $y = 59.77 + 2.16s$ ,  $r^2 = 0.79$ ,  $F_{(1, 38)} = 4.34$ ,  $p = 0.04$ ; Figure 2.4A, day 136).

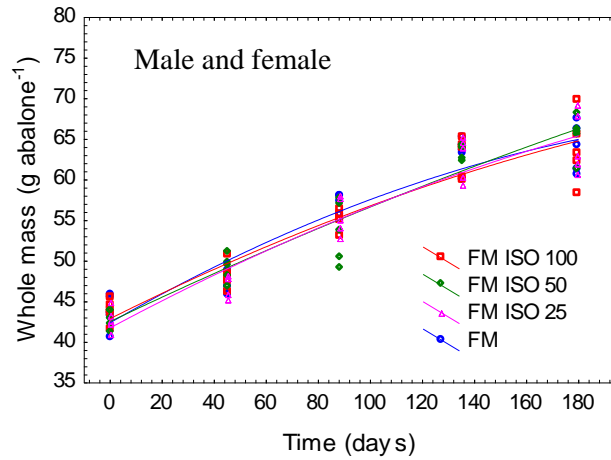
The inclusion of soya had no influence on whole mass in both males and females from day 0 to day 45 (MSR,  $p = 0.31$  and  $p = 0.20$ , respectively; Figure 2.4B, day 0 and day 45), however, by day 88 whole mass had decreased significantly as a function of increasing dietary soya concentration ( $c$ ), from  $55.46 \pm 1.36$  g abalone<sup>-1</sup> for abalone fed 25 % soya inclusion to  $53.79 \pm 0.26$  g abalone<sup>-1</sup> for those fed 100 % soya (MSR,  $y = 56.40 - 0.005c$ ,  $p = 0.01$ ,  $r^2 = 0.31$ ; Figure 2.4B, day 88). Female whole mass increased as a function of increasing dietary soya concentration ( $c$ ) on day 136 (MSR,  $y = 60.30 + 0.007c$ ,  $r^2 = 0.34$ ,  $p = 0.007$ ; Figure 2.4B, day 136). At the end of the trial, the mean male whole body mass was greater than in females with an increase in soya levels, increasing from  $65.22 \pm 1.93$  g abalone<sup>-1</sup> for abalone fed 25 % soya inclusion rate to  $69.00 \pm 2.48$  g abalone<sup>-1</sup> for abalone fed FM with 100 % soya, while female whole mass was similar in all treatments with an overall mean of  $63.46 \pm 0.79$  g abalone<sup>-1</sup> (Figure 2.4B, day 180).

### *Meat mass*

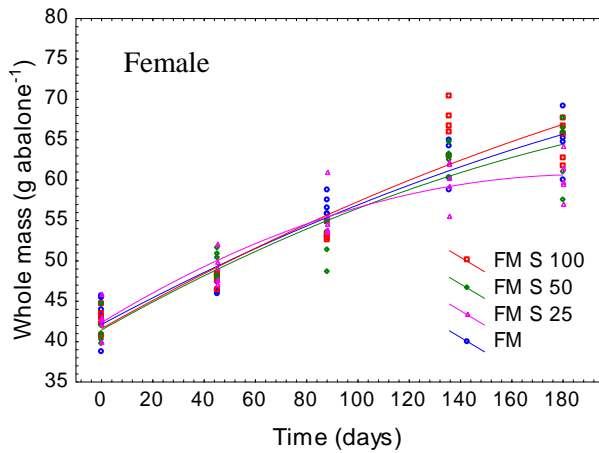
The increase in meat mass over time ( $t$ ) was not affected by sex or concentration of ISO (MSR,  $y = 27.62 + 0.07t$ ,  $r^2 = 0.85$ ,  $F_{(1, 98)} = 558.28$ ,  $p < 0.001$ ; Table 2.4). Meat mass thus increased similarly in all treatments from  $27.50 \pm 0.24$  g abalone<sup>-1</sup> at the start of the study to an overall mean of  $38.23 \pm 0.45$  g abalone<sup>-1</sup> at day 180 (Figure 2.5A).

Meat mass for animals fed diets that included soya increased over time ( $t$ ) and this variable was significantly influenced by the interaction between time and soya concentration ( $tc$ ) and soya concentration ( $c$ ) (MSR,  $y = 28.10 + 0.05t + 0.00002tc - 2.80\log(c)$ ,  $r^2 = 0.83$ ,  $F_{(5, 94)} = 94.40$ ,  $p < 0.001$ ; Table 2.4), where abalone meat mass differed between treatments towards the end of the study (Figure 2.5B).

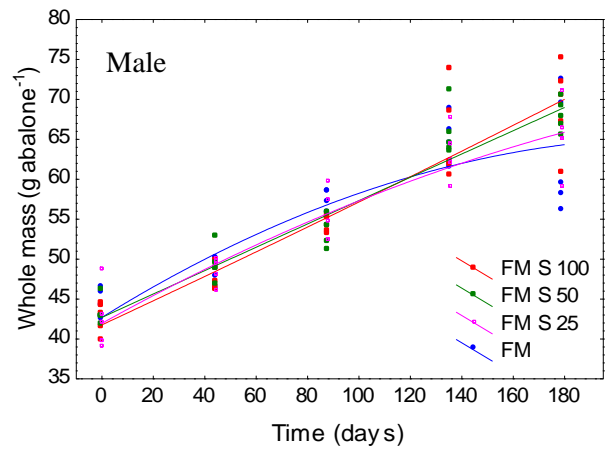
A – Fishmeal + crystalline isoflavone



B – Fishmeal + graded levels of soya

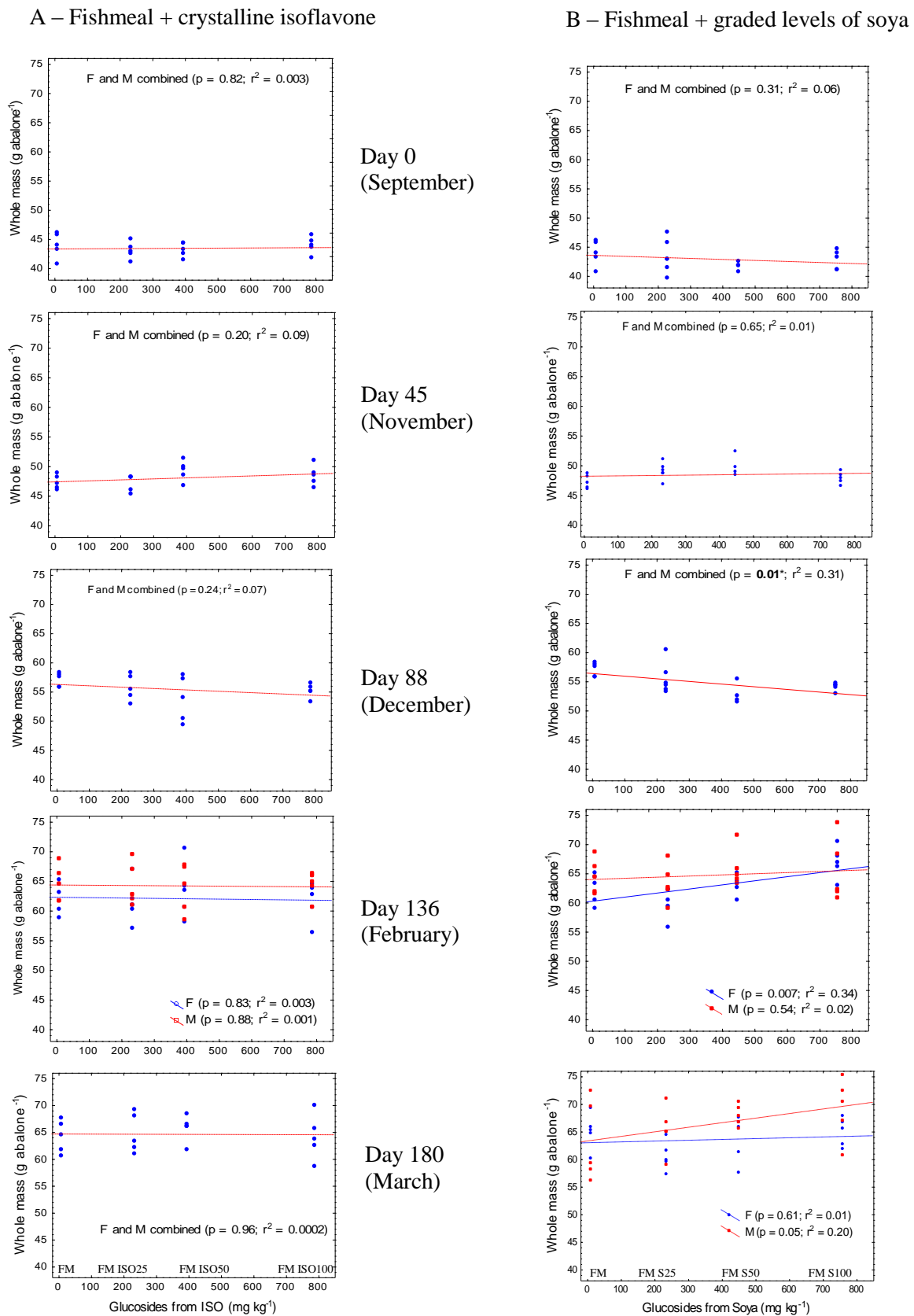


C – Fishmeal + graded levels of soya

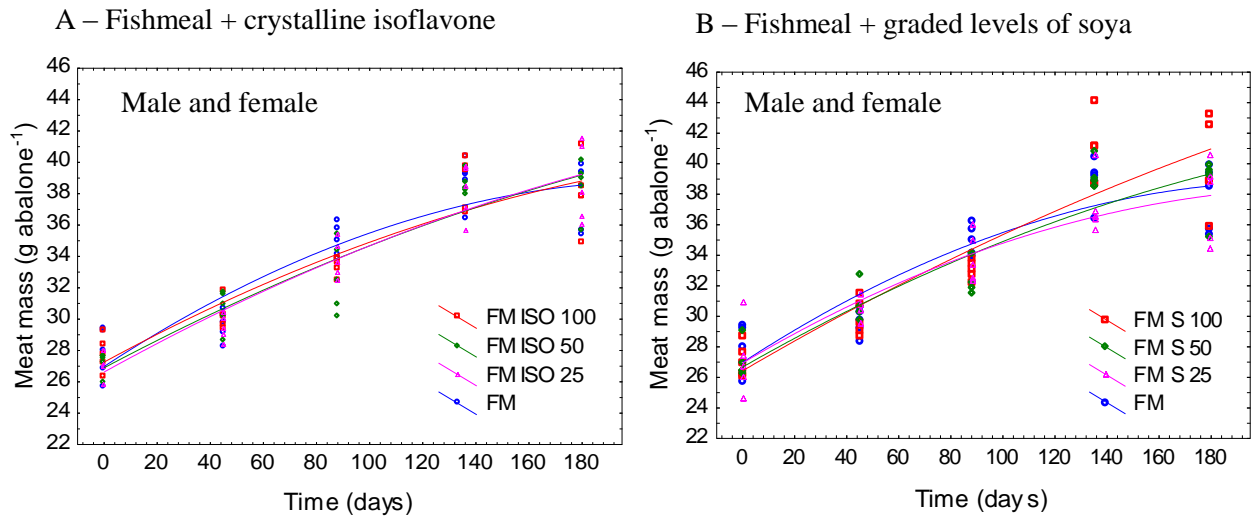


**Figure 2.3:** The average whole mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

(A) FM:  $y = 42.42 + 0.18x - 0.0003x^2$ ; FM ISO 100:  $y = 42.93 + 0.16x - 0.0002x^2$ ; FM ISO 50:  $y = 42.57 + 0.15x - 0.0001x^2$ ; FM ISO 25:  $y = 41.78 + 0.17x - 0.0002x^2$ . (B) FM:  $y = 42.13 + 0.16x - 0.0002x^2$ ; FM S 100:  $y = 41.63 + 0.17x - 0.0002x^2$ ; FM S 50:  $y = 41.45 + 0.17x - 0.0003x^2$ ; FM S 25:  $y = 42.36 + 0.19x - 0.0005x$ . (C) FM:  $y = 42.70 + 0.19x - 0.0004x^2$ ; FM S 100:  $y = 41.77 + 0.149x + 3.92 \times 10^{-5}x^2$ ; FM S 50:  $y = 42.70 + 0.14x - 2.96 \times 10^{-6}x^2$ ; FM S 25:  $y = 41.96 + 0.17x - 0.0003x^2$ .



**Figure 2.4:** The mean whole mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).



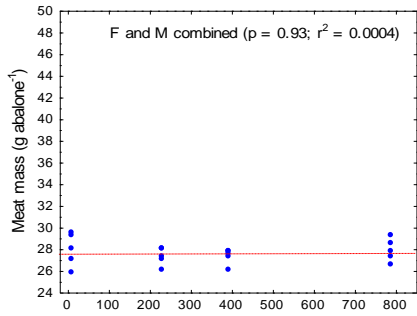
**Figure 2.5:** The average meat mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

(A) FM  $y = 26.97 + 0.11x - 0.0003x^2$ ; FM ISO 100:  $y = 27.26 + 0.09x - 0.0002x^2$ ; FM ISO 50:  $y = 26.89 + 0.08x - 0.0001x^2$ ; FM ISO 25:  $y = 26.62 + 0.09x - 0.0001x^2$ . (B) FM:  $y = 26.97 + 0.11x - 0.0003x^2$ ; FM S 100:  $y = 26.43 + 0.099x - 0.0001x^2$ ; FM S 50:  $y = 26.68 + 0.097x - 0.0001x^2$ ; FM S 25:  $y = 26.94 + 0.1004x - 0.0002x^2$ .

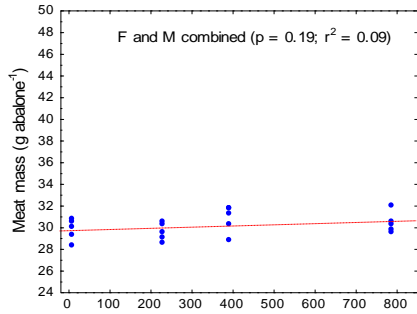
There was no correlation between the level of ISO and meat mass at each of the sampling times (MSR,  $p > 0.05$ ; Figure 2.6A). Meat mass of animals fed graded levels of soya illustrated similar results as whole mass, where soya inclusion had no effect on meat mass from day 0 to day 45, followed by a decrease in meat mass as a function of dietary soya concentration ( $c$ ) at day 88 (MSR,  $y = 34.62 - 0.003c$ ,  $r^2 = 0.34$ ,  $p = 0.01$ ; Figure 2.6B, day 88). Meat mass decreased from  $35.00 \pm 0.45$  g abalone<sup>-1</sup> for abalone fed the FM-only based diet to  $33.02 \pm 0.28$  g abalone<sup>-1</sup> for abalone fed FM with 100 % soya inclusion level. At day 136, female meat mass increased with increasing dietary soya concentration ( $c$ ) from  $37.75 \pm 0.48$  g abalone<sup>-1</sup> for abalone fed the FM-only based diet to  $41.00 \pm 0.44$  g abalone<sup>-1</sup> for abalone fed FM with 100 % soya inclusion (MSR,

$y = 36.56 + 0.005c$ ,  $r^2 = 0.28$ ,  $p = 0.02$ ; Figure 2.6B, day 136), while male meat mass was not correlated to soya inclusion rate with an overall meat mass of  $39.54 \pm 0.50$  g abalone<sup>-1</sup> (MSR,  $p = 0.44$ ; Figure 2.6B, day 136). At the end of the trial, the mean meat mass of abalone fed graded levels of soya was significantly affected by the interaction between sex and soya concentration (sc) (MSR,  $y = 39.28 + 0.004sc$ ,  $r^2 = 0.13$ ,  $F_{(2, 37)} = 3.90$ ,  $p = 0.003$ ), where male meat mass was on average higher than that of females with increasing soya levels (Figure 2.6B, day 180). At 25 % soya inclusion rate, male meat mass ( $38.48 \pm 0.77$  g abalone<sup>-1</sup>) was higher than female meat mass ( $36.77 \pm 0.78$  g abalone<sup>-1</sup>), however, the difference in meat mass increased further when abalone were fed FM with 100 % soya for males ( $41.80 \pm 1.12$  g abalone<sup>-1</sup>) and females ( $37.90 \pm 0.48$  g abalone<sup>-1</sup>; Figure 2.6B, day 180).

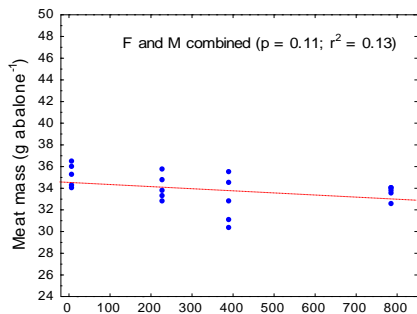
A – Fishmeal + crystalline isoflavone



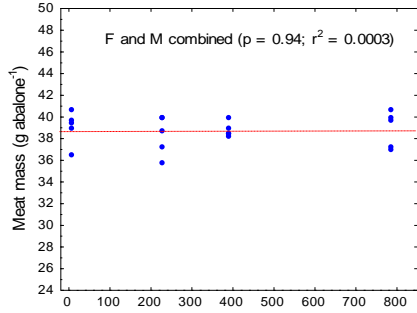
Day 0  
(September)



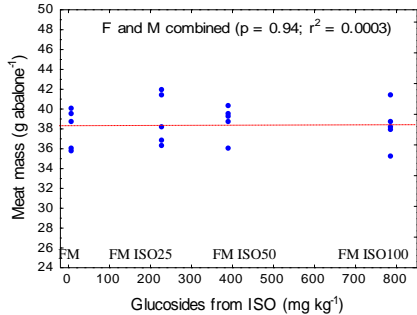
Day 45  
(November)



Day 88  
(December)

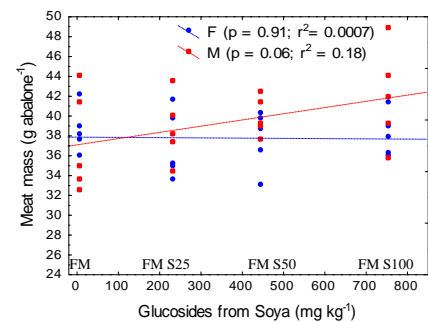
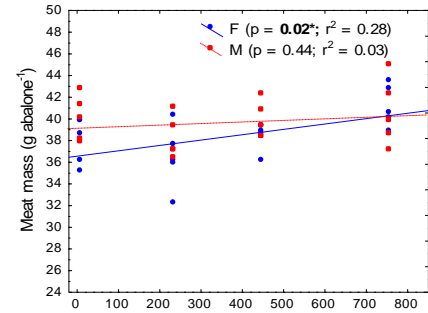
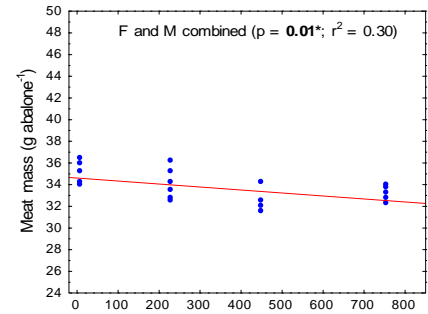
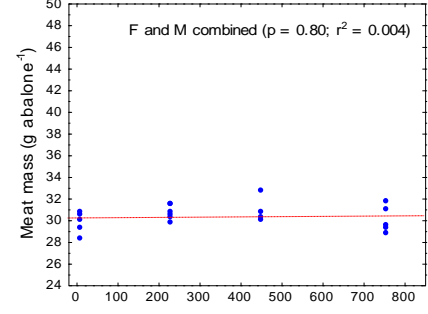
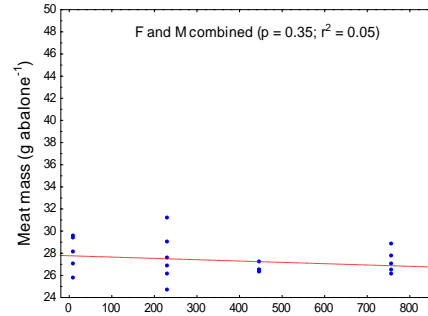


Day 136  
(February)



Day 180  
(March)

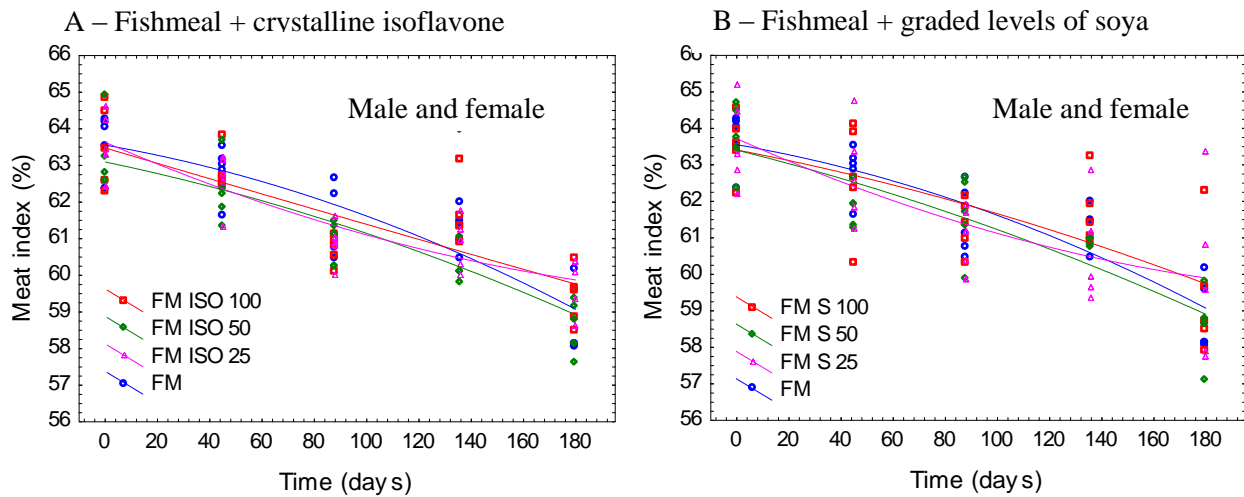
B – Fishmeal + graded levels of soya



**Figure 2.6:** The average meat mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

### Meat index

Meat index was not influenced by the inclusion of ISO and soya with the average value of all treatments decreasing as a function of time (MSR,  $p < 0.05$ ; Table 2.4). The mean meat index for abalone in all treatments was  $63.55 \pm 0.20$  % at the start of the experiment and  $59.20 \pm 0.29$  % after 180 d (Figure 2.7).



**Figure 2.7:** The mean meat index (%) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

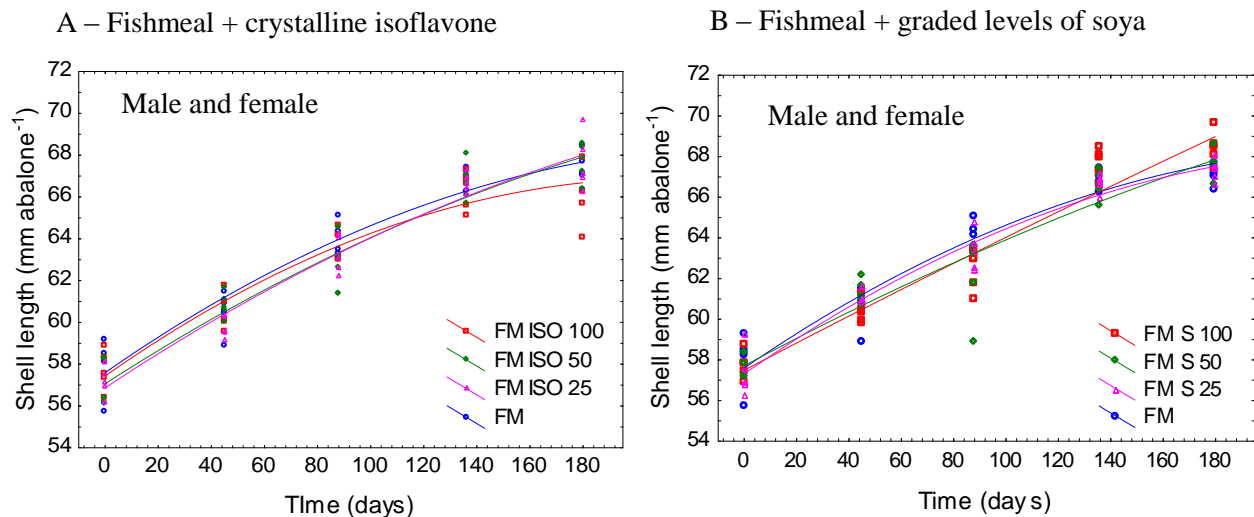
(A) FM:  $y = 26.97 + 0.11x - 0.0003x^2$ ; FM ISO 100:  $y = 27.26 + 0.0918x - 0.0002x^2$ ; FM ISO 50:  $y = 26.89 + 0.08x - 0.0001x^2$ ; FM ISO 25:  $y = 26.62 + 0.09x - 0.0001x^2$ . (B) FM S 100:  $y = 26.43 + 0.099x - 0.0001x^2$ ; FM S 50:  $y = 26.68 + 0.097x - 0.0001x^2$ ; FM S 25:  $y = 26.94 + 0.1004x - 0.0002x^2$ .

### Shell length

The increase in shell length over time ( $t$ ) was influenced by the interaction between time and ISO concentration ( $tc$ ) (MSR,  $y = 57.62 + 0.05t - 0.00001tc$ ,  $r^2 = 0.91$ ,  $F_{(3, 96)} = 355.18$ ,  $p < 0.001$ ; Table 2.4), due to the sex by ISO concentration effect at day 88 (Figure 2.9A, day 88), where the

differences in male and female shell length increased with increasing ISO concentration. Males fed the diet containing 100 % ISO inclusion had a higher mean shell length ( $64.09 \pm 2.38$  mm abalone<sup>-1</sup>) than females ( $63.09 \pm 0.40$  mm abalone<sup>-1</sup>) at day 88. The initial mean shell length of the abalone fed FM with ISO inclusion was  $57.48 \pm 0.23$  mm abalone<sup>-1</sup> with an overall mean of  $67.26 \pm 0.28$  mm abalone<sup>-1</sup> after 180 d (Figure 2.8A).

Shell length for animals fed soya diets increased with time (t) and this was influenced by an interaction of time and soya concentration (tc) (MSR,  $y = 58.01 + 0.04t + 0.0001tc$ ,  $r^2 = 0.92$ ,  $F_{(2, 97)} = 576.02$ ,  $p < 0.001$ ; Table 2.4), where from day 88 onwards, mean shell length was different for abalone fed the graded levels of soya (Figure 2.9B, day 88 to day 180).



**Figure 2.8:** The average shell length of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

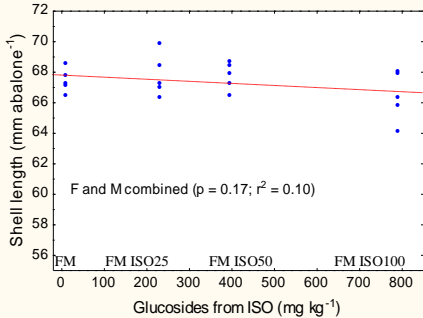
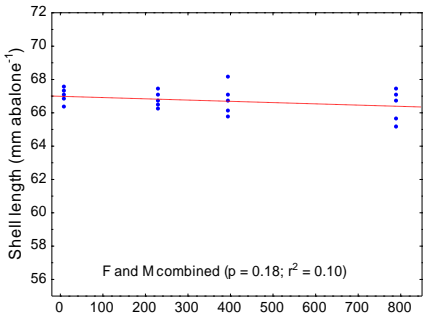
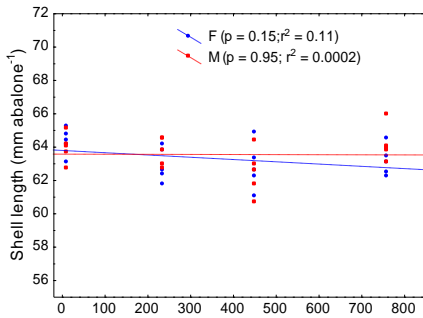
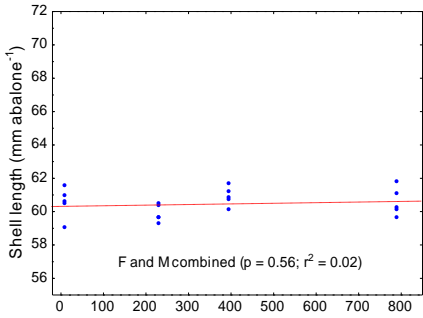
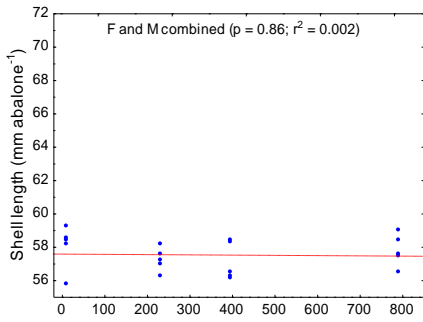
(A) FM:  $y = 57.60 + 0.08x - 0.0002x^2$ ; FM ISO 100:  $y = 57.43 + 0.08x - 0.0002x^2$ ; FM ISO 50:  $y = 57.06 + 0.08x - 0.0001x^2$ ; FM ISO 25:  $y = 56.87 + 0.0839x - 0.0001x^2$ . (B) FM S 100:  $y = 57.49 + 0.06x - 1.89 \times 10^{-5}x^2$ ; FM S 50:  $y = 57.71 + 0.06x - 6.9674 \times 10^{-5}x^2$ ; FM S 25:  $y = 57.31 + 0.08x - 0.0002x^2$ .

The mean shell length of abalone fed soya was influenced by soya concentrations from day 88 to the end of the trial (MSR,  $p < 0.05$ ; Figure 2.9). At day 88, mean shell length decreased with increasing soya inclusion rate from  $64.07 \pm 0.32$  mm abalone<sup>-1</sup> for abalone fed the FM-only based diet to  $62.39 \pm 0.44$  mm abalone<sup>-1</sup> for abalone fed FM with 100 % S inclusion. Mean shell length at day 136 and 180 was significantly affected by sex and by soya concentration (MSR,  $p = 0.02$  and  $p = 0.01$ , respectively; Figure 2.9B day 136 and day 180), where female mean shell length increased with increasing soya levels on day 136 and male mean shell length increased with increasing soya levels on day 180. Mean male shell length increased from  $67.03 \pm 0.96$  mm abalone<sup>-1</sup> for abalone fed FM to  $69.00 \pm 0.53$  mm abalone<sup>-1</sup> for abalone fed FM with 100 % soya inclusion, while females were not affected by the increasing soya levels with an overall mean shell length of  $67.52 \pm 0.43$  mm abalone<sup>-1</sup> at day 180 (Figure 2.9B, day 180).

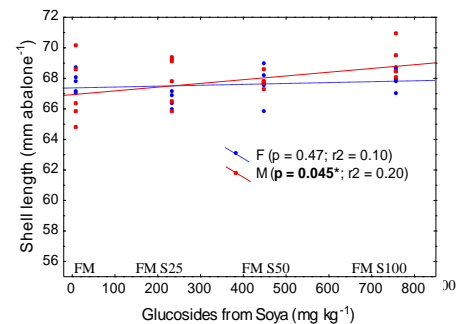
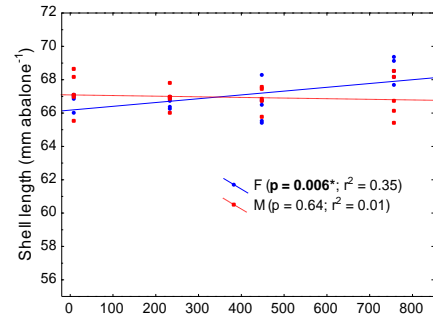
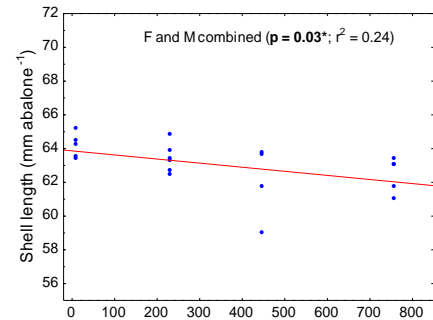
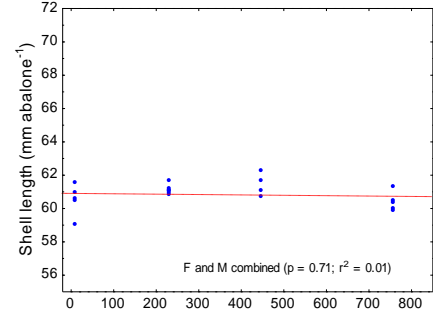
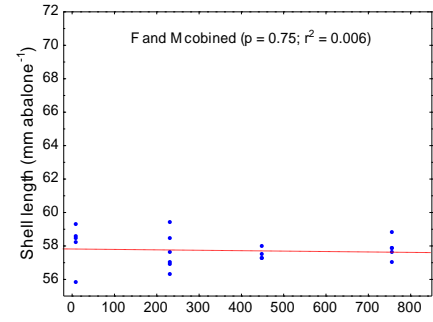
#### *Condition factor*

Condition factor (CF) decreased over time (t) (Figure 2.10A) with no influence of sex and ISO concentration from  $1.33 \pm 0.01$  at the start of the study to an overall mean of  $1.23 \pm 0.01$  (MSR,  $y = 1.28 - 0.0005t$ ,  $r^2 = 0.39$ ,  $F_{(6, 93)} = 11.63$ ,  $p = 0.03$ ; Table 2.4). Condition factor for abalone fed graded levels of soya decreased over time (t) with sex (s) being a significant predictor (MSR,  $y = 1.26 - 0.0005t + 0.02s$ ,  $r^2 = 0.22$ ,  $F_{(3, 196)} = 19.48$ ,  $p < 0.001$ ; Table 2.4). Condition factor of female abalone fed increasing rates of soya decreased from  $1.29 \pm 0.01$  at the start of the study to an overall mean of  $1.20 \pm 0.01$  (Figure 2.10B), while that of males decreased from  $1.32 \pm 0.01$  to an overall mean of  $1.24 \pm 0.02$  (Figure 2.10C).

A – Fishmeal + crystalline isoflavone

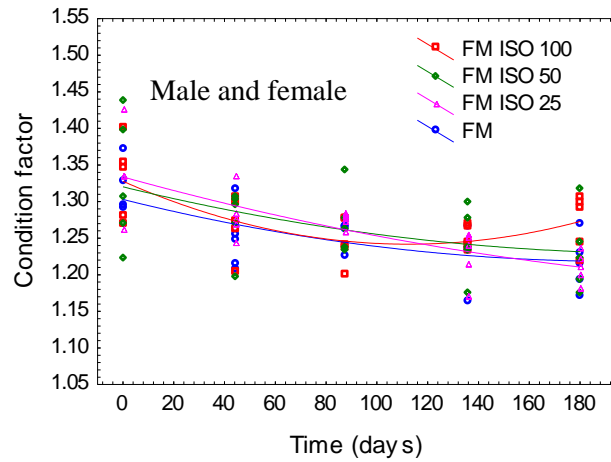


B – Fishmeal + graded levels of soya

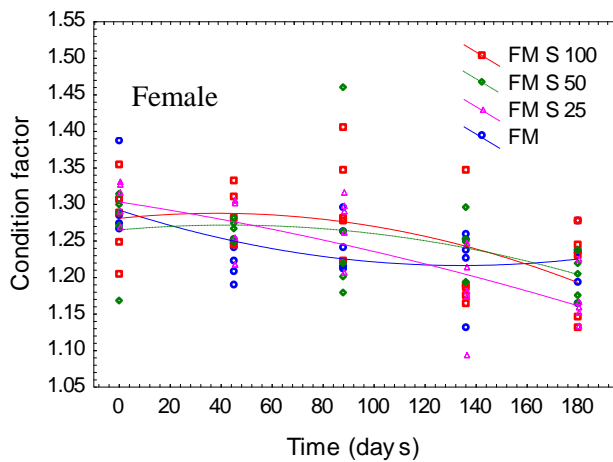


**Figure 2.9:** The average shell length of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

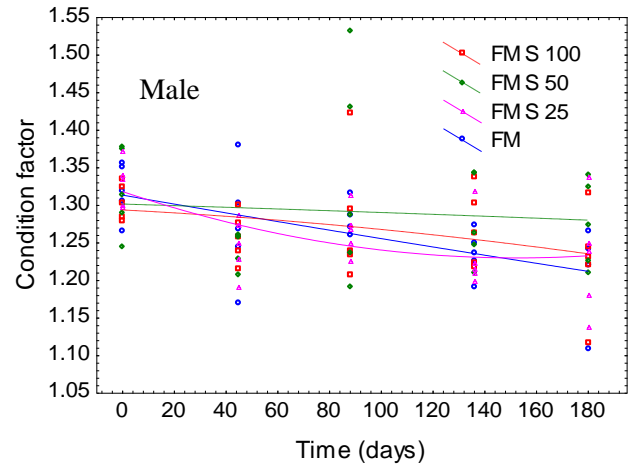
A – Fishmeal + crystalline isoflavone



B – Fishmeal + graded levels of soya



C – Fishmeal + graded levels of soya



**Figure 2.10:** The average condition factor of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

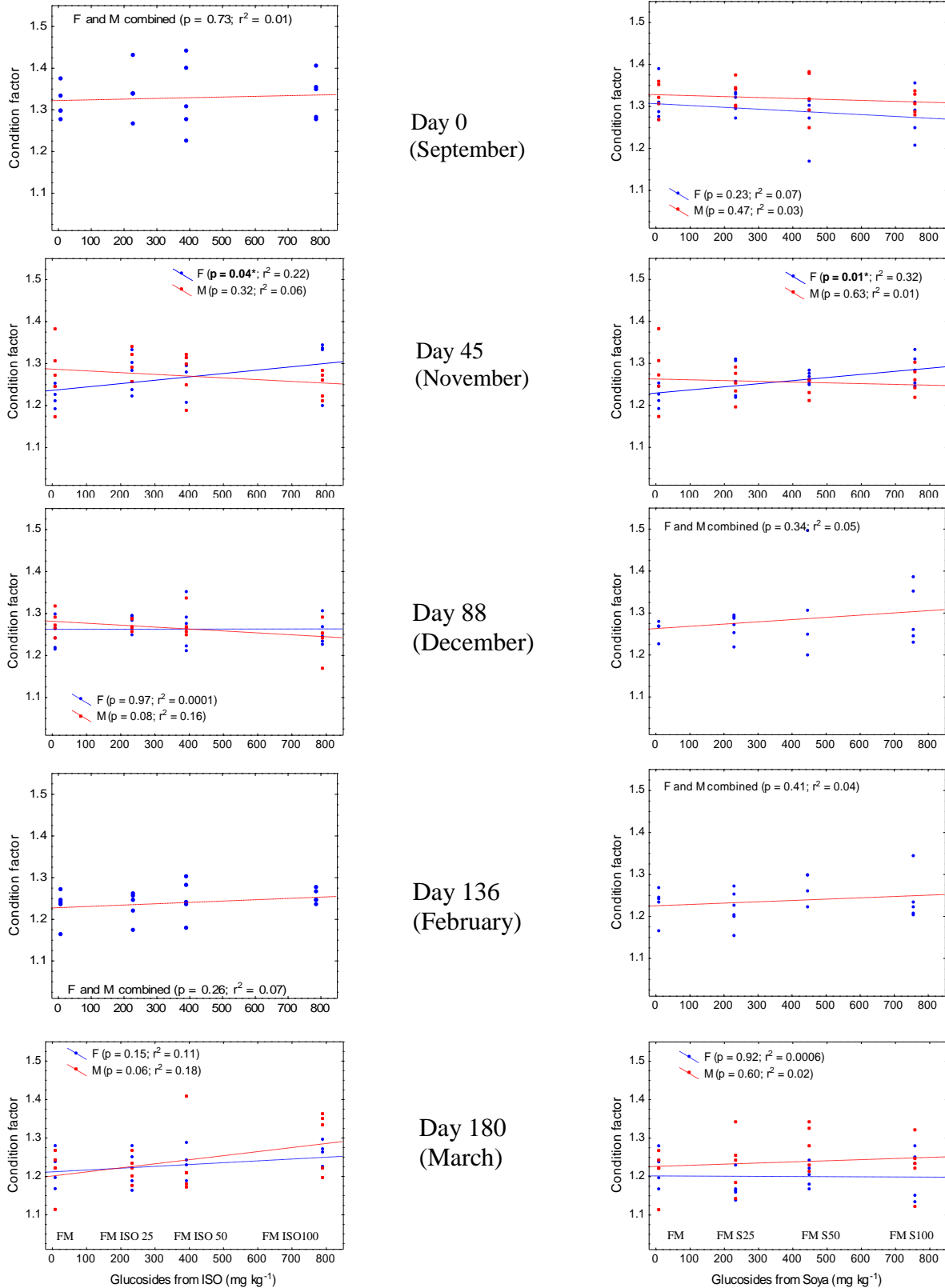
(A) FM:  $y = 1.30 - 0.0009x + 2.20 \times 10^{-6}x^2$ ; FM ISO 100:  $y = 1.33 - 0.002x + 6.80 \times 10^{-6}x^2$ ; FM ISO 50:  $y = 1.32 - 0.0008x + 1.95 \times 10^{-6}x^2$ ; FM ISO 25:  $y = 1.33 - 0.001x + 1.52 \times 10^{-6}x^2$ . (B) FM:  $y = 1.29 - 0.001x + 4.20 \times 10^{-6}x^2$ ; FM S 100:  $y = 1.28 + 0.0004x - 4.72 \times 10^{-6}x^2$ ; FM S 25:  $y = 1.30 - 0.0005x - 1.38 \times 10^{-6}x^2$ . (C) FM:  $y = 1.31 - 0.0006x + 1.93 \times 10^{-7}x^2$ ; FM S 25:  $y = 1.32 - 0.001x + 3.79 \times 10^{-6}x^2$ .

The product of abalone sex and ISO concentration significantly affected the CF of abalone fed ISO diets from day 45 to 88 and on day 180 (MSR,  $p = 0.03$ ,  $p = 0.02$  and  $p = 0.01$ , respectively, Figure 2.11A, day 45, day 88 and day 180), where increasing ISO rates affected males and females differently. However, ISO concentration had no influence on the CF of both sexes at the beginning of the study and on day 136 ( $p > 0.05$ ; Figure 2.11A, day 0 and day 136). At day 45 female CF had increased as a function of ISO concentration (MSR,  $y = 1.24 + 8.02 \times 10^{-5}c$ ,  $r^2 = 0.22$ ,  $p = 0.04$ ; Figure 2.11A, day 45), where female abalone fed FM had a mean CF of  $1.22 \pm 0.01$  compared to females fed FM with the addition of 100 % S with a mean CF of  $1.29 \pm 0.03$ , while the CF of male abalone fed graded levels of ISO was similar to that of abalone fed FM with a mean CF of  $1.27 \pm 0.01$ . At day 180, the differences in mean CF between males and females increased with increasing ISO inclusion rate (Figure 2.11A, day 180).

The average CF for males and females fed graded levels of soya was different at the beginning of the study, independent of treatment, with males having a higher average CF of  $1.32 \pm 0.01$  than females ( $1.29 \pm 0.01$ ). At day 45, female CF increased as a function of soya inclusion rate (MSR,  $y = 1.23 + 7.40 \times 10^{-5}c$ ,  $r^2 = 0.32$ ,  $p = 0.01$ ; Figure 2.11B, day 0), where female abalone fed FM had a mean CF of  $1.22 \pm 0.1$  compared to females fed FM with the addition of 100 % soya ( $1.28 \pm 0.02$ ), while the CF of male abalone was similar for FM and ISO treatments with a mean CF of  $1.25 \pm 0.01$ . At day 180, male abalone had a higher mean CF ( $1.24 \pm 0.02$ ) than females ( $1.20 \pm 0.01$ ; Figure 2.11B, day 180).

**A – Fishmeal + crystalline isoflavone**

**B – Fishmeal + graded levels of soya**



**Figure 2.11:** The average condition factor of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

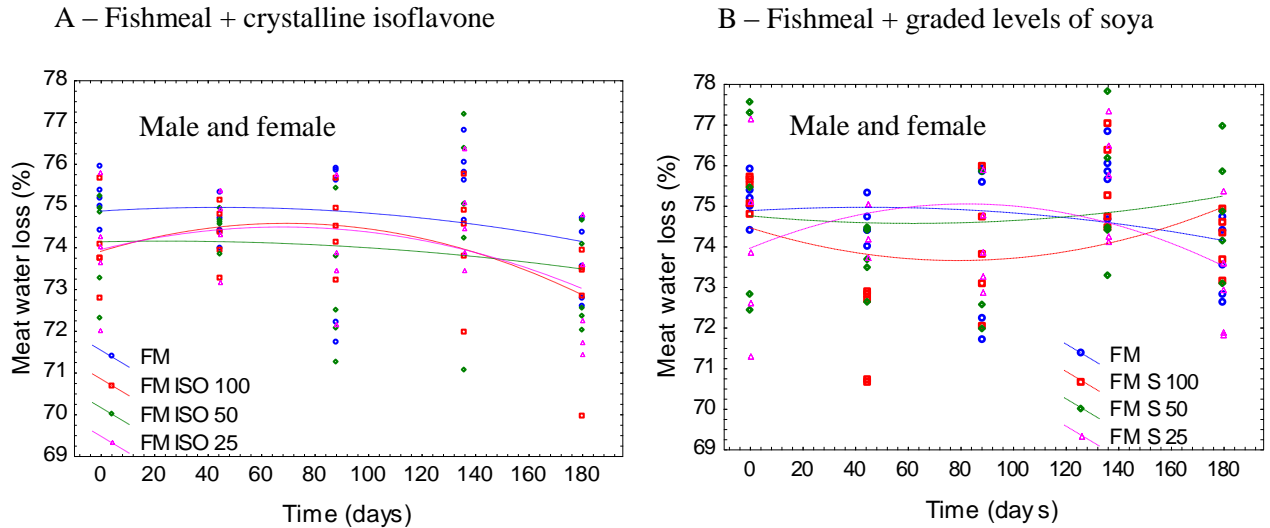
### *Meat water loss*

Meat water loss in animals fed ISO-enriched diets decreased with time (t) with a significant contribution of the product of time and concentration (tc) to the model (MSR,  $y = 87.47 - 0.006t + 0.1\log(tc)$ ,  $r^2 = 0.03$ ,  $F_{(2, 197)} = 3.91$ ,  $p = 0.02$ ; Table 2.4). The sex by concentration interaction significantly contributed to the model of meat water loss in abalone fed ISO-enriched diets on day 0 (Table 2.4), resulting in a significant contribution of time by concentration. Over 180 d. Meat water loss for abalone fed graded levels of soya increased from day 45 to 136, followed by a decrease at the end of the study (Figure 2.12B). The increase in soya level did not significantly contribute to the mean water loss at any sampling time (MSR,  $P > 0.05$ ; Table 2.4).

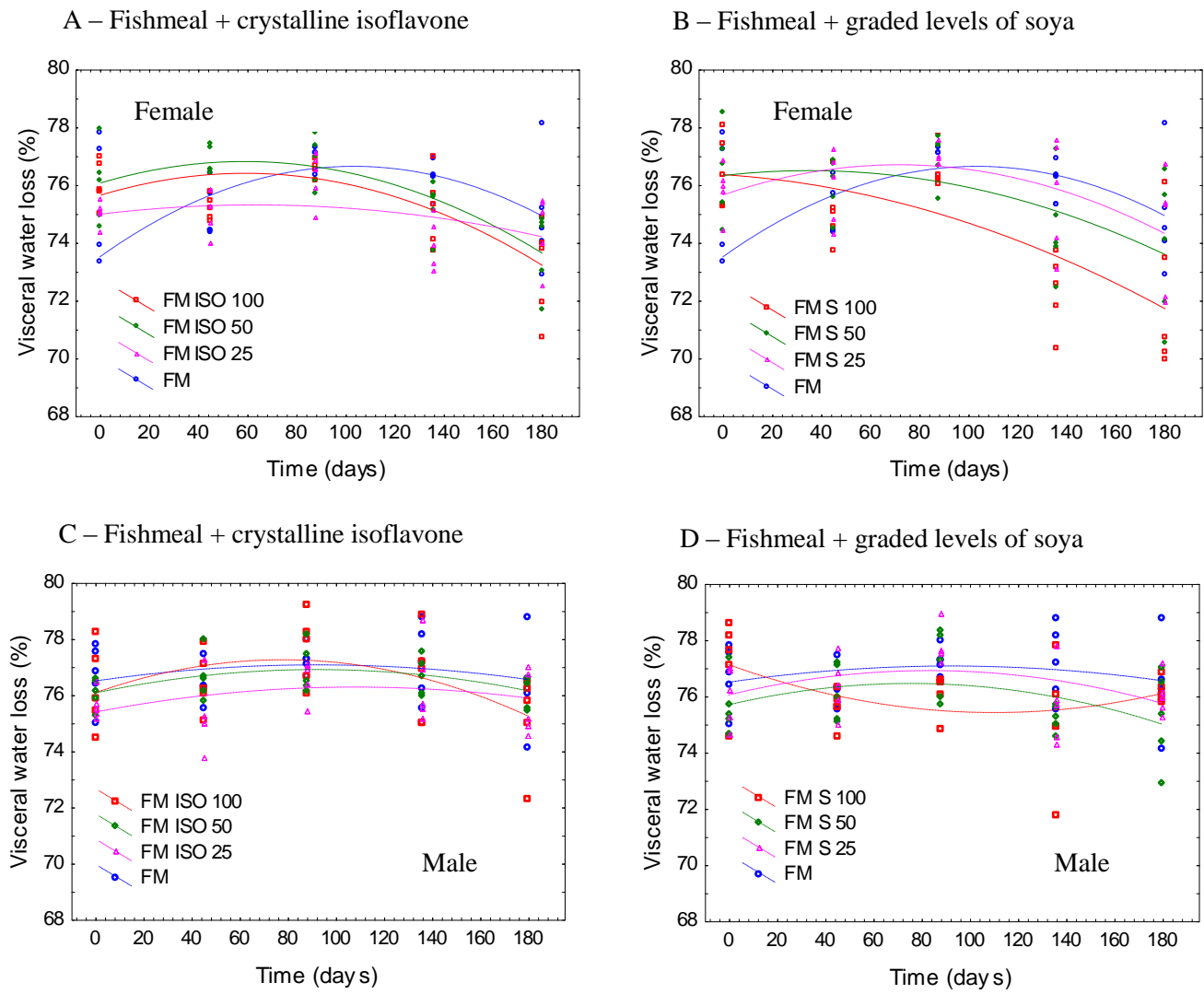
### *Visceral water loss*

The change in visceral water loss over time in animals fed ISO-enriched diets was influenced by sex (s), time by concentration interaction (tc) and sex by concentration interaction (sc) (MSR,  $y = 74.34 + 1.25s - 0.00002tc + 0.003c - 0.61\log(sc)$ ,  $r^2 = 0.22$ ,  $F_{(5, 194)} = 10.56$ ,  $p < 0.001$ ; Table 2.4), where the effect of increasing ISO concentration on visceral water loss was different at different times of the year and dependent on the sex of the abalone. Visceral water loss in females fed ISO-enriched diets increased from day 0 to day 88, and then decreased towards the end of the trial (Figure 2.13A). Males fed the ISO-enriched diets had relatively similar visceral water loss values throughout the study (Figure 2.13C). The change in visceral water loss in animals fed graded levels of soya was influenced by the sex (s) of the abalone and the time by concentration interaction (tc) (MSR,  $y = 74.25 - 0.00003tc + 0.96s$ ,  $r^2 = 0.21$ ,  $F_{(2, 195)} = 14.41$ ,  $p < 0.001$ ; Table 2.4), where water loss in males and females changed differently over time and the effects of increasing soya concentration was sex dependent. Females fed graded levels of soya

also increased in visceral water loss from day 0 to 88, then decreased towards the end of the study (Figure 2.13B). This pattern was not evident in males fed increasing rates of soya (Figure 2.13D).



**Figure 2.12:** The average meat water loss of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).



**Figure 2.13:** The mean visceral water loss of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days from September to March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

(A) FM ISO 100:  $y = 75.66 + 0.02x - 0.0002x^2$ ; FM ISO 50:  $y = 76.09 + 0.02x - 0.0002x^2$ . (B) FM S 100:  $y = 76.37 - 0.01x - 0.0001x^2$ ; FM S 50:  $y = 76.35 + 0.01x - 0.0001x^2$ .

The average visceral water loss was higher in males than in females independent of dietary treatment at all sampling times except on day 0 and 88 for abalone fed soya diets (Figure 2.14).

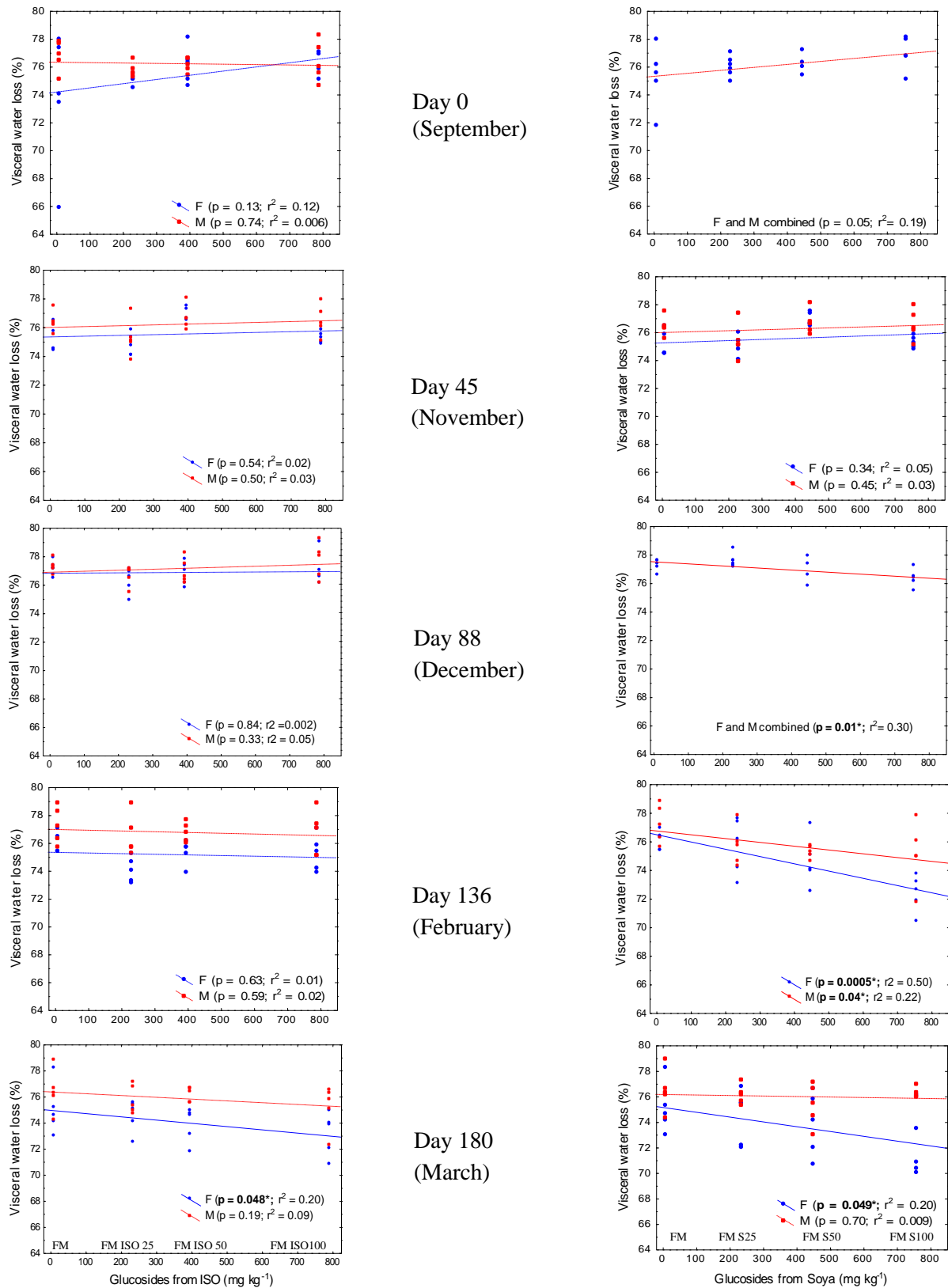
At day 180, visceral water loss in females decreased as ISO concentration (c) increased (MSR,  $y = 74.97 - 0.0025c$ ,  $r^2 = 0.20$ ,  $p = 0.048$ ; Figure 2.14A, day 180) from  $74.98 \pm 0.88$  % in abalone

fed the FM-only based diet to  $73.10 \pm 0.75$  for abalone fed FM with 100 % ISO inclusion, while male visceral water loss was not correlated to ISO inclusion rate with a mean visceral water loss of  $76.18 \pm 0.22$  % (MSR,  $p = 0.19$ ; Figure 2.14A, day 180).

Visceral water loss was similar for both males and females at day 88, with water loss decreasing as dietary soya levels increased (MSR,  $y = 76.48 - 0.003c$ ,  $r^2 = 0.25$ ,  $F_{(2,37)} = 7.60$ ,  $p = 0.002$ ; Table 2.4). However, at day 136, male visceral water loss was higher than that of females, while both sexes had a decrease in visceral water loss as dietary soya level increased (MSR,  $p < 0.05$ ; Figure 2.14B, day 136). By the end of the study, only female visceral water loss decreased as soya concentration ( $c$ ) increased (MSR,  $y = 75.17 - 0.0038c$ ,  $r^2 = 0.20$ ,  $p = 0.049$ ; Figure 2.14B, day 180) from  $74.98 \pm 0.88$  % for females fed the FM-only based diet to  $72.10 \pm 1.18$  % for females fed FM with 100 % soya inclusion rate. Male abalone fed soya diets had similar visceral water loss to the animals fed the FM-only based diet at day 180 with an overall mean of  $75.97 \pm 0.27$  % (MSR,  $p = 0.70$ ; Figure 2.14B, day 180).

A – Fishmeal + crystalline isoflavone

B – Fishmeal + graded levels of soya



**Figure 2.14:** The mean visceral water loss of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Table 2.4:** Growth of abalone fed a fishmeal-only based diet and fishmeal with increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya in the commercial feed, Abfeed@S34 at sampling intervals and over 180 days. The inclusion of the following variables indicates a significant contribution to the model. c = concentration, s = sex, sc = sex by concentration, t = time, tc = time by concentration, log = log transformed data. A dash reflects a lack of contribution of all variables to the model (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

Dependent variable	Day	ISO model	r <sup>2</sup>	Soya Model	r <sup>2</sup>
Whole mass (g abalone <sup>-1</sup> )	0-180	$y = 42.17 + 0.13t$	0.86	$y = 43.51 + 0.12t + 0.00004tc + 1.40s - 1.07\log(sc)$	0.86
Meat mass (g abalone <sup>-1</sup> )	0-180	$y = 26.89 + 0.07t$	0.78	$y = 28.1 + 0.05t + 0.00002tc - 2.80\log(c)$	0.77
Shell length (mm abalone <sup>-1</sup> )	0-180	$y = 57.62 + 0.05t - 0.00001tc$	0.88	$y = 58.01 + 0.04t + 0.00001tc$	0.90
Condition factor	0-180	$y = 1.28 - 0.0005t$	0.29	$y = 1.26 - 0.0005t + 0.02s$	0.22
Meat index (%)	0-180	$y = 63.50 - 0.02t$	0.55	$y = 63.61 - 0.03t$	0.48
	0	-		-	
	45	-		-	
	88	-		-	
	136	-		-	
	180	-		-	
Meat water loss (%)	0-180	$y = 87.47 - 0.006t + 0.18\log(tc)$	0.03	-	
	0	$y = 74.55 + 1.33s - 1.14\log(sc)$	0.24	-	
	45	-		-	
	88	-		-	
	136	-		-	
	180	-		-	
Visceral water loss (%)	0-180	$y = 74.34 + 1.25s - 0.00002tc + 0.003c - 0.61\log(sc)$	0.19	$y = 74.25 - 0.00003tc + 0.96s$	0.21

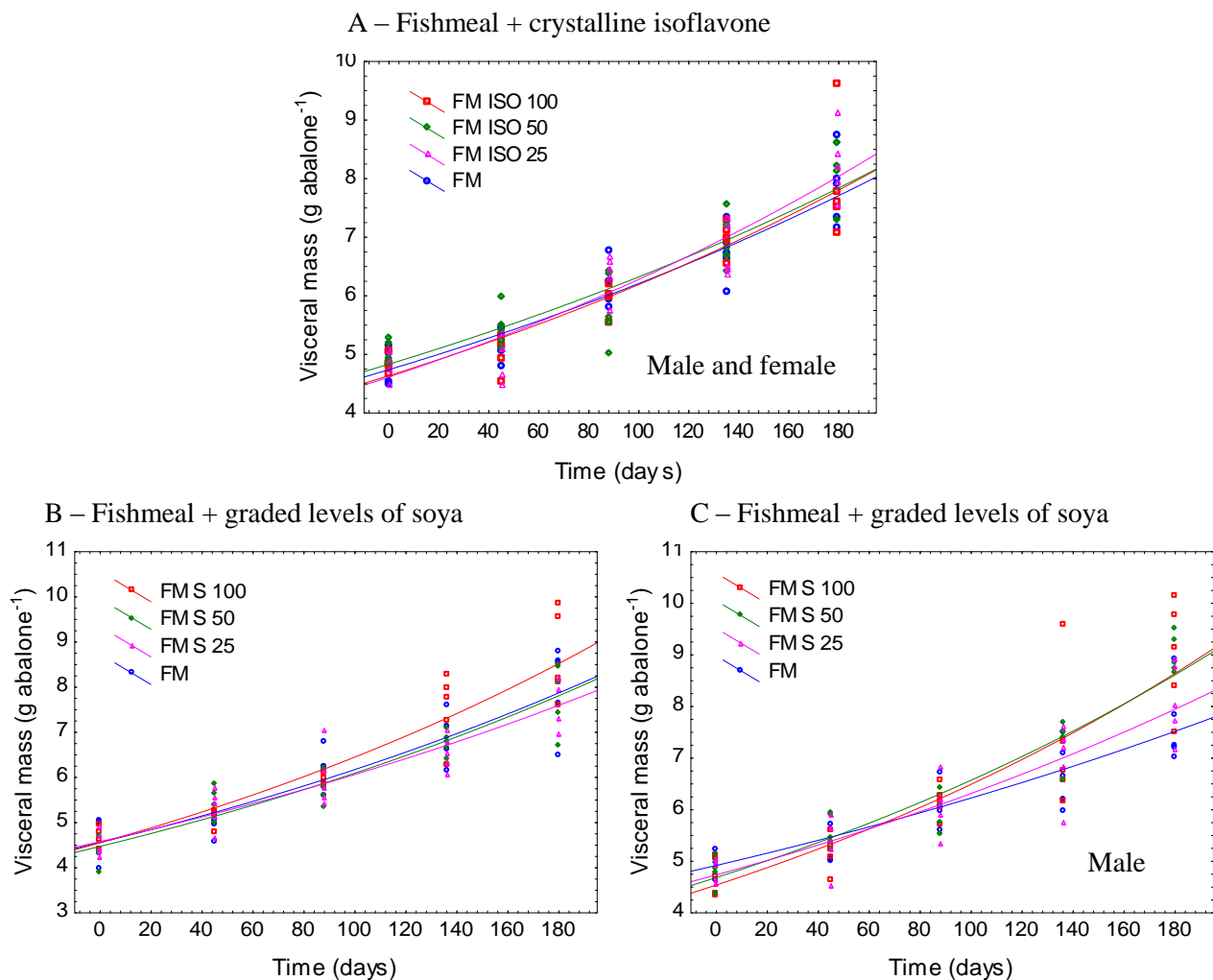
### *Gonad development*

### *Visceral mass*

The increase in visceral mass over time (t) was similar for both sexes (MSR,  $y = 4.61 + 0.02t$ ,  $r^2 = 0.82$ ,  $F_{(3, 196)} = 300.3$ ,  $p < 0.001$ ; Table 2.5) and was not influenced by ISO concentration from  $4.88 \pm 0.05$  g abalone<sup>-1</sup> at the start of the study to an overall mean of  $8.03 \pm 0.15$  g abalone<sup>-1</sup> on day 180 (Figure 2.15A). Visceral mass growth over time for abalone fed soya was influenced by both the product of time and soya concentration (tc) and sex (s) (MSR,  $y = 4.47 + 0.02t + 0.00001tc + 0.21s$ ,  $r^2 = 0.84$ ,  $F_{(5, 194)} = 205.13$ ,  $p < 0.001$ ; Table 2.5), where male and

female visceral mass increased differently over time and the effect of soya concentration on mean visceral mass was different at different times. A difference in visceral mass for the soya treatments was observed from day 88 to the end of the study for both females and males (Figure 2.15B and Figure 2.15C, respectively).

At the start of the experiment there was a significant sex effect (s), which was independent of treatment for abalone fed ISO-enriched diets (MSR,  $y = 4.46 + 0.27s$ ,  $r^2 = 0.15$ ,  $F_{(1, 38)} = 8.06$ ,  $p =$



**Figure 2.15:** The mean visceral mass of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March. Data were analysed with a multiple forward stepwise regression analysis at an error level of 5 %.

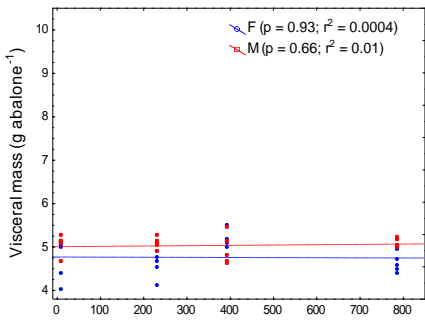
(A) FM:  $y = 4.7e^{0.0027x}$ ; FM ISO 100:  $y = 4.63e^{0.0029x}$ ; FM ISO 50:  $y = 4.83e^{0.0027x}$ ; FM ISO 25:  $y = 4.61e^{0.0031x}$ . (B) FM:  $y = 4.55e^{0.003x}$ ; FM S 100:  $y = 4.55e^{0.0035x}$ ; FM S 50:  $y = 4.47e^{0.0031x}$ ; FM S 25:  $y = 4.51e^{0.0028x}$ . (C) FM:  $y = 4.92e^{0.0024x}$ ; FM S 100:  $y = 4.54e^{0.0036x}$ ; FM S 50:  $y = 4.68e^{0.0034x}$ ; FM S 25:  $y = 4.73e^{0.0029x}$ .

0.007; Figure 2.12A, day 0) as well as abalone fed graded levels of soya (MSR,  $y = 4.36 + 0.51s$ ,  $r^2 = 0.18$ ,  $F_{(4, 35)} = 3.20$ ,  $p = 0.005$ ; Figure 2.16A, day 0), with males having a higher average visceral mass than females. For animals fed ISO-enriched diets, this sex (s) difference was still present by day 45 with no influence of the ISO concentration on visceral mass (MSR,  $y = 4.74 + 0.27s$ ,  $r^2 = 0.08$ ,  $F_{(1, 38)} = 4.57$ ,  $p = 0.04$ ; Figure 2.16A, day 45). From day 88 to 180, visceral mass was similar for both sexes independent of ISO concentrations (MSR,  $p > 0.05$ ; Figure 2.16A).

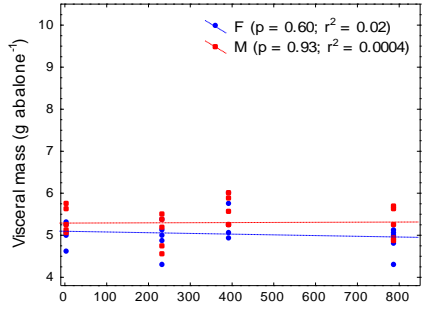
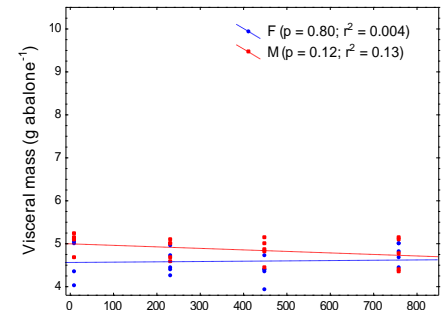
There was a significant sex effect in abalone fed graded levels of soya on day 45 (MSR,  $y = 10.53 + 0.49s$ ,  $r^2 = 0.11$ ,  $F_{(1, 38)} = 5.86$ ,  $p = 0.02$ ; Figure 2.16B, day 45) and by day 88, both sexes had similar average visceral mass values in all treatments with no effect of soya concentrations (MSR;  $p = 0.61$ , Figure 2.12b, Day 88). At day 136, visceral mass had increased as a function of dietary soya concentration (c) ( $y = 62.10 + 0.005c$ ,  $p = 0.046$ ;  $r^2 = 0.20$ ; MSR; Figure 2.12B, day 136), but by the end of the trial, males and females were influenced differently by soya inclusion rate (sc) (MSR,  $y = 7.70 + 0.001sc$ ,  $r^2 = 0.25$ ,  $F_{(1, 38)} = 14.32$ ,  $p = 0.0005$ ; Figure 2.16B, day 180,) with only male visceral mass increasing as a function of dietary soya levels (c) (MSR,  $y = 7.73 + 0.002c$ ,  $r^2 = 0.35$ ,  $p = 0.006$ ; Figure 2.16B, day 180). At day 180, male abalone visceral mass increased from  $7.64 \pm 0.35$  g abalone<sup>-1</sup> for males fed the FM-only based diet to  $9.00 \pm 0.47$  g abalone<sup>-1</sup> for abalone fed FM with 100 % soya, while female abalone fed graded levels of soya had similar mean visceral mass values as females fed the FM-only based diet with a mean of  $8.04 \pm 0.19$  g abalone<sup>-1</sup> (Figure 2.16B, day 180).

A – Fishmeal + crystalline isoflavone

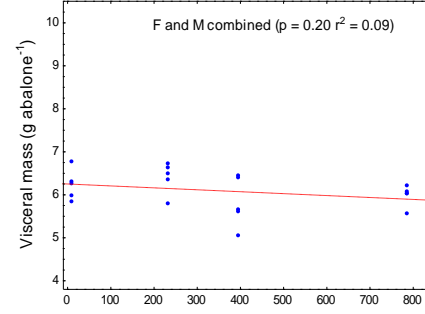
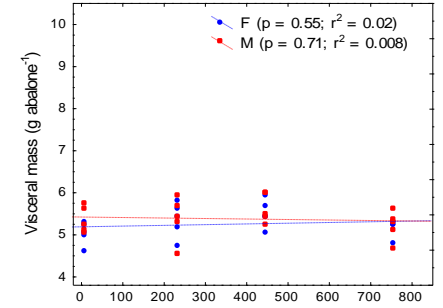
B – Fishmeal + graded levels of soya



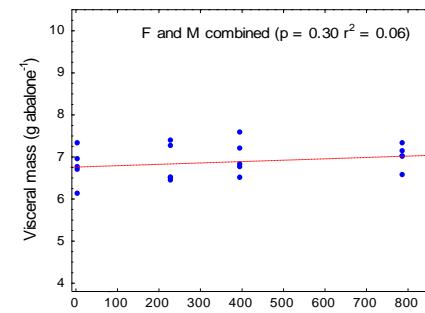
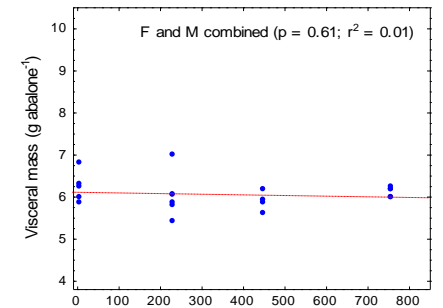
Day 0  
(September)



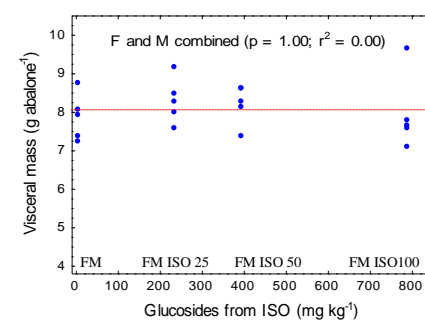
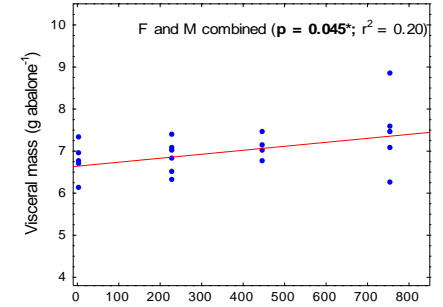
Day 45  
(November)



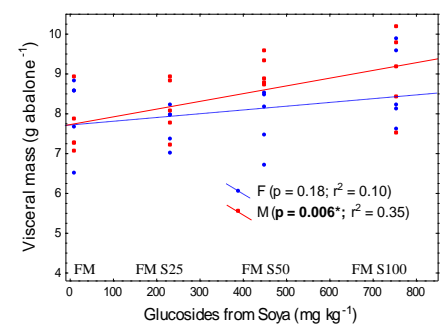
Day 88  
(December)



Day 136  
(February)



Day 180  
(March)



**Figure 2.16:** The mean visceral mass abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed@S 34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

### *Visceral index*

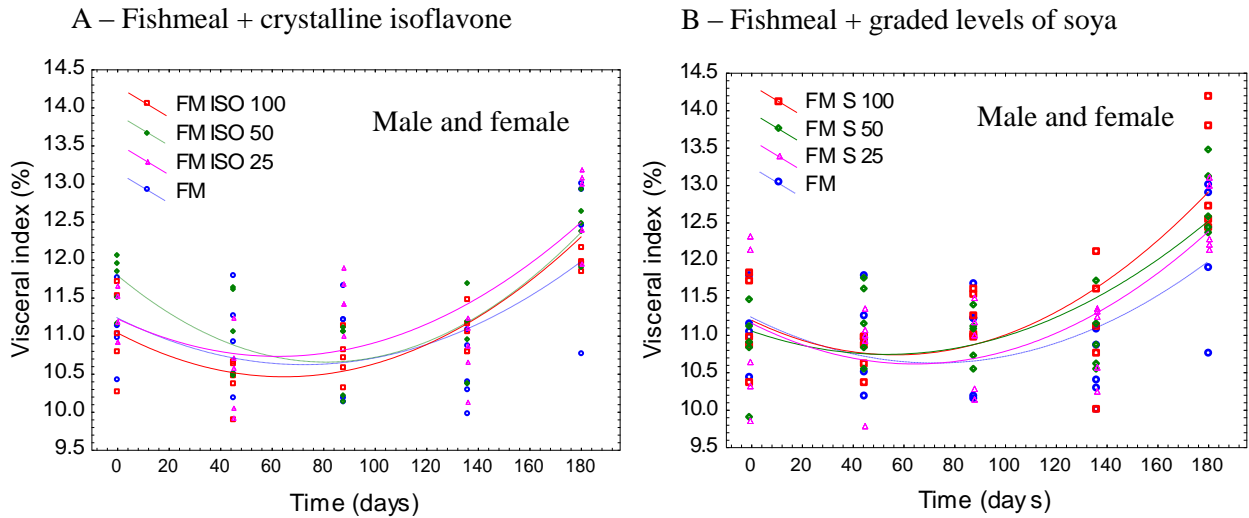
The change in mean visceral index of animals fed ISO-enriched diets over the 180 days (t) was not affected by the inclusion of ISO from  $11.27 \pm 0.11$  % at the start of the study to  $12.43 \pm 0.15$  % at day 180 (MSR,  $y = 11.07 + 0.01t$ ,  $r^2 = 0.32$ ,  $F_{(2, 197)} = 46.9$ ,  $p < 0.001$ ; Table 2.5). The mean visceral index was similar from day 0 to day 136 with an increase at the end of the study (Figure 2.17A).

The mean visceral index of abalone fed increasing rates of soya increased over time (t) with a significant contribution of time by concentration (tc) to the model (MSR,  $y = 10.64 + 0.01t$ ,  $r^2 = 0.36$ ,  $F_{(4, 195)} = 20.1$ ,  $p < 0.001$ ; Table 2.5), whereby the visceral index was similar from day 0 to 136 with differences in visceral index on day 180 as soya levels increased (Figure 2.17B).

On day 0, the average visceral index of males was higher than that for females. This was independent of treatment for abalone fed ISO-enriched diets (MSR,  $y = 10.53 + 0.49s$ ,  $r^2 = 0.11$ ,  $F_{(1, 38)} = 5.86$ ,  $p = 0.02$ ; Figure 2.18A, day 0) and abalone fed soya diets (MSR,  $y = 10.26 + 0.51s$ ,  $r^2 = 0.7$ ,  $F_{(1, 38)} = 4.16$ ,  $p = 0.048$ ; Figure 2.18B, day 0). By day 45, both males and females had similar average visceral index values with no influence of ISO and soya levels (MSR,  $p = 0.07$  and  $p = 0.61$ , respectively; Figure 2.18A, Figure 2.18B, day 45). At day 88, sex (s) significantly contributed towards the model (MSR,  $y = 10.35 + 0.47s$ ,  $r^2 = 0.10$ ,  $F_{(2, 37)} = 3.24$ ,  $p = 0.05$ ; Figure 2.18A), however, for the remaining period of the study the ISO concentration had no influence on the visceral index ( $p > 0.05$ ).

Animals fed soya diets exhibited similar indices from day 45-136, with no influence of dietary soya levels (MSR,  $P > 0.05$ ; Figure 2.18B). At day 180, average visceral index values had increased as a function of dietary soya concentration (c) from  $12.21 \pm 0.41$  % to  $13.10 \pm 0.35$  %

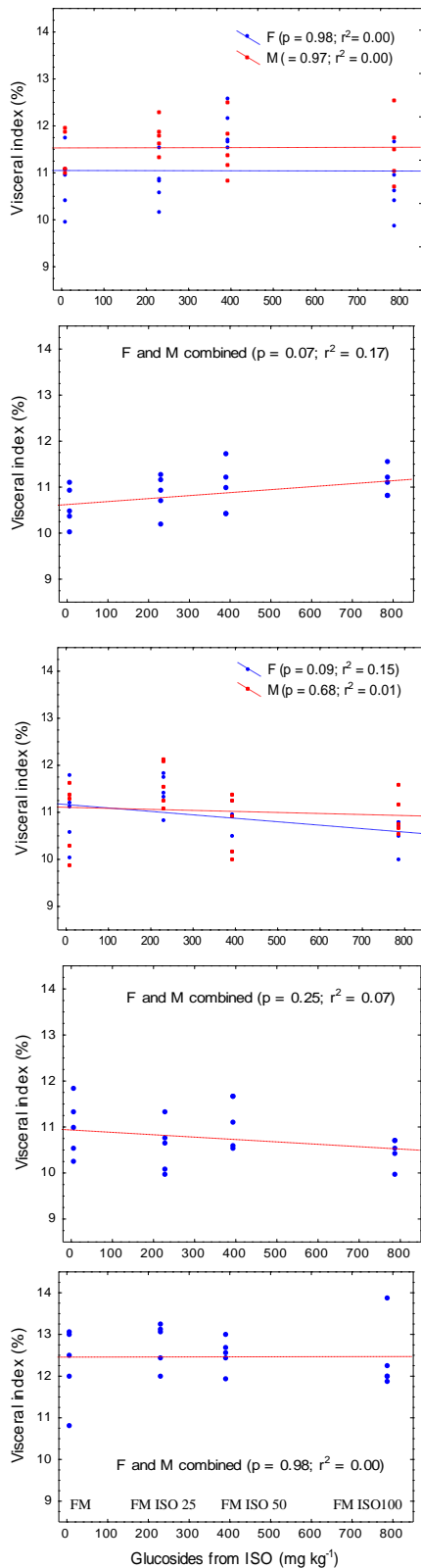
for abalone fed the FM-based diet and FM with 100 % soya, respectively ( $MSR, y = 12.24 + 0.001c, r^2 = 0.23, p = 0.03$ ; Figure 2.18B, day 180).



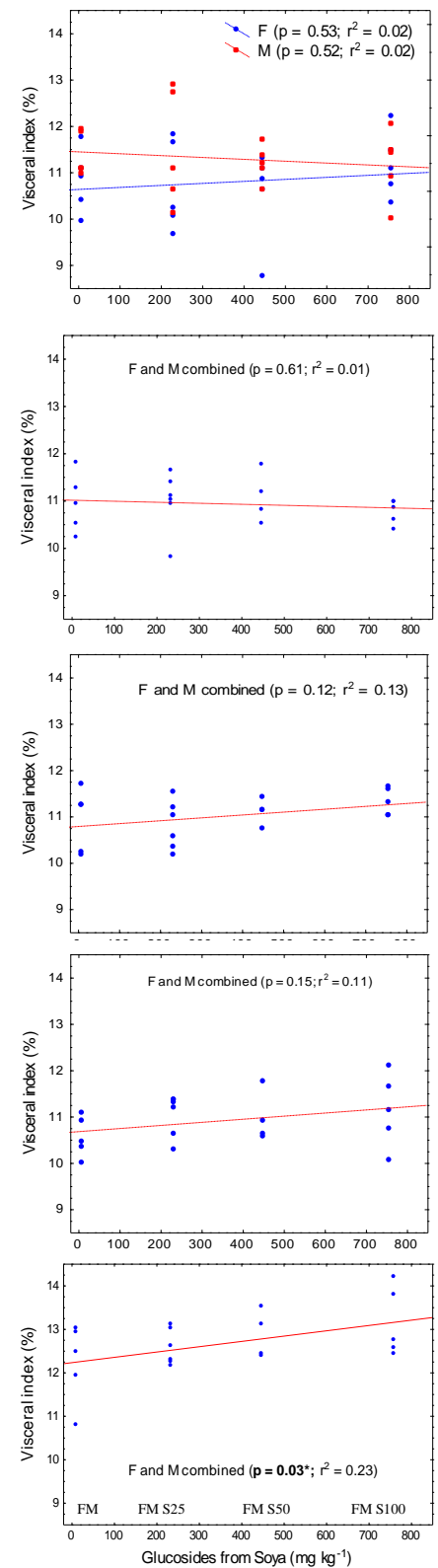
**Figure 2.17:** The change in mean visceral index of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

(A) FM:  $y = 11.23 - 0.017x + 0.0001x^2$ ; FM ISO 100:  $y = 11.04 - 0.0178x + 0.0001x^2$ ; FM ISO 50:  $y = 11.79 - 0.02x + 0.0002x^2$ ; FM ISO 25:  $y = 11.22 - 0.01x + 0.0001x^2$ . (B) FM S 100:  $y = 11.20 - 0.016x + 0.0001x^2$ ; FM S 50:  $y = 11.05 - 0.01x + 0.0001x^2$ ; FM S 25:  $y = 11.16 - 0.01x + 0.0001x^2$ .

A – Fishmeal + crystalline isoflavone



B – Fishmeal + graded levels of soya



**Figure 2.18:** The mean visceral index of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0,25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

### *Gonad bulk index*

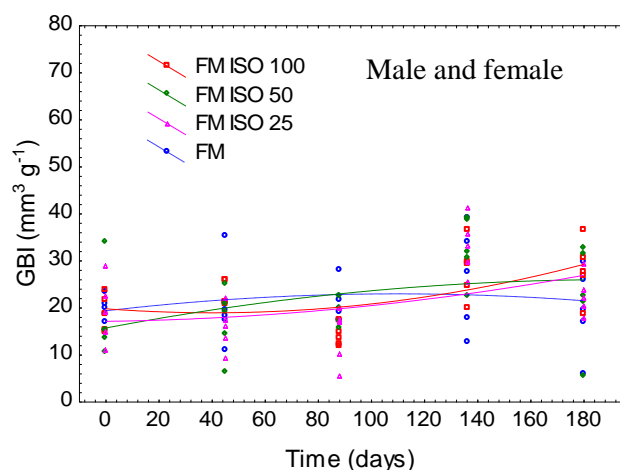
The change in mean gonad bulk index (GBI) in abalone fed ISO-enriched diets over time (t) was not significantly influenced by ISO concentration or sex, ranging from  $19.04 \pm 1.30 \text{ mm}^3 \text{ g}^{-1}$  at the start of the study to  $23.32 \pm 1.80 \text{ mm}^3 \text{ g}^{-1}$  after 180 d (MSR,  $y = 11.07 + 0.01t$ ,  $r^2 = 0.38$ ,  $F_{(2, 97)} = 30.79$ ,  $p < 0.001$ ; Table 2.5). The mean GBI slightly decreased, but not significantly from day 0 to 88, followed by an increase by day 138 and then decreased at the end of the study (Figure 2.19A).

The GBI of animals fed graded levels of soya over time was significantly affected by the time by concentration interaction (tc), sex by concentration (sc) and concentration (c) (MSR,  $y = 11.85 + 0.0002tc - 0.03c + \log 18.66\log(sc)$ ,  $r^2 = 0.20$ ,  $F_{(4, 195)} = 13.60$ ,  $p < 0.001$ ; Table 2.5), where males and females fed graded levels of soya increased in GBI differently over time as they were affected by soya levels differently at different sampling times. Some males and females fed the soya diets had very high GBI values of up to  $75 \text{ mm}^3 \text{ g}^{-1}$  at day 136 and 180, while abalone fed FM with ISO had a maximum value of only  $40 \text{ mm}^3 \text{ g}^{-1}$  at day 136. Females fed the soya diets exhibited a gradual increase in GBI from day 0 to 136, with a decrease at the end of the study (Figure 2.19B). Males fed graded levels of soya exhibited a drop in GBI on day 88, followed by an increase on day 136, after which the values decreased by the end of the study (Figure 2.19C).

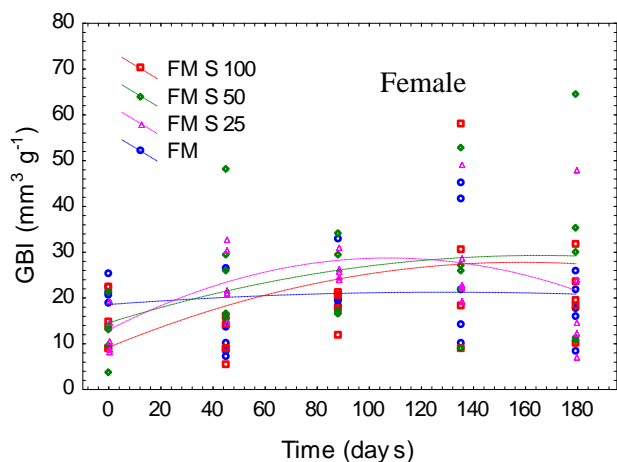
The difference in sex (s) was also evident, independent of treatment for average GBI, where male GBI was higher in ISO treatments (MSR,  $y = 7.77 + 7.51s$ ,  $r^2 = 0.17$ ,  $F_{(1, 38)} = 9.27$ ,  $p = 0.004$ ; Figure 2.20A, day 0) as well as in abalone fed the soya diets at day 0 (MSR,  $y = 13.68 + 4.69s$ ,  $r^2 = 0.20$ ,  $F_{(2, 37)} = 5.85$ ,  $p = 0.006$ ; Figure 2.20B, day 0). The difference between sexes continued until day 45 in both ISO and soya treatments. From day 136 to 180 animals fed ISO

diets were not affected by the inclusion rate of ISO (MSR,  $p = 0.94$  and  $p = 0.10$ , respectively; Figure 2.30A, day 136 and day 180). At day 88, GBI for soya fed animals was similar for both sexes with no influence of soya concentration, but from day 136, males had higher GBI values than females (MSR,  $= 27.34 + 0.01s$ ,  $r^2 = 0.86$ ,  $F_{(1, 38)} = 4.68$ ,  $p = 0.04$ ; Figure 2.20B). At the end of the study, only male GBI had increased as a function of dietary soya concentration ( $c$ ) (MSR,  $y = 21.93 + 0.03c$ ,  $r^2 = 0.21$ ,  $p = 0.04$ ; Figure 2.20B, day 180) from  $21.80 \pm 5.26 \text{ mm}^3 \text{ g}^{-1}$  to  $42.70 \pm 9.82 \text{ mm}^3 \text{ g}^{-1}$  for abalone fed the FM-only based diet and FM with 100 % soya inclusion rate, respectively.

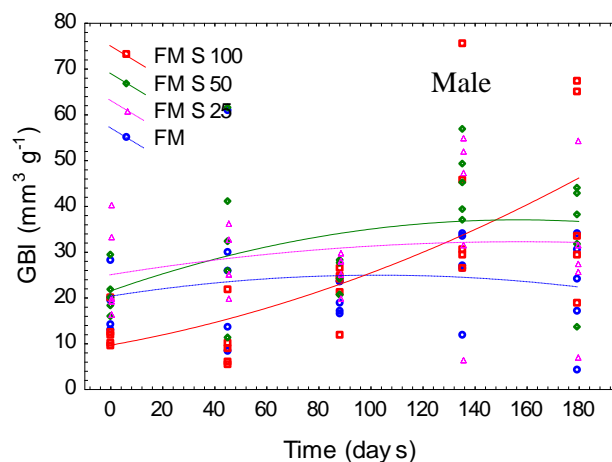
A – Fishmeal + crystalline isoflavone



B – Fishmeal + graded levels of soya

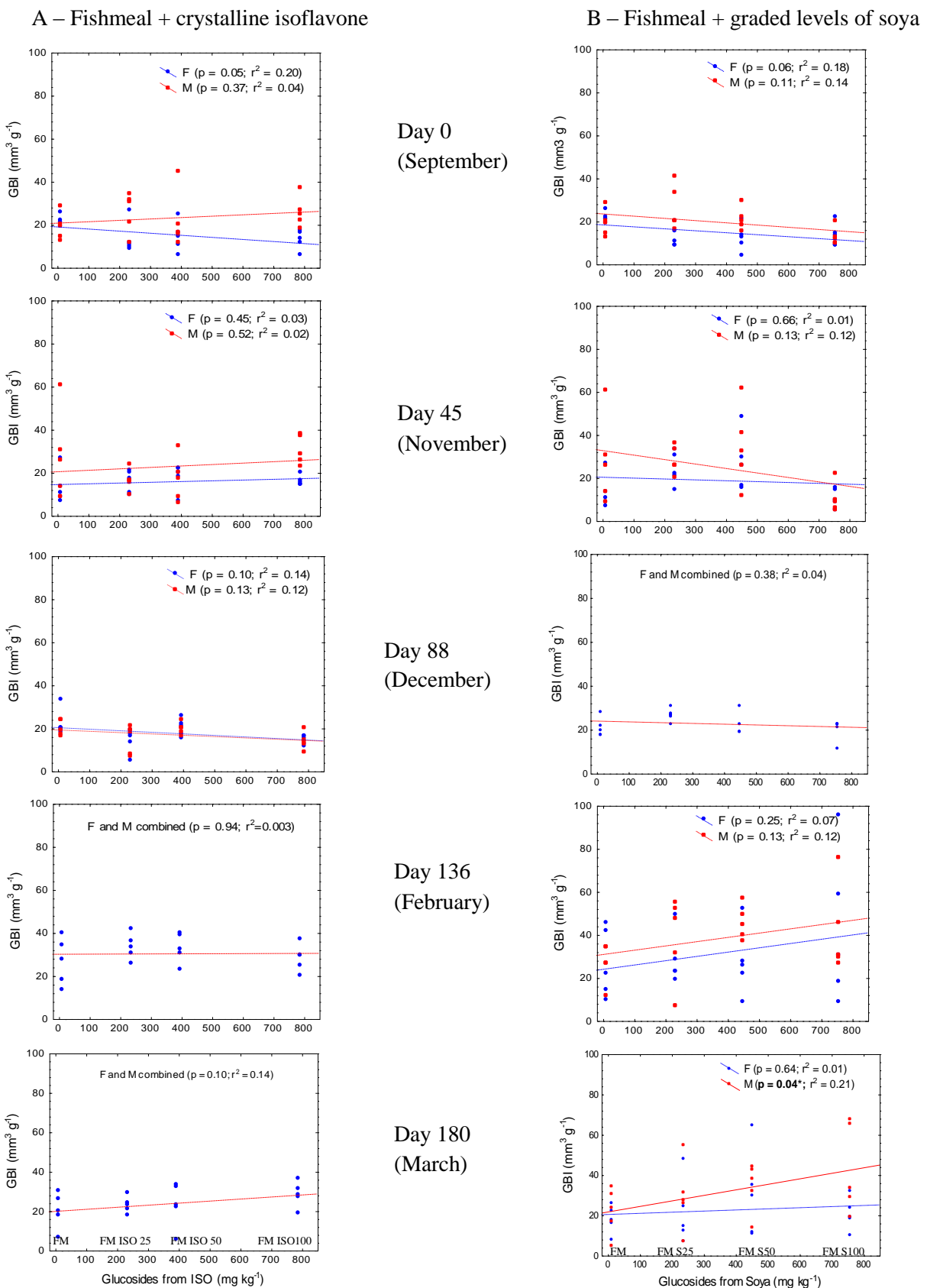


C – Fishmeal + graded levels of soya



**Figure 2.19:** The mean gonad bulk index (GBI) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

(A) FM ISO 100:  $y = 19.83 - 0.04x + 0.0005x^2$ ; FM ISO 50:  $y = 15.75 + 0.10x - 0.0003x^2$ ; FM ISO 25:  $y = 17.17 + 0.01x + 0.0003x^2$ . (C) FM S 100:  $y = 9.70 + 0.10x + 0.001x^2$ ; FM S 50:  $y = 21.56 + 0.19x - 0.0006x^2$ .



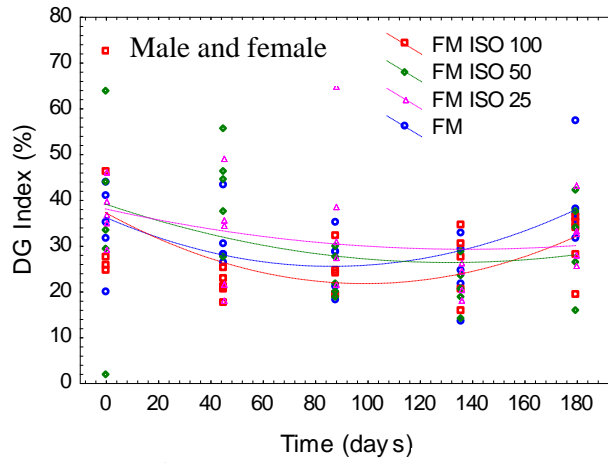
**Figure 2.20:** The mean gonad bulk index (GBI) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

### *Digestive gland index*

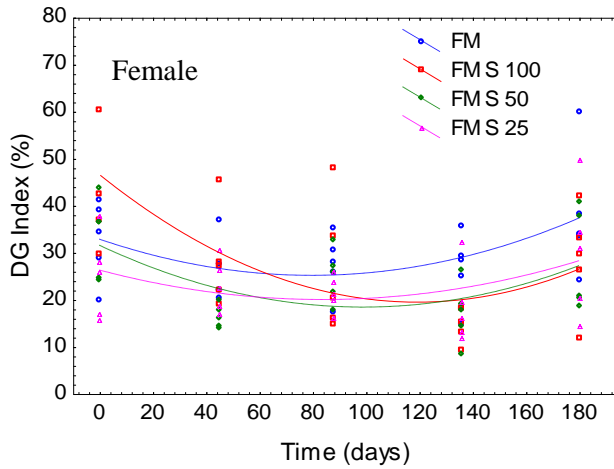
The change in digestive gland (DG) index over time (t) for abalone fed FM with the addition of ISO was affected by an interaction of time and concentration (tc) (MSR,  $y = 35.91 - 1.65\log(tc)$ ,  $r^2 = 0.07$ ,  $F_{(1, 98)} = 8.18$ ,  $p = 0.005$ ; Table 2.5), where the differences in DG index due to ISO concentration were dependent on the time of sampling. The DG index of abalone fed FM with ISO inclusion ranged from  $36.30 \pm 3.36$  % at day 0 to  $23.18 \pm 1.35$  % after 136 d, followed by an increase to  $33.53 \pm 1.98$  % at day 180. (Figure 2.21A). The change in DG index of abalone fed graded levels of soya over time (t) was influenced by the product of time and concentration (tc), concentration (c) and sex (s) of the abalone (MSR,  $y = 43.98 - 4.42\log(tc) + 0.08t + 0.02c - 3.71s$ ,  $r^2 = 0.20$ ,  $F_{(5, 194)} = 14.86$ ,  $p < 0.001$ ; Table 2.5). The DG index of female and male abalone fed graded levels of soya decreased, but not significantly, from day 0 ( $40.28 \pm 1.93$  % and  $34.43 \pm 2.79$  %, respectively) to day 136 ( $23.23 \pm 1.90$  % and  $19.36 \pm 1.73$  %, respectively) followed by an increase at day 180 ( $40.95 \pm 3.14$  % and  $31.12 \pm 2.68$  %, respectively); Figure 2.21A and Figure 2.21B, respectively).

The inclusion of ISO had no significant effect on the DG index at any sampling time (MSR,  $p > 0.05$ , Figure 2.22A). The effect of sex was evident in abalone fed increasing rates of soya on day 45 and at the end of the study, with females ( $40.95 \pm 3.14$  %) having the higher DG index than males ( $31.12 \pm 2.68$  %; Figure 2.22B).

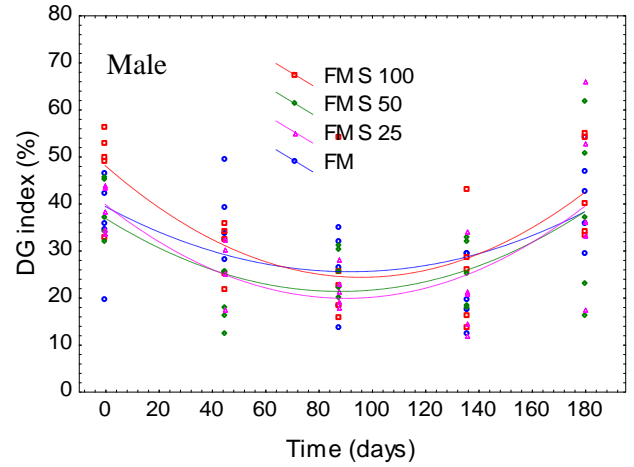
A – Fishmeal + crystalline isoflavone



B – Fishmeal + graded levels of soya



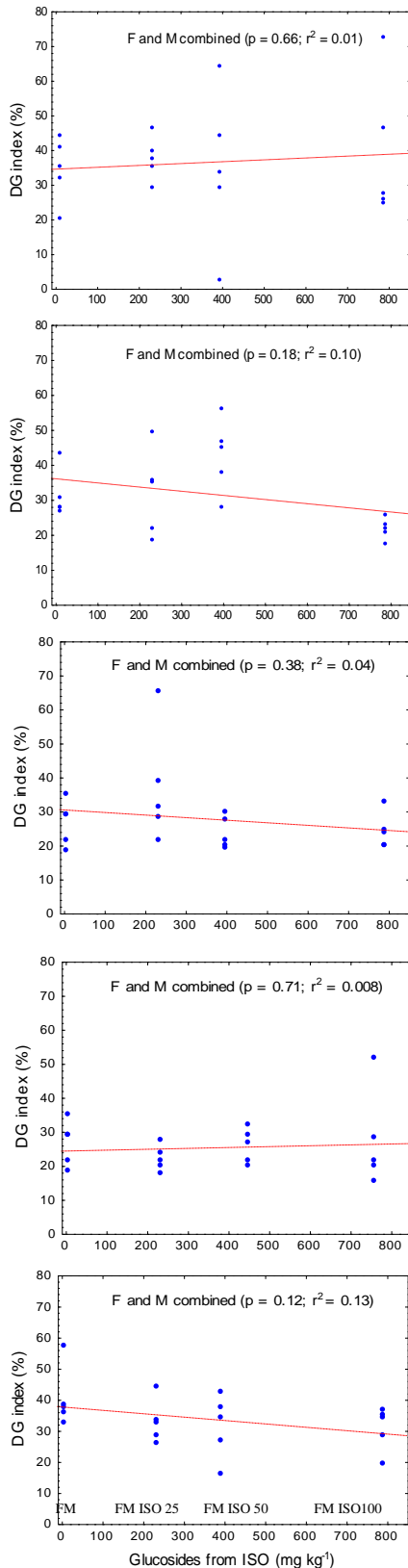
C – Fishmeal + graded levels of soya



**Figure 2.21:** The mean digestive gland (DG) index of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) over 180 days between September and March (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

(C) FM S 100:  $y = 46.63 - 0.45x + 0.0019x^2$ .

A – Fishmeal + crystalline isoflavone



Day 0  
(September)

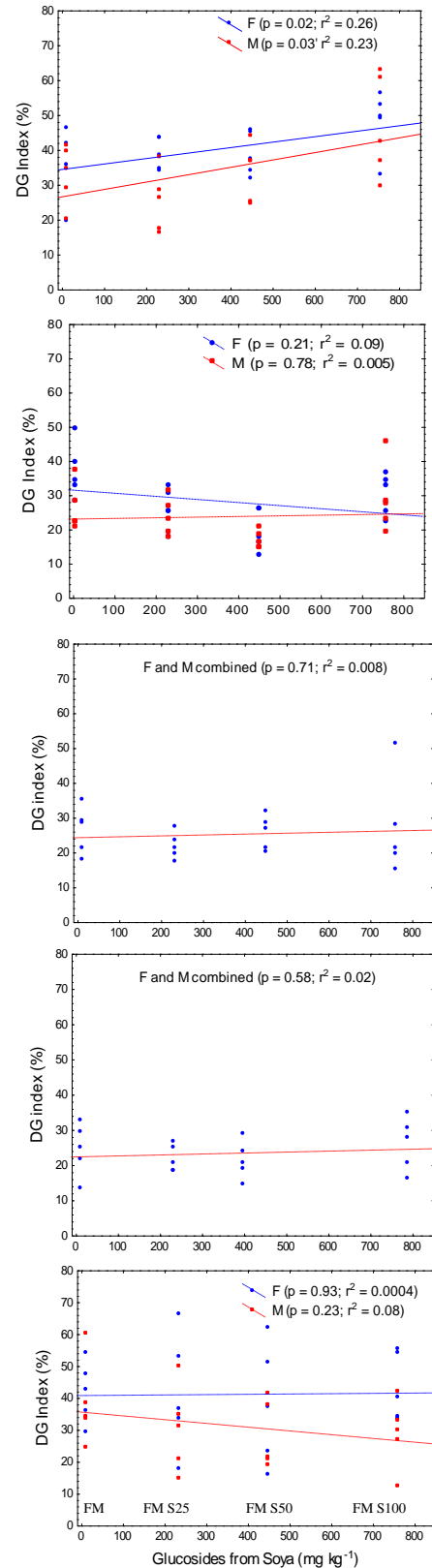
Day 45  
(November)

Day 88  
(December)

Day 136  
(February)

Day 180  
(March)

B – Fishmeal + graded levels of soya



**Figure 2.22:** The mean digestive gland (DG) index of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of the soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forward stepwise regression analysis,  $p \leq 0.05$ ).

**Table 2.5:** Gonad development of abalone fed fishmeal only and increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya in the commercial feed (Abfeed ® S34, Marifeed Pty (Ltd), Hermanus) over 180 days. The inclusion of the following variables indicates a significant contribution to the model over time. c = concentration, s = sex, sc = sex and concentration interaction, t = time, tc = time and concentration interaction, log = log transformed data. Data was analysed with a multiple forward stepwise regression analysis with an error level of 5 %.

	Day	ISO Model	r <sup>2</sup>	Soya Model	r <sup>2</sup>
Visceral mass (g abalone <sup>-1</sup> )	0-180	$y = 4.62 + 0.02t$	0.82	$y = 4.47 + 0.02t + 0.00001tc + 0.21s$	0.84
Visceral index (%)	0-180	$y = 11.07 + 0.01t$	0.32	$y = 10.64 + 0.01t + 0.00001tc$	0.34
Gonad bulk index (mm <sup>3</sup> g <sup>-1</sup> )	0-180	$y = 14.31 + 0.06t$	0.08	$y = 11.85 + 0.0002tc - 0.03c + 18.66\log(sc)$	0.20
Digestive gland index (%)	0-180	$y = 35.91 - 1.65\log(tc)$	0.07	$y = 43.98 - 4.42\log(tc) + 0.08t + 0.02c - 3.71s$	0.26

#### *Feed conversion ratio*

Feed conversion ratio (FCR) was significantly higher for animals fed the FM-only based diet on day 45 of the study (Tukey's post-hoc analysis;  $p < 0.05$ ; Table 2.6). By day 88, animals fed the diet with a 50 % soya inclusion rate had a significantly lower FCR, while by day 136, animals fed 100 % soya inclusion rate had a significantly higher FCR (Tukey's post-hoc analysis,  $p < 0.05$ ; Table 2.6). The FCR values from day 136 to 180 are not reported as animals lost weight over that period.

**Table 2.6:** Feed conversion ratio of animals fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed ® S34, Marifeed Pty (Ltd), Hermanus) from November to February. Means with different superscripts within each row and column indicate significant differences (RM-ANOVA,  $p \leq 0.05$ ).

Treatment	Day					
	45		88		136	
FM	2.23	± 0.19 <sup>b</sup>	1.06	± 0.19 <sup>ab</sup>	1.72	± 0.38 <sup>ab</sup>
FM S 100	1.28	± 0.11 <sup>ab</sup>	1.19	± 0.10 <sup>ab</sup>	2.25	± 0.33 <sup>b</sup>
FM S 50	2.84	± 1.12 <sup>ab</sup>	0.84	± 0.06 <sup>a</sup>	1.76	± 0.26 <sup>ab</sup>
FM S 25	1.49	± 0.38 <sup>ab</sup>	1.15	± 0.06 <sup>ab</sup>	1.56	± 0.31 <sup>ab</sup>
FM ISO 100	2.02	± 0.58 <sup>ab</sup>	1.41	± 0.18 <sup>ab</sup>	1.88	± 0.18 <sup>ab</sup>
FM ISO 50	1.41	± 0.19 <sup>ab</sup>	1.23	± 0.36 <sup>ab</sup>	1.80	± 0.24 <sup>ab</sup>
FM ISO 25	1.29	± 0.13 <sup>ab</sup>	0.96	± 0.04 <sup>ab</sup>	1.36	± 0.24 <sup>ab</sup>

#### *Water quality*

There was no significant difference in water temperature ( $16.97 \pm 0.07^\circ \text{C}$ ), dissolved oxygen ( $7.67 \pm 0.02 \text{ mg L}^{-1}$ ), pH (8.13) and total ammonia nitrogen ( $26.33 \pm 14.49 \mu\text{g L}^{-1}$ ) between treatments (RM-ANOVA,  $p > 0.05$ ; Table 2.7).

**Table 2.7:** Mean ( $\pm$  standard deviation) water temperature, oxygen (O<sub>2</sub>), pH and total ammonia nitrogen (TAN) of abalone fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (RM-ANOVA,  $p > 0.05$ ).

	Treatment	Mean	Minimum	Maximum
Water Temperature (°C)	FM	17.03 $\pm$ 1.50	17.03	20.10
	FM S 100	16.92 $\pm$ 1.49	13.80	20.10
	FM S 50	17.00 $\pm$ 1.59	14.00	19.70
	FM S 25	16.87 $\pm$ 1.50	13.90	19.60
	FM ISO 100	16.95 $\pm$ 1.57	13.90	19.70
	FM ISO 50	16.97 $\pm$ 1.58	14.20	19.80
	FM ISO 25	17.07 $\pm$ 1.49	14.20	19.90
O <sub>2</sub> (mg L <sup>-1</sup> )	FM	7.66 $\pm$ 0.40	7.66	8.30
	FM S 100	7.70 $\pm$ 0.43	6.76	8.56
	FM S 50	7.66 $\pm$ 0.42	6.77	8.26
	FM S 25	7.67 $\pm$ 0.40	6.67	8.37
	FM ISO 100	7.67 $\pm$ 0.40	6.57	8.23
	FM ISO 50	7.68 $\pm$ 0.42	6.81	8.39
	FM ISO 25	7.64 $\pm$ 0.45	6.27	8.42
pH	FM	8.10	7.80	8.31
	FM S 100	8.11	7.88	8.56
	FM S 50	8.17	7.82	8.90
	FM S 25	8.09	7.66	8.37
	FM ISO 100	8.17	7.73	8.31
	FM ISO 50	8.14	7.90	8.90
	FM ISO 25	8.10	7.16	8.90
TAN ( $\mu$ g L <sup>-1</sup> )	FM	24.72 $\pm$ 11.54	3.38	47.35
	FM S 100	27.81 $\pm$ 13.41	5.33	62.01
	FM S 50	24.66 $\pm$ 10.88	4.35	47.35
	FM S 25	27.05 $\pm$ 11.88	8.26	58.10
	FM ISO 100	27.72 $\pm$ 11.81	8.26	52.24
	FM ISO 50	27.13 $\pm$ 12.00	2.40	59.01
	FM ISO 25	24.96 $\pm$ 15.12	5.33	91.33

## DISCUSSION

### *Crystalline isoflavone inclusion*

The objective of this study was to compare the growth and gonad development of farmed abalone fed isonitrogenous and isoenergetic diets with graded levels of soya, graded levels of crystalline ISO without the inclusion of soya and a diet without the inclusion of both ISO and soya. These objectives were met through the successful inclusion of crystalline ISO in FM-only based diets. The crystalline ISO concentrations were similar to those of the ISO concentrations in their equivalent soya diets at the graded levels of 25, 50 and 100 % of the soya present in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus). In addition, the leaching rates of ISO in the crystalline form and in the plant-derived form in the soya were independent of the source and treatment. Therefore, all dietary treatments were compared.

The concentrations of ISO in soya were variable and it was suggested that this is dependent on the processing procedures (Coward *et al.*, 1998). The total isoflavone concentration, as reported by Ayres (2013) (64.14 mg 100 g<sup>-1</sup>) was between the 50 % (45.20 mg 100 g<sup>-1</sup>) and 100 % (76.00 mg 100 g<sup>-1</sup>) soya inclusion rate.

### *Abalone growth*

The growth of abalone fed ISO-enriched diets was not affected by ISO levels as there were no concentrated-related changes in monthly weight and length gain. However, the monthly weight gain of male abalone increased as a function of dietary soya inclusion level. Ayres (2013) fed abalone, *H. midae* two isonitrogenous and isoenergetic diets with protein sources from FM as the sole protein source and a combination of soya and FM. Diets containing soya resulted in faster

weight and length gain (Ayres, 2013). However, the effect of sex on growth was not reported (Ayres, 2013). Shipton and Britz (2001) substituted fishmeal in an FM diet with 30 and 50 % soya and found that this had no significant effect on abalone growth. A reason for differences in findings may be due to the use of juvenile abalone ( $10.60 \pm 0.1$  mm shell length) cultured in a laboratory in 2-L plastic jars by Shipton and Britz (2001), whereas Ayres (2013) kept the abalone in a farm-scale flow-through system. In addition, Ayres (2013) and the current study used sexually mature abalone and the reproductive indices such GBI, visceral index, and gonad histology could be quantified. A comparison of these studies suggests that, in sexually mature abalone, formulated diets that contain both plant and animal protein exhibited better growth than diets containing FM as the sole protein source. Similarly, a combination of plant and animal protein lead to better growth than plant protein alone in *H. midae*, *H. fulgens*, *H. discus hannai* and *H. asinina* (Britz, 1996; Guzmán and Vianna, 1998; Kim *et al.*, 1998; Lee, 1998; Bautista-Teruel *et al.*, 2003; Dlaza *et al.*, 2008). As the soya inclusion level in the commercial feed (Abfeed® S34, Marifeed, Pty (Ltd), Hermanus) was reduced, mean monthly weight gain decreased. This correlation was not present in abalone fed the ISO-enriched diets. Length gain was not affected by the increase in soya level, which suggests that energy was invested into somatic growth during the six month study. Therefore, soya, or possibly a compound in soya not excluding plant derived ISO, may have caused the increase in mean monthly weight gain in males.

Weight loss occurred in at least one replicate of each treatment between day 136 (February) and day 180 (March). Abalone meat mass and shell length were similar between days 136 and 180, while visceral mass continued to increase indicating that investment into somatic growth discontinued between these two sampling times. In addition, the decrease in biomass may have

been due to a loss of gametes (Newman, 1968). Up to 10 % loss of the total body mass was reported in wild *H.midae* due to the excretion of gametes (Newman, 1968). Weight loss due to spawning events has been reported in the zebra mussel (*Dreissena polymorpha*), blue mussel (*Mytilus eduli*) and the Atlantic ribbed mussel (*Geukensia demissa*) (Garton and Haag, 1992; Reimer, 1999; Jost and Helmuth, 2007). Abalone fed the FM treatment had the highest percentage of biomass loss (7.56 %), while abalone fed FM with 100 % soya inclusion had the lowest percentage of biomass loss (0.79 %). However, the frequency of tanks with reported weight loss was highest for abalone fed FM with 100 % soya inclusion, and the number of tanks with weight loss decreased as soya inclusion rate decreased. These findings have applications since this will not only affect the meat yields for the canned products, but also the biomass of exported live abalone. Due to the duration of the study, it is uncertain whether how long this decrease in biomass may have continued and whether the biomass loss may increase as the decrease was only observed between the last two sampling intervals.

The whole body mass of abalone fed the FM-only based diet and ISO-enriched diets were similar throughout the study, while the inclusion of graded levels of soya influenced males and females differently dependent on time. At day 88 (December), the whole body mass of abalone had decreased as a function of dietary soya level due to the decrease in the mean meat mass and mean shell length. Thus, abalone fed the FM-only based diet grew the best, while the more soya was added, the less investment was made into meat and shell growth. From day 45 (November) to day 88 (December) abalone consuming FM with the addition of 50 % soya grew better than abalone fed FM with 100 % soya while consuming less feed. Ayres (2013) reported no effect of diet on FCR, however, FCR values were higher during the apparent spawning season. In addition, abalone fed a combination of FM and soya grew better than abalone fed the FM-only

based diet at every sampling time over the 12-months period in both sexes (Ayres, 2013). At day 136 and day 180, the effect of increasing soya levels on the whole body mass was dependent on the sex of the abalone. At day 136, only female whole body mass increased as a function of dietary soya level with a higher FCR for abalone fed FM with 100 % soya inclusion. On day 180, the mean whole body mass of males was greater than that of females and it increased with increasing soya level. The correlations on day 136 and 180 were due to the mean meat mass and mean shell length increase as a function of soya level. Although the female mean whole body mass and mean meat mass positively correlated with dietary soya on day 136, female abalone fed FM with 100 % soya inclusion performed similarly to males fed the FM-only based diet and FM with graded levels of soya. By the end of the study, only mean male abalone whole mass was positively correlated to dietary soya level, due to the increase in mean meat mass. However, the meat index of abalone was not affected by the inclusion of graded levels of ISO and soya. The meat mass as a percentage of whole body mass decreased similarly over time for all treatments. These findings support those reported by Ayres (2013), where meat mass index was similar between diets during periods of gonad growth. Feeding abalone a FM-only based diet at certain times of the year may be beneficial as this dietary treatment yielded higher whole body mass and meat mass at those times of year. However, in order for females to obtain maximum growth at the current soya inclusion rate, more feed may be required during those times.

The sex of the abalone significantly contributed to the CF in abalone fed ISO-enriched diets and FM with the addition of soya diets. Therefore, the investment of energy into tissue was different between males and females, which was supported by sex-related meat mass and shell length changes at different sampling times. Only at day 45 (November) was the CF affected by the increase of both ISO concentration and soya concentration, where only female CF increased as a

function of crystalline ISO and dietary soya levels. This correlation was however, not present for the rest of the study. By the end of the study, males had a higher CF than females independent of dietary treatment. Ayres (2013) reported a similar CF with no sex effect for abalone fed a FM-only based diet and a combination of FM and soya. Therefore, the inclusion of crystalline ISO and soya at graded levels had no effect on the weight-length relationship of farmed abalone, *H. midae*.

#### *Water loss*

Meat water loss was similar for all treatments throughout the study. These findings are similar to those reported by Ayres (2013), where meat water loss was independent of dietary treatment with and without soya. Webber (1970) suggested that the foot was the nutritive storage tissue, decreasing in size as gonad size increases, while the concentration of the metabolite, glycogen, decreased with decreasing foot mass. In the current study, meat mass decreased at the end of the study, coinciding with a decrease in meat water loss. Due to the length of the study, it cannot be established whether the decline would have continued. Glycogen is the major storage form of energy in abalone tissues (Laas and Vosloo, 2010) and for every gram of glycogen stored, three to four grams of water are stored (Kreitzman *et al.*, 1992). Since water loss in the meat was similar, this suggests that the glycogen levels in animals in all treatments were probably similar although meat glycogen levels were not measured in this study.

The inclusion of crystalline ISO affected the visceral water loss of females at certain times of the year, while the effect of graded levels of soya on visceral water loss was more prominent. The inclusion of ISO only had a significant effect on female visceral water loss at the end of the study, when visceral water loss decreased as ISO-levels increased. Visceral water loss began to

decrease with increasing soya levels from day 88 until the end of the study. The inclusion of soya at graded levels affected the visceral water retention in both males and females, but by the end of the study, only females were affected by soya inclusion level. The decrease in visceral water loss in females at day 88 coincided with the increase in visceral index and GBI values. These results differed from those of Ayres (2013), where visceral water loss was independent of diet. Abalone fed a FM-only based diet and abalone fed a combination of FM and soya exhibited similar visceral water loss, with more water loss after spawning and decreasing water loss during the spawning season (Ayres, 2013). Thus, the decrease in visceral water loss between day 136 and day 180 may be indicative of spawning events. The visceral water loss in the current study was similar to the water loss reported by Webber (1970), Bilbao *et al.* (2012), Laas and Vosloo (2010) and Ayres (2013) for *H. cracheroidii*, *H. tuberculata coccinea* and *H. midae*, respectively. Riddin (2013) reported that testes contained less lipid than ovaries with a higher moisture content. Liyana-Pathirana *et al.* (2002) also reported an increase in gonad moisture content with a decrease in lipid content in the green sea urchin, *Strongylocentrotus droebachiensis*. Lipid is the nutritive storage product of oocytes and has been reported to be significantly higher in females than in males (Webber, 1970; Huang *et al.*, 2008; Riddin, 2013). Although gonad lipid content was not assessed in the current study, this may support the fact that male viscera on average lost more water than females within all treatments. The negative correlation of both crystalline ISO and soya concentration with visceral water loss suggests that lipid deposition, resulting in water loss was dependent on the treatments. This remains to be tested.

### *Gonad development*

The gonad forms a large component of abalone viscera and it is not possible to accurately separate gonad tissue from visceral tissue (Tutschulte and Connell, 1981) and, as such, changes in the visceral mass are sometimes used as an indicator of changes in gonad development in abalone (Ayres, 2013; Riddin, 2013). The visceral mass of abalone fed ISO-enriched diets was not affected by ISO level, but by soya inclusion rate. For the first 45 days, male abalone had a higher mean visceral mass than females, independent of dietary treatments. For the rest of the study, visceral mass in abalone fed FM and ISO-enriched diets increased similarly. At day 88 (December) mean visceral mass was the same for all treatments, while mean meat mass and mean shell length decreased as a function of soya levels. In addition, the mean visceral mass continued to increase between day 136 and 180, despite the discontinuation in mean meat mass and shell length gain, suggesting that gonad mass increase was relatively faster. As a result, visceral index increased between days 136 and 180. By day 136, female meat mass and visceral mass increased as a function of dietary soya level, while in males only visceral mass increased as a function of soya inclusion level. This suggests that females were investing energy in both meat and visceral mass with increasing soya level, while males invested energy into visceral mass. By the end of the study, only male visceral mass was increasing with increasing soya inclusion rate, suggesting that some females had spawned, since this correlation occurred in females at the previous sampling time. In addition, necrotic oocytes were present on day 180 (Chapter 3), an occurrence that follows a spawning event (Gurny and Mundy, 2004). At the end of study, meat index increased with increasing soya levels, which supports the findings by Ayres (2013), where abalone fed diets containing soya had higher visceral index values. Therefore, as the soya inclusion rate in the commercial feed used in this study (Abfeed® S34, Marifeed Pty (Ltd),

Hermanus) was reduced, visceral mass decreased. Abalone farmers may benefit from feeding a diet that produces the smallest gonads, while maximizing meat gain. The smallest gonads may be obtained by feeding abalone a FM-only based diet without compromising meat mass in females. However, this is based on the results obtained at the end of the study. A longer study may be able to determine the long-term effects of feeding abalone graded levels of soya. If abalone were exported live, then a FM with the addition of 100 % soya would be required as larger gonads would contribute to the weight of the product. For males, a compromise would have to be made, as both mean visceral mass and mean meat mass increased with dietary soya levels. Therefore, abalone used for live export would benefit from the FM with 100 % soya, as would abalone that were used for canning as meat mass was greatest when the diet contained the full complement of soya.

The mean GBI of abalone fed ISO-enriched diets were not affected by ISO levels, while mean GBI was correlated to increasing soya levels dependent on sex. The mean GBI of abalone in all treatments decreased between day 136 (February) and day 180 (March), but in abalone fed ISO-enriched diets values reached a maximum of around  $40 \text{ mm}^3 \text{ g}^{-1}$ , while in abalone fed FM with soya GBI increased to approximately  $80 \text{ mm}^3 \text{ g}^{-1}$ . Males had higher GBI values than females for the first 45 days and on day 136, independent of treatment. These findings support those reported by Ayres (2013), where males had on average higher GBI values than females. At the end of the study, only male GBI values increased as a function of dietary soya level, which coincide with the increase in only mean male visceral mass. A sudden decrease in GBI values is an indicator of spawning in abalone (Wood and Buxton, 1996; Gurny and Mundy, 2004). However, the interpretation of gonad indices may be confounded when necrosis occurs within the ovary (Lleonart, 1992; Gurny and Mundy, 2004). Lleonart (1992) observed a reduction in gonad

volume as a result of reabsorption of oocytes. Therefore, the drop in gonad index as reported by Leonart (1992) was likely a result of prolonged necrosis and not a spawning event. Ayres (2013) was able to determine spawning peaks as his study was conducted over 12 months (July 2012 – June 2013). Mean GBI dropped after October and peaked again in January, with FM-fed animals exhibiting lower peaks compared to animals fed a combination of FM and soya (Ayres, 2013). The current study was conducted over six months (September 2013 to March 2014) and the aim was not directed at determining spawning peaks, but testing the effects that ISO and graded levels of soya had on somatic growth and gonad growth. Riddin (2012) found that GBI of animals fed diets that included soya exhibited higher GBI values compared to abalone fed kelp and a FM-only based diet. Since the mean GBI for abalone in all treatments decreased from day 136 to 180, the results should be interpreted by including the fact that necrotic oocytes were observed at the end of the study (Chapter 3). Therefore, minimizing mean GBI values may be obtained by decreasing soya levels, but this would affect meat mass gain in males. The abalone used for live export would not be affected as the gonads form part of the product, but for abalone used for canning, abalone farmers would have to make a compromise as to whether they want to obtain maximum meat gain or to minimize gonad size.

The inclusion of ISO had no significant effect on digestive gland size over the 180 days or at any of the sampling times while a sex effect was evident in abalone fed graded levels of soya, with females having higher mean DG index values. Digestive gland size has been found to vary seasonally (Hooker and Creese, 1995; Carefoot *et al.*, 1998; Ayres, 2013), decreasing in size as gonad tissue mass increased during the reproductive season. The digestive gland is the chief organ involved in energy transformation in abalone (Carefoot *et al.*, 1998) and it is the place of nutrient storage (Litaay and De Silva, 2003). Therefore as maturation proceeds, nutrients are

drawn from the digestive gland resulting in a decrease in digestive gland size (Litaay and De Silva, 2003). In addition, the maximum relative size of the digestive gland and its metabolic activity would be expected to occur beside gametogenesis (Carefoot *et al.*, 1998). In the current study, DG index decreased from day 0 to 136 with an increase towards the end of the study, suggesting that a relatively smaller quantity of nutrients was being transported to the gonad. This is supported by the gradual decrease in GBI from day 136 to 180 in some treatments. Hahn (1981) found that the digestive gland increased in size just before or immediately after spawning, which occurred in the current study between days 136 and 180, further suggesting that some females had spawned. In contrast, Webber (1970) reported relatively constant DG sizes throughout the reproductive cycle, indicating the unlikeliness of the DG gland being a nutrient storage. However, Webber (1970) used wild abalone that fed on macro algae in its environment, compared to farmed abalone fed a high protein diet. Although there are variations in DG size, Hooker and Creese (1995) concluded that the variation of the DG should not influence GBI. Ayres (2013) reported that the DG index of abalone fed FM-only based diets was significantly higher than in abalone fed graded levels of soya, indicating a lower proportion of gonad tissue in relation to the size of the digestive gland. Similar results were found in the current study, where the DG index values in males fed FM-only based diets were higher than in those fed soya diets towards the end of the study. Differences in DG index were not evident in females over the six-month period. Females had higher DG index values at the beginning and end of the study, indicating that females had less gonad tissue mass than males. This is supported by males having higher visceral mass, visceral index and GBI at the beginning and at the end of the study, where only male visceral mass, visceral index and GBI values were increasing as a function of dietary

soya levels at day 180. Therefore, it is hypothesised that at those sampling times males were still drawing nutrients from the DG, hence the lower DG index values.

The dietary treatments were isonitrogenous and isoenergetic, therefore it is unlikely that it was the protein and energy content in the diets that resulted in the differences in growth in abalone fed the soya diets. Ingredients in the soya diets at graded levels varied in order to produce isonitrogenous and isoenergetic diets, therefore the amino acid profiles for these diets may have differed. Abalone fed ISO-enriched diets were fed the same FM-only based diet, which most likely had the same amino acid profile, which may be the reason why growth and gonad development for abalone fed ISO-enriched diets were similar. In order to test the effects of ISO present in soya on abalone growth and gonad development, the ISO in soya had to be isolated and used in a diet that did not contain soya. The use of crystalline ISO on its own, failed to promote growth and gonad development, but the reasoning behind this is still unknown. However, this may be linked to species, considering the effectiveness of crystalline ISO on various fish species. In addition, the stage of maturation of the abalone used may have also had an effect. Dietary lysine levels have been found to correlate with growth in *H. midae* (Shipton *et al.*, 2002) and since the growth and gonad development of abalone was dose dependent on soya levels, measuring the amino acid profiles may be beneficial. This was done, but it was beyond the scope of this thesis to include these data as part of the current work.

### *Conclusion*

The inclusion of crystalline isoflavone did not promote larger gonad development in farmed, *H. midae*. Although evidence suggested that mollusks have pathways of steroid binding that are functional, the effect of crystalline isoflavones on growth in mollusks and their reproduction has

not been published. The current study cannot conclude that it was not the isoflavones in the soya that was promoting gonad growth, but the form (crystalline) in which the isoflavone was administered did not promote gonad development. The increase in dietary soya levels lead to an increase in meat mass and visceral mass for both males and females. A sex effect was evident at certain times of the year, possibly due to spawning and allocation of nutrients for reproduction as indicated by the significant contribution of sex to the models predicting whole body mass, meat mass, CF, visceral mass, GBI and DG index values. Overall, only male monthly weight gain increased with dietary soya level, possibly due to some females having spawned. Weight loss was observed at the end of the study due to lack of investment into meat and shell growth, however, visceral mass continued to increase. Between day 88 and 136, abalone fed FM with 100 % soya inclusion level had a significantly higher FCR suggesting that in order to obtain higher tissue mass, abalone had to consume more food, possibly prior to spawning. Minimising visceral mass may be obtained by decreasing the soya inclusion level prior to investing energy into gonad growth, but not without compromising meat mass gain.

## CHAPTER 3

### THE EFFECT OF PHYTOESTROGENS ON OOGENESIS

#### INTRODUCTION

Different methods have been used for reproductive biology studies in abalone. Of these, histological examinations are the most accurate way to clarify maturity stages (Litaay and De Silva, 2003). This chapter will outline the histological evaluation of oogenesis in farmed female abalone, *Haliotis midae* that were fed diets with fishmeal (FM) as the only protein source and FM with increasing rates of isoflavone (ISO) equivalent to diets with either 0, 25, 50 or 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus).

Giving a broad description of oocyte maturity stages cannot be reliable for staging gametogenesis as cells are constantly dividing and differentiating into developmental stages (Roux *et al.*, 2013). Detailed descriptions of oogenesis based on cytological structures, staining and oocyte morphometrics have assessed the reproductive cycle of *Haliotids* (Young and DeMartini, 1970; Georgi and DeMartini, 1977; Ault, 1985; Nujmudeen, 2008, Ayres, 2013; Roux *et al.*, 2013). The reproductive cycle is most commonly determined in female abalone as it is easier to monitor the maturation process than in males (Gurny and Mundy, 2004). When comparing gonad development, the criteria of cell structures from the same species should be used as differences in species were found due to differences in author criteria (Roux *et al.*, 2013). Roux *et al.* (2013) divided oogenesis into nine stages in farmed *H. midae* based on ultrastructure in farmed abalone, *H. midae*. Cell types were divided into oogonia, previtellogenic oocytes, vitellogenic oocytes and mature oocytes (Roux *et al.*, 2013).

To date, no studies have evaluated the effect of isoflavones (ISO), a class of phytoestrogens on oogenesis in mollusks, while numerous studies have been conducted on fish species with variable results (Bennetau-Pellissero *et al.*, 2001; Brown *et al.*, 2014; Jourdehi *et al.*, 2014). However, xeneoestrogens such as bisphenol-A and octylphenol have been found to increase embryo production in the freshwater mud snail, *Potamopyrgus antipodarum* (Duft *et al.*, 2003; Jobling *et al.*, 2003). In addition, enhancement of oocyte production and spawning mass production were observed in the freshwater snail, *Marisa cornuarietis* and the marine prosobranch, *Nucella lapillus* when exposed to bisphenol-A and octylphenol (Oehlmann *et al.*, 2000). The inclusion of soya resulted in an increase in plasma vitellogenin in the Siberian sturgeon, *Acipenser baeri* when compared to fish fed a FM diet (Pelissero *et al.*, 1991). Oogenesis in farmed abalone, *H. midae* was influenced differently in abalone fed a diet that included soya compared to an FM-only diet (Ayres, 2013). Abalone fed a diet that included soya contained higher proportions of stage 8 oocytes that were ready to be spawned, compared to FM-fed abalone that contained majority stage 7 oocytes (Ayres, 2013).

To date, only one study has evaluated the effect of soya on the oocyte development in farmed abalone, *H. midae* (Ayres, 2013). This study is a continuation of the research by Ayres (2013). The aim of this study was to determine the effect of phytoestrogens on oocyte development of farmed *H. midae*. The aim was achieved by addressing the following objectives, which were to:

- 1) produce a diet with FM as the only major protein source and similar diets that also include 25, 50 and 100 % of the soya present in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus);

- 2) produce a diet FM as protein source with the addition of crystalline ISO that reflect the concentrations of natural, plant derived isoflavones in the soya diets at the same rate as those described in objective 1;
- 3) describe the various stages of oocyte development and compare their frequency distributions between treatments; and
- 4) determine the correlation between oocyte development and ISO and soya inclusion rate.

## **MATERIALS AND METHODS**

### *Experimental system and animals*

Animals used for histological analysis were the same animals, subject to the same dietary treatments and kept in the same system described in Chapter 2.

### *Data collection*

Of the four males and four females that were randomly selected from each tank for GBI determination, as outlined in Chapter 2, one male and one female per replicate were used for histological analysis at the end of the study. Animals were placed in labelled mesh bags, and water temperature was lowered to 10° C. Animals were shucked (removal of the shell), and the viscera (digestive gland and gonad) were separated from the foot. The wet whole mass, shucked mass, meat mass and visceral mass were obtained using an electronic balance (Kern PLS 4200-2F, serial number: WIC1200486) and measured to the nearest 0.01 g. Shell length was determined to the nearest 0.01 mm using vernier callipers. Whole viscera were placed in 40 ml plastic bottles containing Davidson's fixative (20 % formalin, 10 % glycerol, 10 % acetic acid,

30 % absolute ethanol and 30 % salt water) for 24 h after which time the solution was replaced with 70% ethanol.

### *Slide preparation*

Fixed samples were dehydrated in ethanol at 70, 96 and 100 % dilution, for 180 min each, cleared with xylene for 150 min and embedded in paraffin wax at 60° C for 120 min. Samples were sectioned with a microtome at 4 – 5 µm thickness and incubated at 60° C in an autoclave for 60 min. Sections were rinsed at 100, 90 and 70 % dilution of ethanol for 2 min each and washed in distilled water for two min. The sections were immersed in Lily Mayer's Haemotoxylin for 10 min and rinsed in water until all residual haemotoxylin was washed off. The sections were differentiated in acid alcohol and placed under a running tap for 8 min to stain blue and then rinsed in 70 % ethanol for two min. The sections were stained with Eosin for 2 min and dehydrated with 90 % ethanol for 4 min, followed by three min in 100 % ethanol and then cleared in xylene for 1 – 2 min. Subsequently, sections were mounted on microscope slides (25 x 75 x 1 mm) with di-n-butyl phthalate in xylene mounting medium and covered with a cover slide.

Slides were viewed under a light microscope (UOP, UB200i compound microscope, serial number: 201101137) at 40 and 100 X magnification. A photograph (UOP, Microscope camera DCM900) was taken randomly from the mid-section at 40 X magnification. From the photograph, each oocyte was measured and staged using SIGMASCAN® PRO 5 (Systat Software, San Jose, CA, USA). A photograph was also taken of a calibration slide with a one mm scale bar for each observed magnification to measure oocyte dimensions. Once every oocyte in the photograph was measured and assigned a maturity stage, a frequency distribution (%) was

determined with Equation 9, where the total number of oocytes for any of the nine developmental stages ( $O_s$ ) was divided by the total number of oocytes in the photograph ( $E_T$ ), and multiplied by 100. In addition, the number of oocytes  $\text{mm}^{-2}$  was determined with Equation 10, where the total number of oocytes in the photograph ( $E_T$ ) was divided by the total area in the photograph ( $A_T$ ).

$$\text{Frequency distribution (\%)} = \frac{O_s}{E_T} \times 100 \quad (9)$$

$$\text{Number of oocytes per } \text{mm}^2 = \frac{E_T}{A_T} \quad (10)$$

### *Histological assessments*

The development of oocytes was divided into 10 stages, including oogonia and necrotic oocytes, based on the descriptions and illustrations of Georgi and DeMartini (1977), Wood and Buxton (1996), Najmudeen (2008), Roux *et al.* (2013) and Ayres (2013). 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.

### *Statistics*

All data were tested for homogeneity of variance (Levene, 1960) and for normal distribution of residuals (Shapiro and Wilk, 1965). If data did not meet the assumptions they were log-transformed before analysis. Stage 7 oocytes were isolated for analysis as this was the most abundant mature stage that could be used to determine gonad development. The effect that ISO

concentration and soya concentration had on stage 7 oocyte distribution (%) and the number of oocytes  $\text{mm}^{-2}$  was analysed using multiple forward stepwise regression analysis (MSR). The ISO concentration and number of oocytes that contributed to the models were abbreviated in the regression models as “c” and “o”, respectively. All analyses were performed using the Statistica 12® software package.

## **RESULTS**

Oocytes began small and round with a large nucleus and as they matured they became polygonal and then round again, close to ovulation. However, well-developed ovaries contained fully mature oocytes that remained polygonal due to the compactness of the full ovary. In addition to the nine oocyte stages, necrotic oocytes were also observed. Necrosis is the autolysis and degeneration of unspawned oocytes (Hahn, 1989). All developmental stages were present in all treatments. They were identified according to the following criteria.

### **Oogenesis**

#### ***Oogonia***

Oogonia were oval or round and 15 – 20  $\mu\text{m}$  in diameter (Figure 3.1A). The cytoplasm of Oogonia stained dark blue indicating the presence of ribosomes. Similarly, nucleoli also stained dark-blue with haematoxylin & eosin, indicating strong basophilic properties. Oogonia were surrounded by flat, squamous follicular cells that lined up against the trabeculae, separating the gonad into small compartments.

### ***Stage 1 Oocyte***

Stage 1 oocytes were oval shaped cells, 21 – 30  $\mu\text{m}$  in length with a large round nucleus of approximately 12  $\mu\text{m}$  in diameter (Figure 3.1B). The cytoplasm of Stage 1 oocytes remained dark-blue when stained with haematoxylin-eosin, indicative of strong basophilic properties. Similarly, the nucleus was basophilic. The nucleus was stained light-blue with a densely packed chromatin network. Stage 1 oocytes adhered closely to the trabeculae and were found in clusters.

### ***Stage 2 Oocyte***

Stage 2 oocytes were round or scallop-shaped, 31 – 40  $\mu\text{m}$  in diameter with a large, round, light-blue-stained nucleus of approximately 20  $\mu\text{m}$  in diameter (Figure 3.1C). The nucleus was strongly basophilic, approximately six  $\mu\text{m}$  in diameter. The uncoiling of the chromatic network was visible in the light-blue-stained nucleus. The cytoplasm was dark-blue, with small lipid droplets. Stage 2 oocytes were found in clusters adhering to trabeculae.

### ***Stage 3 Oocyte***

Stage 3 oocytes were scallop-shaped cells measuring 41 – 55  $\mu\text{m}$  in length with a large round nucleus of approximately 25  $\mu\text{m}$  in diameter (Figure 3.1D). The nucleus was light-blue with uncoiling chromatin, and slightly lighter than that in stage 2. The dark-blue cytoplasm contained a greater number of lipid droplets with a slight increase in lipid droplet size relative to Stage 2. Stage 3 oocytes were still in clusters, attached to trabeculae, however these oocytes had larger distances between them compared to Stage 2 oocytes, due to the formation of the chorion.

### ***Stage 4 Oocyte***

Stage 4 oocytes were columnar or pear-shaped, 60 – 80  $\mu\text{m}$  in length, attached to the trabeculae at the base of the cell (Figure 3.1E). The round nucleus of approximately 30  $\mu\text{m}$  contained uncoiling chromatin. The cytoplasm was stained dark-blue and contained lipid droplets that were

more numerous and larger than in Stage 3 oocytes. The chorion became more visible in Stage 4 oocytes.

### ***Stage 5 Oocyte***

Stage 5 oocytes were columnar shaped, 100 – 160  $\mu\text{m}$  in length, with an oval nucleus that had increased to approximately 60  $\mu\text{m}$  (Figure 3.1F). The cytoplasm was stained pink due to the presence of yolk platelets and it contained many lipid droplets of similar size. The chorion was clearly visible in addition to the jelly coat formation. The attachment of Stage 5 oocytes to the trabeculae began to form a cytoplasmic stalk.

### ***Stage 6 Oocyte***

Stage 6 oocytes presented as clearly teardrop-shaped cells of 160 – 250  $\mu\text{m}$  in length. The evident characteristic of this stage was the thin cytoplasmic stalk still attached to the trabeculae (Figure 3.2A). The oval nucleus of approximately 55  $\mu\text{m}$  in length was stained pink with an enlarged and lightened round nucleolus of approximately 25  $\mu\text{m}$  in diameter. The cytoplasm was stained pink due to the presence of yolk platelets and contained lipid droplets of variable sizes. Each oocyte was encased in a thick jelly coat layer, separating cells into loose clusters.

### ***Stage 7 Oocyte***

Stage 7 oocytes were polygonal, 200 – 250  $\mu\text{m}$  in length containing a pink oval nucleus of approximately 80  $\mu\text{m}$  in length (Figure 3.2B). The cytoplasmic stalk, which had presented in Stage 6 was detached from the trabeculae and the cells were situated freely in the lumen, surrounded by the chorion and a thick jelly coat. The cytoplasm stained pink with numerous white lipid droplets.

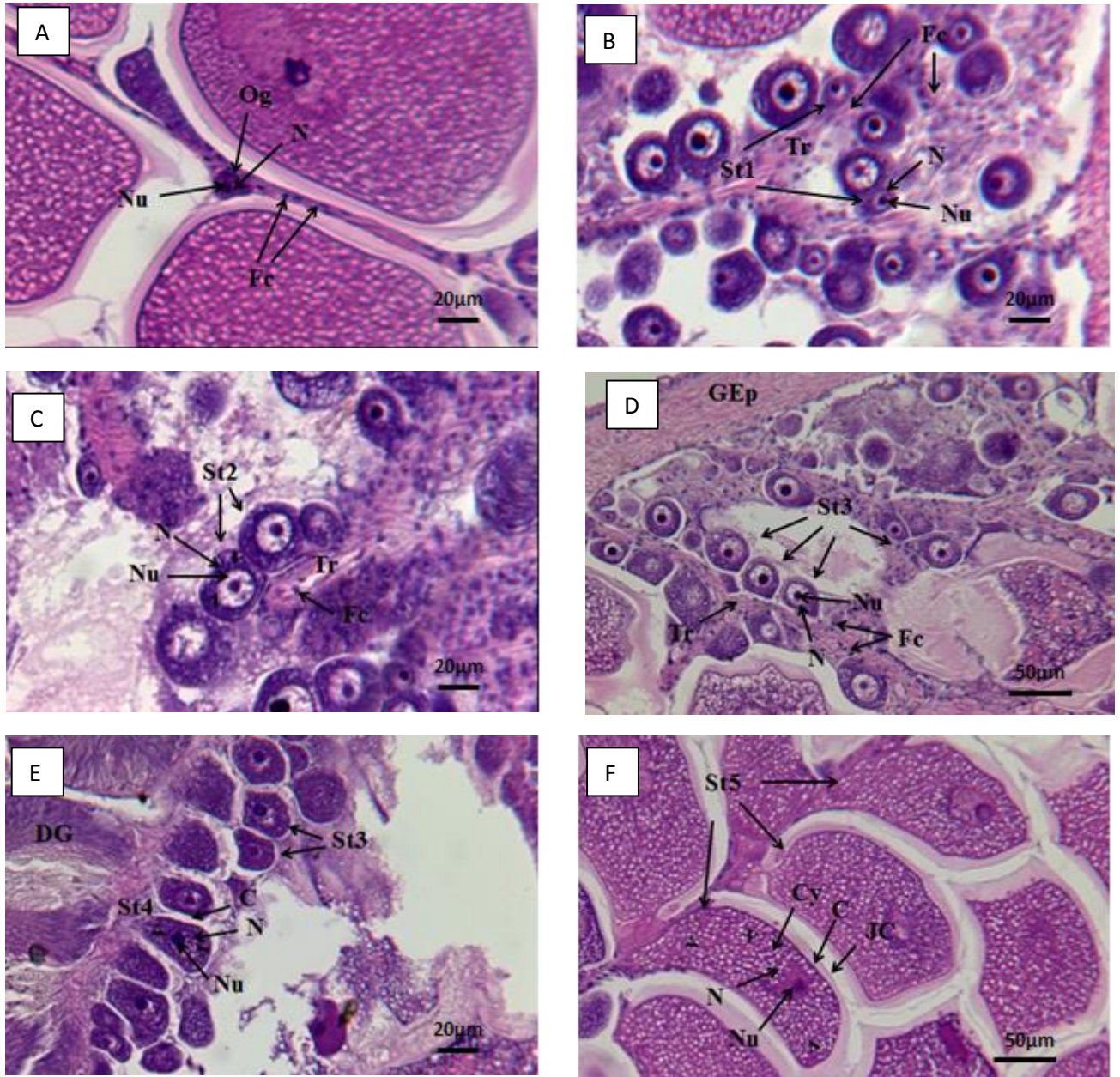
### *Stage 8 Oocyte*

Stage 8 was the final stage of oogenesis. Oocytes were polygonal, due to mature gonads that resulted in compacted oocytes (Figure 3.2C). Stage 8 oocytes measured 250 – 300  $\mu\text{m}$  in diameter and contained an oval nucleus of approximately 90  $\mu\text{m}$  in diameter. The cytoplasm was stained pink and contained lipid droplets grouped in clusters. The chorion and jelly coat became thinner as cells became more compressed.

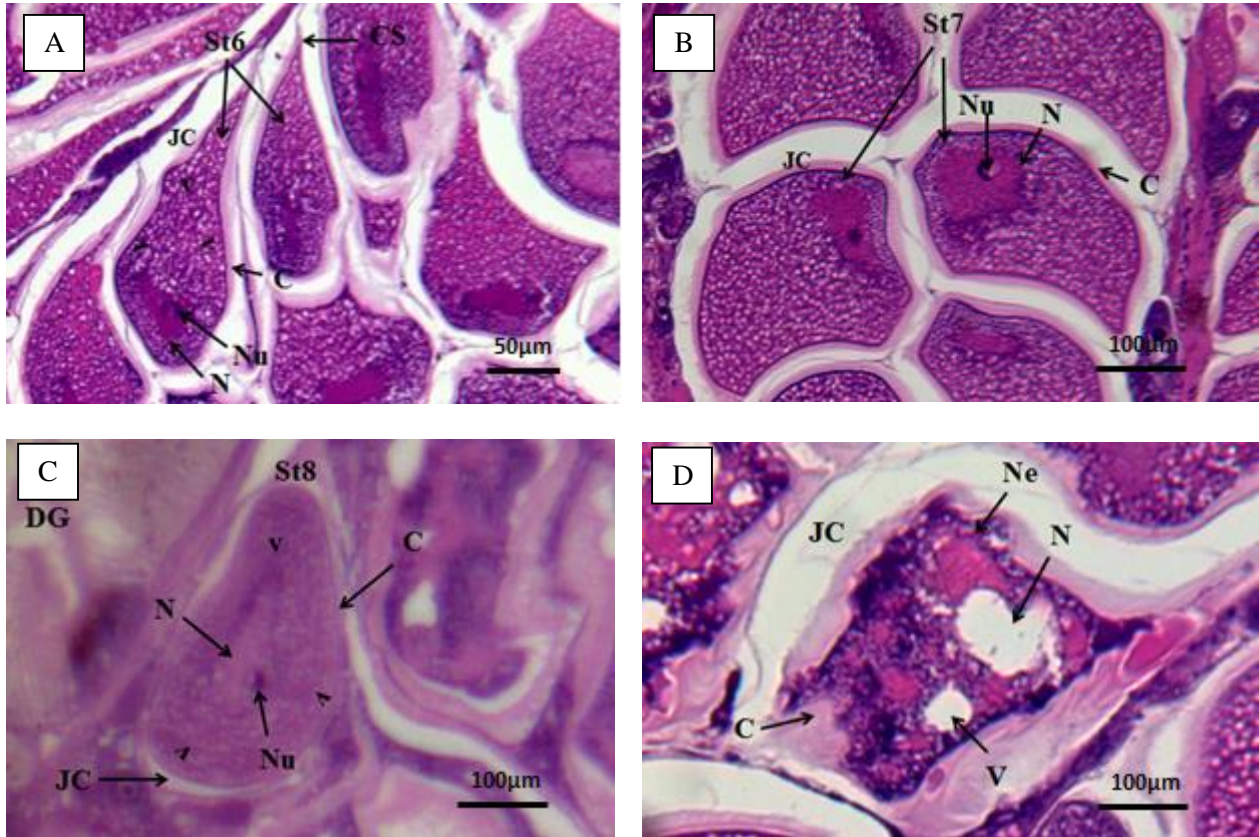
### *Necrotic oocyte*

Necrosis occurred in cells larger than 150  $\mu\text{m}$ , which contained numerous small vacuoles in the cytoplasm (Figure 3.2D). The nucleus appeared to have broken down, shrunk or disappeared. The cell membrane appeared to have disappeared with a ruptured chorion.

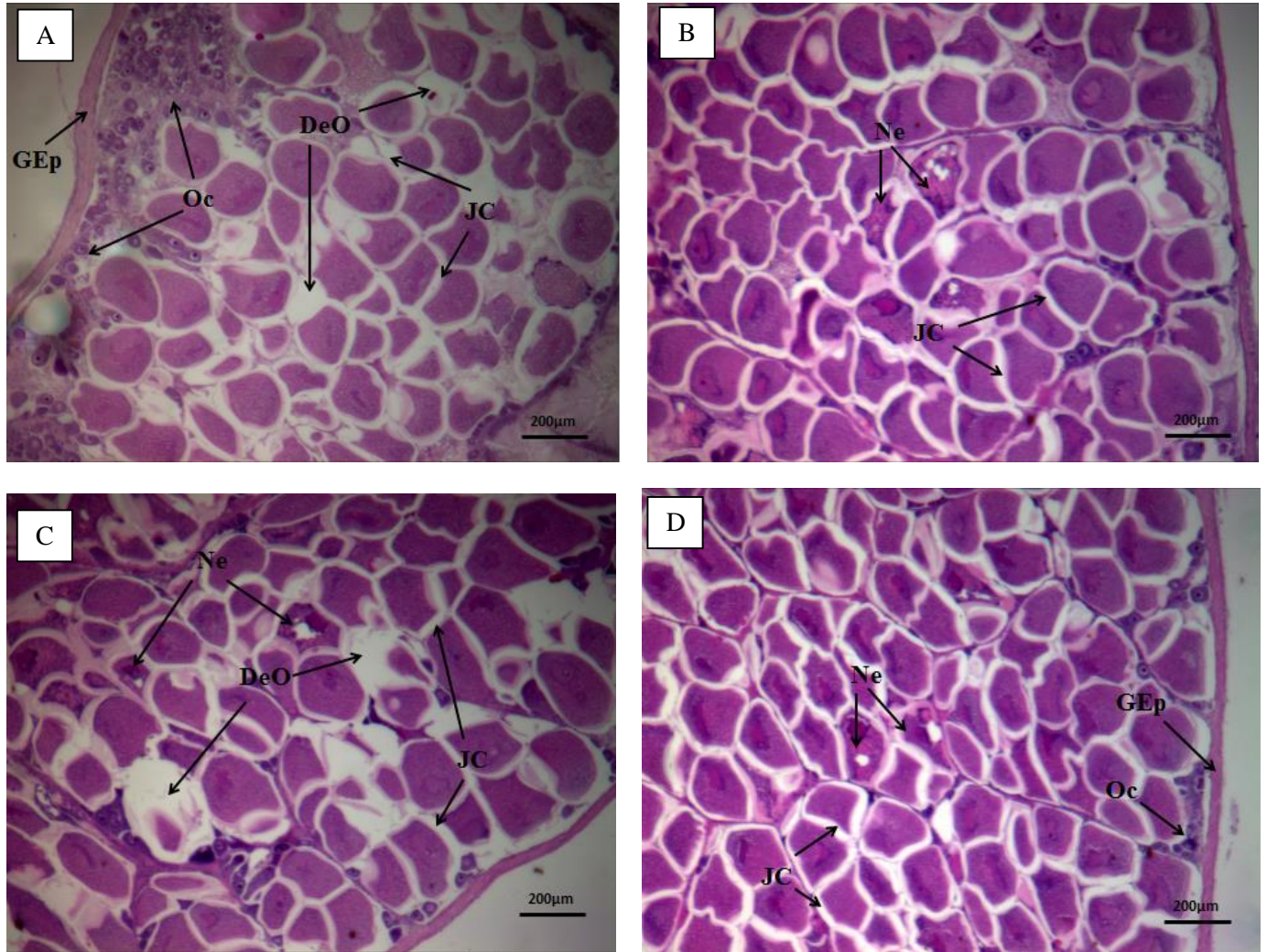
Subsamples of abalone fed the FM-only based diets illustrated gonads with areas that were devoid of Stage 7 and Stage 8 oocytes, resulting in large white spaces in the lumen (Figure 3.3A and 3.3B). In addition, ovaries contained more clusters of early maturity stage oocytes along the gonad epithelium of abalone fed the FM-only based diets (Figure 3A). Abalone fed diets containing soya, independent of inclusion rate, contained fewer ovaries with areas that were devoid of Stage 7 and Stage 8 oocytes within the lumen. The jelly coat of oocytes in abalone fed diets with the inclusion of soya was thinner and distances between oocytes were more uniform (Figure 3.3B and 3.3D) compared to oocytes of abalone fed the FM-only based diet (Figure 3A and 3C).



**Figure 3.1:** Transverse sections of the mid-section of the conical appendage of a female *Haliotis midae*. (A) Oogonia (Og) stained dark blue; (B) stage 1 oocytes (St1) attached to the trabeculae (Tr) containing squamous follicular cells (Fc); (C) scallop-shaped stage 2 oocytes (St2) in clusters; (D) larger stage 3 oocytes (St3) with a light blue-stained nucleus (N) and a dark nucleolus (Nu); (E) columnar stage 4 oocyte (St4) adhering to the trabeculae alongside the digestive gland (DG); and (F) large columnar stage 5 oocytes (St5) with pink cytoplasm (Cy) containing numerous lipid droplets (v) a prominent jelly coat (JC) and chorion (C).



**Figure 3.2:** Transverse sections of the mid-section of the conical appendage in a female *Haliotis midae* depicting mature oocyte development. **(A)** Teardrop-shaped stage 6 oocyte (St6) with long cytoplasmic stalk (CS) and cytoplasm containing numerous lipid droplets (v); **(B)** stage 7 oocytes (St7) surrounded by a thick chorium (C) and jelly coat (JC); **(C)** stage 8 oocyte (St8) alongside the digestive gland (DG) surrounded by a thin jelly coat and thin chorium; and **(D)** necrotic (Ne) oocyte with a large vacuole (V), collapsed chorium (C) and disappearance of the nucleus (N). In all stages except in necrotic oocytes the nucleolus (Nu) was stained light-blue.

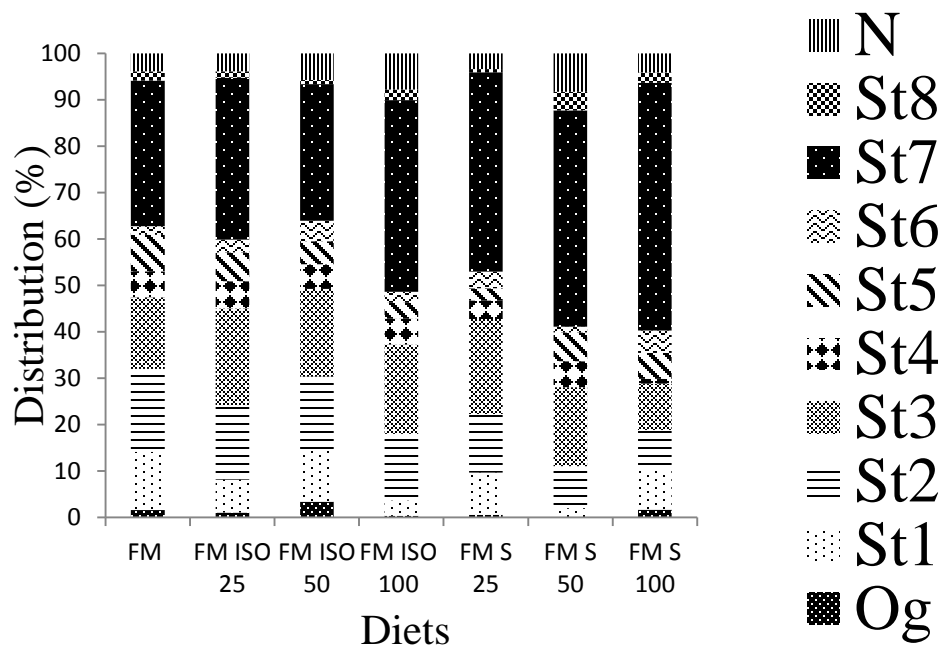


**Figure 3.3:** Transverse sections of the mid-section of the conical appendage in female *Haliotis midae*, depicting oocytes of animals fed different diets. **(A)** Animal fed a fishmeal (FM) only based diet with clusters of early maturity stage oocytes (Oc) and areas of the lumen that were devoid of oocytes (DeO); **(B)** animal fed the diet containing 100 % soya inclusion level, illustrating necrotic oocytes and a thick jelly (JC); **(C)** animal fed the FM diet with the inclusion of 100 % isoflavones, illustrating necrotic oocytes (Ne) and areas devoid of oocytes (DeO) with a thick jelly coat (JC) surrounding all oocytes; and **(D)** animal fed the FM diet with 25 % soya inclusion level, illustrating a cluster of immature oocytes along the gonad epithelium (GEp) and the presence of necrotic oocytes (Ne).

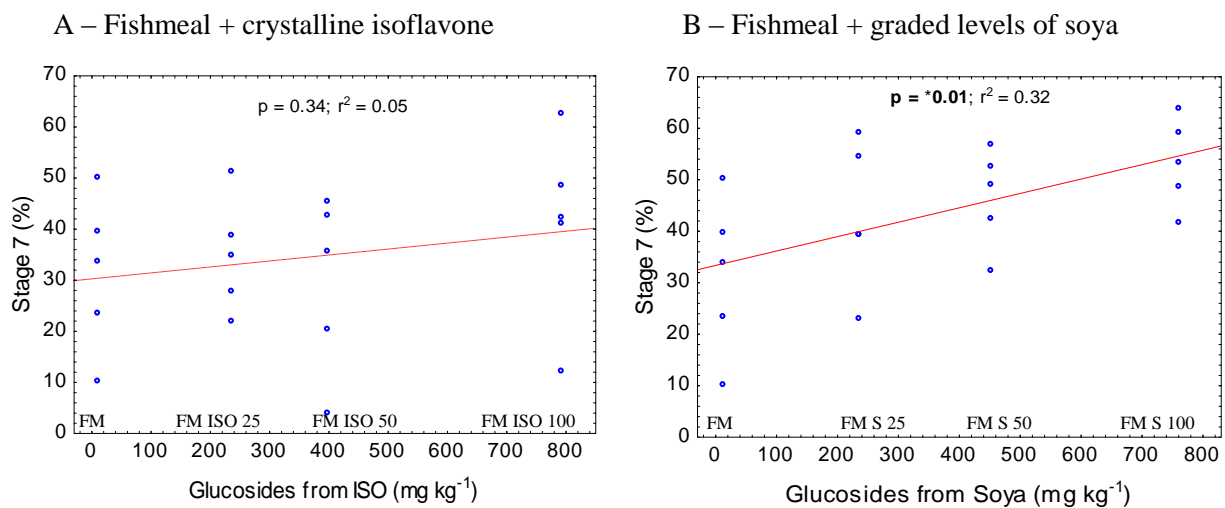
All stages of oocyte maturation were present in all treatments. Fifty-three-percent of the oocytes in the gonads of female abalone fed FM-only based diets were made up of immature (Oogonia to Stage 4) oocytes. The gonads of female abalone fed increasing levels of soya (i.e. 25, 50 and 100 % soya diets) were characterised by a decrease in proportion of immature oocytes in the gonad (47, 34 and 30 % in the, respectively). Mature oocytes (Stage 5 to 8) in females fed the FM-only based diets made up 43 % of the oocytes in the gonad, and 45, 40 and 49 % of the oocytes in abalone fed increasing levels of ISO were mature. Female abalone fed FM with increasing levels of soya contained mature oocytes that made up 50, 58 and 66 % of the oocytes in the gonad.

The predominant stage in all treatments was Stage 7, which made up 31 % of the oocytes in the gonads of abalone fed the FM-only based diets. At increasing rates of ISO inclusion, female gonads were made up of 35, 30 and 41 % of Stage 7 oocytes, respectively. The gonads of female abalone fed increasing levels of soya were characterised by an increase in proportion of Stage 7 oocytes in the gonad (43, 47 and 53 %, respectively). The gonads of females fed the FM-only based diet contained 2 % of Stage 8 oocytes, and the gonads of females fed ISO-enriched diets contained 1.4, 0.8 and 2.3 % of Stage 8 oocytes at increasing ISO inclusion rate. Necrotic oocytes were present in the gonads of female fed the FM-only based diet at a rate of 3.9 %, and at increasing ISO inclusion levels, necrotic oocytes made up 3.9, 5.8 and 7.8 % of the oocytes in the gonad. At increasing soya inclusion rate, female gonads made up were made up of 3.4, 8.3 and 4.1 % of necrotic oocytes (Figure 3.4).

The predominant oocyte maturity stage, Stage 7, was not influenced by the increase in ISO levels (MSR,  $p = 0.34$ ; Figure 3.5A), while Stage 7 oocyte numbers increased as dietary soya increased (MSR,  $y = 33.38 + 0.03c$ ,  $r^2 = 0.32$ ,  $F_{(1, 18)} = 8.52$ ,  $p = 0.01$ ; Figure 3.5B).



**Figure 3.4:** Average distribution of oocyte development in female abalone, *Haliotis midae* fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either no soya, 25, 50 and 100 % of the soya (S) in the commercial feed, equivalent to 12, 236, 452 and 760 mg kg<sup>-1</sup>. N=necrotic; St = stage; Og = oogonia.



**Figure 3.5:** Stage 7 oocytes as a percentage of the total number of oocytes in the gonads of females fed a fishmeal (FM) only based diet and FM with increasing rates of isoflavones (ISO) equivalent to diets with either 0, 25, 50 and 100 % of soya (S) in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) (Multiple forwards stepwise regression analysis,  $p \leq 0.05$ ).

### *Oocyte density*

Oocyte density in the gonads of abalone fed ISO-enriched diets was not significantly related to ISO inclusion levels with an average of  $50.01 \pm 4.54$  oocytes  $\text{mm}^{-2}$  (MSR,  $p = 0.79$ ). Similarly, an increase in dietary soya did not significantly affect oocytes density, with an average of  $44.96 \pm 3.01$  oocytes  $\text{mm}^{-2}$  (MSR,  $p = 0.58$ ). However, oocytes density decreased as the percentage of Stage 7 oocytes increased in abalone fed ISO-enriched diets (MSR,  $y = 58.28 - 0.48o$ ,  $r^2 = 0.38$ ,  $F_{(1, 18)} = 12.51$ ,  $p = 0.002$ ), while oocytes density was not influenced by soya inclusion (MSR,  $p = 0.08$ ).

## **DISCUSSION**

### *Endocrine disrupting compounds*

The aim of this study was to determine if the isoflavones (ISO) present in soya were responsible for larger gonads reported in farmed *H. midae* (Ayres, 2013; Riddin, 2013). The concentration of ISO used in the current study was based on the amount of ISO in the commercial feed, Abfeed® S34 (Marifeed Pty (Ltd), Hermanus), and was comparable to the work by Ayres (2013). The dietary treatment including soya had a total ISO concentration of  $64.14 \text{ mg } 100 \text{ g}^{-1}$  (Ayres, 2013), which in the current study fell between the 50 and 100 % soya inclusion rate of  $45.20 \text{ mg } 100 \text{ g}^{-1}$  and  $76 \text{ mg } 100 \text{ g}^{-1}$ , respectively. Therefore the aim of the study could be met by the successful inclusion of crystalline ISO in the various dietary treatments.

To date, no studies have been published on the effects of isoflavones on molluscan reproduction, however, studies using xenoestrogens, such as bisphenol-A and octylphenol have been

conducted (Duft *et al.*, 2003; Jobling *et al.*, 2003). These studies produced varying results with variable concentrations of bisphenol-A and octylphenol, using different administration methods. For example, the freshwater mud snail, *Potamopyrgus antipodarum* was exposed to sediment mixed with bisphenol-A and octylphenol at 1, 10, 30, 100 and 300  $\mu\text{g kg}^{-1}$  of dry sediment, with increased embryo production after just two weeks of exposure to a bisphenol-A concentration between 30 and 300  $\mu\text{g kg}^{-1}$ . After 4 weeks, the increases became more distinct followed by an increase in embryo production in all treatments after eight weeks of exposure (Duft *et al.*, 2003). The use of octylphenol showed effects on embryo production after two weeks at concentrations as low as 1  $\mu\text{g kg}^{-1}$  (Duft *et al.*, 2003). In a separate study freshwater mud snails, *Potamopyrgus antipodarum*, were exposed to 1, 5, 25 and 100  $\mu\text{g L}^{-1}$  of bisphenol-A and octylphenol and exhibited increased embryo production from both bisphenol-A and octylphenol at 5  $\mu\text{L}^{-1}$  after just three weeks of exposure (Jobling *et al.*, 2003). Although xenoestrogens have been found to influence embryo production in mollusks, evaluations have not included abalone, therefore there remains many unanswered questions in this field.

The use of pure genistein, in crystalline and powder form has been used to demonstrate the oestrogenic effects of the natural, plant derived isoflavone genistein (Bennetau-Pelissero *et al.*, 2001; El-Sayed *et al.*, 2012; Jourdehi *et al.*, 2014). However, these studies were conducted on various fish species, including the rainbow trout (*Oncorhynchus mykiss*), beluga (*Huso huso*) and Nile tilapia (*Oreochromis niloticus*). The ineffectiveness of the crystalline ISO used in the present study may be due to a number of reasons. For example, the endocrine functioning of abalone were not suitable to respond to crystalline ISO because of the absence of functional steroid receptors in mollusks (Scott 2012; Scott 2013), or due to the slow growing nature of abalone, a longer study may have yielded results. To date, no studies have been published on the

effects of isoflavones on abalone gonad development, therefore the results from this study generates many questions still to be answered.

### *Oocyte development*

The inclusion of soya was probably responsible for accelerated oocyte development in farmed *H.midae*. The frequency of occurrence of the predominant stage, Stage 7, in abalone fed ISO-enriched diets was not influenced by ISO concentration, while the percentage of Stage 7 oocytes in abalone fed FM with soya increased as a function of soya level. Stage 7 oocytes were isolated for analysis as this was the most abundant mature stage that could be used to determine gonad development. The histological assessments of females fed ISO-enriched diets were in alignment with results obtained in Chapter 2, where gonad growth was similar in abalone fed FM and those fed ISO-enriched diets. The effect that diet had on the percentage distribution of the various stages of oogenesis was similar to findings of Ayres (2013), where oocytes in females fed a diet that included soya contained more developed oocytes than abalone fed diets without soya. Therefore, the inclusion of soya had a significant effect on oogenesis in female abalone, both in this study and in the study on the same species by Ayres (2013), while the inclusion of ISO did not show this effect. Due to the length of the study, it is not possible to determine whether the crystalline ISO may have had an effect after prolonged exposure, although the enrichment of diets with genistein has been reported to exhibit effects on fish after just two and three weeks (Bennetau-Pelissero *et al.*, 2001; Jourdehi *et al.*, 2014). This may not be comparable as the effect of phytoestrogen is dependent on the species and its reproductive status (El-Sayed *et al.*, 2012; Anderson *et al.*, 2003). The current study was 20 weeks long, so it is unlikely that the result was confounded by the length of the study.

Oogenesis in mollusks can be divided into two phases, the generative (proliferative) phase and the vegetative (growth) phase (Anderson, 1974). The generative phase involves oogonial cells that increase by mitotic multiplication and the vegetative phase involves oocyte growth due to vitellogenesis and requires uptake and production of nutritive materials (Pipe, 1987). This leads to the hypothesis that differences in the percentage of Stage 7 oocytes were due to the uptake and production of nutrients. Gonad bulk index and visceral mass did not increase with increasing soya levels at the end of the study, but the distribution of developmental stages was different in the ovaries of abalone fed soya from that of abalone fed FM with increasing levels of ISO.

Turker and Bozcaarmulta (2009) reported an increase in plasma vitellogenin levels in both male and female common carp with an increase in total isoflavone concentrations. In addition, Siberian sturgeon, *Acipenser baeri*, fed a soya-based diet showed increased plasma vitellogenin levels compared to fish fed a FM diet (Pelissero *et al.*, 1991). In contrast, in juvenile tilapia vitellogenin levels in the plasma decreased in fish fed a soya diet compared to a FM diet (Davis *et al.*, 2010). This inconsistency may be due to the species and reproductive stage of the animals.

The inclusion of soya has been reported to have enhanced reproductive effects on farmed *H. midae*, where it was suggested that isoflavones present in soya were responsible for this occurrence (Ayres, 2013; Riddin, 2013). Since the inclusion of crystalline ISO had no significant effect on oocyte distribution, this suggests that possibly other compounds in soya might be responsible for the effect that soya had on oogenesis. Thus, the inclusion of soya in farmed abalone diets resulted in physiological differences associated with reproduction and these differences were enhanced with an increase in soya inclusion, just as it does with some fishes. However, the use of crystalline ISO failed to verify the effects of ISO present in soya on reproduction of farmed abalone.

Differences in nutrient content between the soya and fishmeal diets might be responsible for the differences in oocyte development that was observed here. The abundance of food supply has been correlated to gonad growth and gamete production in various *Haliotis* species (Shepherd and Laws, 1974; Georgi and De Martini, 1977). Gamete production is energy-expensive in both males and females (Ault, 1985). Proteins, lipids and fatty acids are essential for gamete production. Proteins that are linked with essential fatty acids and are usually mobilised to the gonads are important components for reproductive functions (Bautista-Teruel *et al.*, 2001). Highly unsaturated fatty acids (HUFAs) are important nutrients for reproductive functions of fish and crustaceans (Millemena *et al.*, 1986; Millamena and Quinitio, 2000; Huang *et al.*, 2008). Higher lipid requirement in female abalone is attributed to the accumulation of lipids in the ovary before spawning (Huang *et al.*, 2008). In addition, amino acids have been reported to vary seasonally, coinciding with gonad development, such as increasing glycine, which has been hypothesised to increase the viability of sperm and assist fertilisation in the sea anemone, *Bundusoma cavernata* (Kasshau and McCommas, 1982). Correlations between glycine and reproduction in *Mytilus spp.* was reported, dependent on the location of the mussels, indicating the role of environmental factors on glycine concentration in the tissue of animals (Kube *et al.*, 2007). The amino acid profile in the eggs and larval samples in the blacklip abalone *Haliotis rubra*, have been reported to change during embryonic development (Littay *et al.*, 2001). In the current study there were no differences in total protein and total lipid between treatments; however, there were probably differences in amino acid and fatty acid profiles since the fishmeal only and fishmeal-soya diets had different amino- and fatty-acid profiles. These differences were measured, but the results were not presented here and will form part of another study. The

differences in the distribution of Stage 7 oocytes in abalone fed graded levels of soya compared to the other treatments may have been attributed to one of these factors.

Necrotic oocytes were present in the ovaries of abalone of all treatments. High numbers of necrotic oocytes may lead to an incorrect interpretation of a spawning event (Gurny and Mundy, 2004); however, the abundance of necrotic oocytes in the current study were relatively low ranging between 3.42 % and 8.29 %. Oocyte degeneration is a process commonly observed in mollusks (Dorange and Le Penec, 1989) and it was first observed in the red abalone, *H. rufescens* by Young and DeMartini (1970). Wood and Buxton (1996) observed the breakdown and resorption of all unspawned eggs immediately after spawning, which continued even during early recrudescence as ova were maturing again. The oocyte size at which necrosis occurs is variable and can be seen in oocytes as small as 50 µm up to 170 µm in *H. varia*, and it was only seen in large oocytes during incomplete spawning in *H. rufescens* (Georgi and DeMartini, 1977). Similarly, the spent ovaries of the sea urchin, *Arbacia punctulata* and the bivalve, *Macoma balthica* have exhibited such oocyte degeneration (Harvey, 1956; Caddy, 1967). The necrotic oocytes in the current study were all mature oocytes from Stage 6 upwards. Necrosis of oocytes in mollusks is brought about by a variety of conditions, such as temperature extremes or low levels of nutrition (Dorange and Le Penec, 1989; Najmudeen, 2008). In the current study, environmental variables such as water temperature were within the abalones optimal temperature range (Britz *et al.*, 1997) and were similar in all treatments. In addition, animals were fed to satiation, therefore the presence of necrotic oocytes was probably not due to nutritional deficiencies.

The occurrence of necrotic oocytes that were seen in this study was possibly due to a spawning event. This was supported by the drop in GBI values and an increase in the DG index at the end

of the study (Chapter 2). Abalone fed the FM-only based diet contained large white spaces in the lumen due to the absence of mature oocytes. Partly spawned ovaries in the turban shell, *Turbo torquatus* contained empty spaces in the lumen from the spawning of some mature oocytes (Ward and Davis, 2002). Williamson and Steinberg (2002) reported necrotic oocytes in the sea urchin, *Holopneustes purpurascens* that were surrounded by spaces in the lumen where ova may have previously been released. Although the ovaries of females fed the soya diets did not contain spaces in the lumen, necrotic oocytes were still present, coupled with the drop in GBI and increase in DG index, suggesting the occurrence of a spawning event. Due to the length of the study, it is uncertain whether the GBI values continued to decrease and the DG index continued to increase, which would further support the occurrence of a spawning event.

Abalone fed FM with the inclusion of soya contained Stage 7 and Stage 8 oocytes with thinner jelly coat layers and distances between oocytes were uniform, whereas the oocytes in females fed the FM-only based diets were loosely packed with thick jelly coat layers. These results support the findings by Ayres (2013), where the thickness of the jelly coat was used to distinguish the ovaries from abalone fed the FM only diet and the ovaries of abalone fed a combination of FM and soya. The jelly coat is a gelatinous layer that forms around the chorion of mature eggs and decreases in thickness from Stage 7 to Stage 8 (Wood and Buxton, 1996). There have been numerous studies on the function of the jelly coat, including protection from mechanical stress (Thomas *et al.*, 1999), increasing motility of sperm (Inamdar *et al.*, 2007), prevention of polyspermy (Schuel, 1984), enhancing the rates of sperm-egg collision by increasing the target size of the egg (Farley and Levitan, 2001; Levitan, 1993) and enhancing fertilisation (Podolsky, 2001). Organisms that adopt external fertilisation, such as abalone are considered sperm-limited (Levitan and Irvine, 2001; McAlister and Moran, 2012). Levitan and Irvine (2001) reported that

the eggs of the sand dollar, *Dendraster excentricus* with larger cells and jelly coats were preferentially fertilised under sperm-limited lab environments. However, investment into the jelly coat uses resources that could be invested into larger or additional ova (Podolsky, 2004). A large volume of jelly coat can constitute 10–20 % of the material cost of an egg (Podolsky, 2002). Since all the abalone were fed isoenergetic diets, this suggests that abalone fed FM and FM with the inclusion of ISO were investing the energy into jelly coat formation. In addition, oocyte density in FM and ISO-fed animals decreased as the percentage of stage 7 oocytes increased. However, oocyte density was not affected by the inclusion of ISO, which supports similar findings by Ayres (2013), suggesting that these large eggs coupled with the thicker jelly coats occupy more space within the lumen. Oocyte density in soya-fed animals was similar for all treatments, suggesting that although Stage 7 increased with increasing soya levels, the number of oocytes produced was not affected.

### *Conclusion*

The aim of this study was to determine the effect of isoflavones on gametogenesis in farmed abalone. With the lack of literature on this subject in mollusks, it was challenging to make comparisons. This was the first study to use crystalline ISO in order to verify the effects of ISO present in soya on oogenesis in farmed *H. midae*. The inclusion of crystalline ISO had no significant effect on oogenesis in female farmed *H. midae*, while the distribution of Stage 7 oocytes was dose-dependent in abalone fed increasing levels of soya. The increase in Stage 7 oocytes in soya-fed animals did not compromise the number of oocytes present within the lumen, while the number of oocytes in abalone fed FM diets decreased with increasing abundance of Stage 7 oocytes, possibly due to the increase in size of the oocytes with thicker jelly coats. Therefore, crystalline ISO alone was not responsible for changes in oocyte distribution. Dietary

soya resulted in changes in the structure of the female gamete, with a greater proportion of developed oocytes at increased levels of soya inclusion. It was not possible to identify the reason for this increase in this study. Soya-derived ISO, or the inclusion of certain amino- or fatty-acids from the soya, or a combination of these may have been responsible for the differences in oocyte maturity stage distribution in farmed *H. midae*.

## CHAPTER 4

### GENERAL DISCUSSION

#### *Isoflavone source*

Although the use of crystalline isoflavone (ISO) has not to our knowledge been tested and verified in mollusks, xenoestrogens such as bisphenol-A and octylphenol have been reported to affect reproductive functioning in mollusks. For example, xenoestrogens were responsible for delaying sexual maturation, lowering gonadosomatic indices, enlarging sex glands and increasing embryo production (Oehlmann *et al.*, 2000; Duft *et al.*, 2003; Jobling *et al.*, 2003; Siah *et al.*, 2003). The concentration of ISO used in the present study fell within the ISO concentrations used on various fish species where fish were fed ISO-enriched diets that had oestrogenic effects on the fish dependent on the species (Bennetau-Pellisero *et al.*, 2001; Jourdehi *et al.*, 2014). Xenoestrogen concentrations used on mollusks were lower than ISO concentrations used in the current study, while exhibiting reproductive effects only two to three weeks after exposure (Duft *et al.*, 2003; Jobling *et al.*, 2003). Thus, the effects of EDCs on mollusks are unlike those of fish and are most likely different for the different synthetic oestrogens.

#### *Somatic growth and gonad development*

The inclusion of ISO in a FM-only based diet had no effect on growth in farmed *H. midae*, as with the rainbow trout, *Oncorhynchus mykiss* and the common carp, *Cyprinus carpio*, and ISO-enriched diets had no effect on gonad development in farmed *H. midae* (Bennetau-Pellisero *et al.*, 2001; Turker and Bozcaarmutlu, 2009). However, both meat and visceral mass correlated with increasing soya level at certain times of the study and this was dependent on the sex of the

abalone during February and March. After three month (December), abalone fed the FM based diet meat and visceral mass and, consequently, the largest whole body mass. However, by February, meat and visceral mass in males was similar independent of the dietary treatments, while females performed best when fed the full complement of soya in the diet. By the end of the study (March), the opposite occurred for the sexes, where female meat and visceral mass were similar independent of treatment, while males grew the best at 100 % soya inclusion. In addition, oogenesis was correlated to soya concentration at the end of the study (March), where stage 7 oocytes increased with soya concentration, however, this did not affect the number of oocytes  $\text{mm}^{-2}$  in the ovary. Abalone fed FM and ISO-enriched diets invested energy into jelly coat formation, which occupied more space within the ovary, which resulted in lower oocyte density as the number of stage 7 oocytes increased. These results support those reported by Ayres (2013). Therefore, adjusting the soya concentration in formulated abalone feed not only affects somatic growth of farmed abalone, but also has implications on the physiology of reproduction.

Although weight was correlated with increasing soya levels, weight loss was observed in all treatments at the end of the study. The weight loss may have been due to a spawning event, a conclusion that was supported by the drop in GBI and digestive gland index between February and March. Although necrotic oocytes were present in all treatments, the ovaries of the abalone were still relatively full of oocytes with some white spaces within the lumen where oocytes had been spawned. This may have been due to the incomplete spawning of females (Georgi and DeMartini, 1977). The lowest biomass loss was in the treatment with soya levels equivalent to that of the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus). In addition, abalone fed FM with 100 % soya inclusion rate contained the most replicate tanks with weight loss. Due to the length of the study, it is unknown how long the loss in weight occurred and whether the

drop in GBI and increase in DG index from February to March would have continued, which would further represent a spawning event. Therefore, in conclusion, meat mass and gonad mass in farmed *H. midae* was dose-dependent when abalone were fed FM diets that included soya and could not be verified by the inclusion of crystalline ISO in a FM-only based diet.

### *Applications*

The aim of this study was to determine the effect of isoflavones, present in soya on the growth and gonad development of farmed *H. midae*, due to the reported decrease in biomass during the reproductive seasons (Ayres, 2013, Riddin, 2013). Although crystalline ISO failed to promote weight gain and larger gonads, a compound in soya is hypothesised to have had an effect on the somatic growth and reproduction in abalone. Since the visceral mass forms part of the product in live abalone export, the investment of energy into larger gonads is of less priority compared to abalone used for canning, where the viscera are being discarded. Therefore, in order to obtain maximum growth in abalone, a few considerations would have to be taken.

Numerous abalone species grow better with the inclusion of both plant and animal protein in the formulated diet (Britz, 1996; Guzmán and Vianna, 1998; Kim *et al.*, 1998; Lee, 1998; Bautista-Terul *et al.*, 2003; Dlaza *et al.*, 2008), however, changing the diet prior to spawning may be beneficial if the investment into gonad development may be directed into somatic growth. Using the current study as an example, abalone could be fed a FM-based diet in September, to obtain the highest meat mass and whole mass without compromising the visceral mass by December, but 100 % of the soya in the current feed would have to be implemented again in February and March, as female meat mass, visceral mass and consequently the whole body mass correlated with increasing soya levels in February and males correlated with soya levels in March. The change back to the full complement of soya in the diet would be beneficial as weight loss that

occurred in March was the highest in abalone fed the FM based diet. In addition, water loss could be minimised since water loss and probably glycogen content was negatively correlated with increasing soya concentration, thus decreasing the amount of weight lost during canning. Therefore a change in diet at certain times of the year may be beneficial for abalone farmers.

#### *Future studies*

Supplementing the formulated feed with fresh kelp, *Ecklonia maxima*, during reproductive seasons may improve growth. Farmed *H. midae*, with a length of  $12.72 \pm 0.02$  mm were fed five FM-only based formulated diets from various manufacturers supplemented with fresh seaweed, *E. maxima* and *Ulva lactuca*, which reportedly enhanced growth in all treatments (Dlaza *et al.*, 2008). However, reproductive performance was not measured as abalone were not sexually mature. It may be worth investigating, whether feeding kelp and ulva can be used as a tool to moderate reproduction in *H. midae*.

The combination of various ingredients used to form the various dietary treatments that were isonitrogenous and isoenergetic may contain different amino acid and essential fatty acid profiles due to the different ratios of each ingredient. These different ratios and profiles may play a role in the growth and reproduction of farmed abalone. Therefore, a study that measures these compounds in line with gonadal development may help to contribute to the gonad development in farmed *H. midae*.

The use of crystalline ISO to verify the effects of ISO present in soya could not demonstrate that ISO in soya was the main cause of the effect of soya on abalone reproduction, however, the use of tamoxifen, an oestrogen antagonist has been reported to decrease the sensitivity of embryos in the sea urchins *Strongylocentrotus purpuratus*, and *Lytechinus anamesus* to endocrine disrupting

compounds at concentrations as low as  $0.2 \text{ ng ml}^{-1}$  (Roepke *et al.*, 2005). Therefore using tamoxifen may be useful in determining the role of ISO present in soya. Alternatively, soya protein, which has been washed in alcohol to remove the ISO could be tested on abalone growth and gonad development. In addition, male gonad histological assessments should be considered in future studies, specifically due to the sex effect that occurred in the current study. Although crystalline ISO had no effect on the growth and gonad development of abalone, alternative ways of demonstrating the effect of ISO present in soya could still be investigated.

### *Conclusion*

The inclusion of crystalline ISO did not promote larger gonads and had no effect on growth, while growth and gonad development was dose dependent when soya was included in the feed. Diets were isonitrogenous and isoenergetic, the inclusion of soya was responsible for the larger gonads and better overall growth.

Reducing the reproductive investment in abalone can be obtained by reducing soya levels.

Weaning abalone onto a FM-only based diet at certain times of year may produce better growth without affecting the visceral mass. However, the inclusion of soya equivalent to the soya in the commercial feed (Abfeed® S34, Marifeed Pty (Ltd), Hermanus) would be required about three months later as both meat and whole body mass were still highest in addition to the larger gonads. Therefore, changing the formulation of the diet may be beneficial for abalone farmers at certain times of the year in order to obtain maximum growth when investment into gonads increases.

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