Aspects of the nutritional physiology of the perlemoen *Haliotis midae* (L.) and red abalone *H. rufescens* (Swainson).

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ABSTRACT

The source of abalone for human consumption has shown a dramatic shift away from wild-capture fisheries in the last 30 years, with over 90% of global production now coming from aquaculture. Farmers initially relied on the natural food of abalone (macroalgae) as a culture feed, though in regions where macroalgae availability was limiting, the need to develop formulated feeds was evident. Extensive research effort has led to the development of a number of formulated feed products currently employed in the industry. These feeds, however, differ markedly from the mixed macroalgal diets that abalone have evolved to utilise, particularly in terms of protein content and carbohydrate structure. The degree to which the nutritional physiology of abalone responds to these novel formulated diets, with and without macroalgal supplementation, was investigated in the current study. A multifaceted approach, combining growth trials, stable isotope nutrient tracers and metabolic experiments, was employed to gain insight into the post-absorption dynamics and utilisation of dietary nutrients under varying dietary regimes of fresh macroalgae and formulated feed.

Growth trials conducted with both *Haliotis rufescens* and *H. midae* showed significantly higher growth and protein utilisation efficiency for abalone fed macroalgal diets compared to formulated feeds. Furthermore, when formulated feeds were supplemented with macroalgae to form combination diets, growth and the utilization of protein was improved compared to the formulated-feed-only diet. The poor utilisation of protein by *H. midae* fed the formulated feed could be traced, using a method combining stable isotope bio-markers with a Bayesian mixing model (SIAR), to the low incorporation of the fishmeal component of protein in the diet. The marked postprandial drop in the O:N ratio on abalone fed formulated feeds indicate that the protein was being diverted into catabolic metabolic pathways. The metabolic cost of digestion, termed specific dynamic action (SDA), was negated as a factor in the improved growth of abalone fed macroalgal diets, with the SDA coefficient 2.1 times that observed for formulated feed. Furthermore, the postprandial haemolymph glucose concentration (HGC) in *H. midae* was elevated when fed formulated feed compared to macroalgae. The high levels of circulating glucose are likely a result of the structure the carbohydrate source in formulated feeds and stimulate the deposition of glycogen through the allosteric control

of glycogen synthase. Formulated feeds produced higher cooked meat yields in canning simulation trials, suggesting that muscle glycogen content may indirectly play a role in increasing canning yields through the displacement of collagen.

The results of these empirical studies are synthesised under key themes, discussed within the context of their potential commercial relevance and future research directions are highlighted.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

List of Abbreviations and Acronyms

AA	Amino Acid
ALA	Alpha-Linolenic Acid
AOAC	Association of Official Analytical Chemists
APD	Apparent Protein Digestibility
aSDA	Apparent Specific Dynamic Action
CD	Combination Diet
CE	Controlled Environment
CF	Condition Factor
CMH	Combination diet Macroalgae High
CML	Combination diet Macroalgae Low
CMM	Combination diet Macroalgae Medium
DAQ	Data Acquisition
DDDF	Diet Derived Discrimination Factor
DHA	Docosahexaenoic Acid
DIT	Diet-Induced Thermogenesis
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DPA	Docosapentaenoic Acid
EER	Energy Efficiency Ratio
EPA	Eicosapentaenoic Acid
FAA	Free Amino Acid
FCR	Feed Conversion Ratio
FD	Formulated Diet
FMD	Foot Muscle Deposition
G6P	Glucose-6-Phosphate
GSase	Glycogen Synthase

HGC	Haemolymph Glucose Concentration
HI	Heat Increment
HIF	Heat Increment of Feeding
HPDE	High Density Polyethylene
ILS	Insulin-Like Substance
IRMS	Isotope-Ratio Mass Spectrometer
L.	Linnaeus
LAN	Limestone Ammonia Nitrate
LGC	Light Green Cell
LGR	Linear Growth Rate
MD	Macroalgae Diet
MIP	Molluscan Insulin-related Peptide
MLND	Mean of the Lowest Normal Distribution
MM	Macroalgae - Multiple sp.
MO_2	Oxygen Consumption Rate
MS	Macroalgae - Single sp.
NH4	Ammonia
$NH4^E$	Ammonia excretion
NNE	Non-Nitrogen Extract
NPN	Non-Protein Nitrogen
O:N	Oxygen:Nitrogen
PAMRL	Port Alfred Marine Research Laboratory
PER	Protein Efficiency Ratio
PER_f	Protein Efficiency Ratio - Formulated component
PFI	Proportional Feed Intake
PPT	Postprandial Thermogenesis
PVC	Polyvinyl Chloride
RAS	Recirculating Aquaculture System

RIA	Radioimmunoassay
RS	Respirometry System
RSA	Republic of South Africa
SD	Standard Deviation
SDA	Specific Dynamic Action
SGR	Specific Growth Rate
SIAR	Stable Isotope Analysis in R
$SIAR_{EC}$	Stable Isotope Analysis in R Estimated Contribution
SMR	Standard Metabolic Rate
TAN	Total Ammonia Nitrogen
TEF	Thermic Effect of Feeding
USB	Universal Serial Bus
UV	Ultraviolet
VB	Visual Basic
$\dot{V}O_2$	Mass-specific Oxygen Consumption Rate
$\dot{V}O_2^D$	Diurnal Mass-specific Oxygen Consumption Rate
$\dot{V}O_2^N$	Nocturnal Mass-specific Oxygen Consumption Rate
WCA	Wild Coast Abalone
WM	Weigh and Measure

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1 INTRODUCTION

Abalone were taxonomically described around 1740 when Linnaeus assigned the name *Haliotis* (meaning "sea-ear") to the genus of Gastropods with ear-shaped shells, although abalone, or at least what are likely to have been abalone, were mentioned as far back as Aristotle's "Historia Animalium" (4thC BC). Thus, began the journey of the formal scientific study of abalone, permanently marked in time by the appendage of the single letter 'L.' behind some extant species names. What followed would essentially be the 'who' phase of investigation, as a period of taxonomic and phylogenetic study of this genus was initiated. With the beasts duly named, thereafter emerged the inevitable 'what' phase of abalone research, as a series of investigators pursued comparative anatomical studies into various aspects including the nervous, circulatory, digestive, renal and reproductive systems. The history of this early effort is neatly detailed in Crofts (1929) monologue "Haliotis", the first comprehensive, English-language anatomical study of the genus. In her seminal work, Crofts (1929) states in the profuse and colonially prejudiced language of the era, that these marine molluscs "… are much sought after because of their decorative shells and the food value and delectable quality of the soft parts. At one time, they made a staple food for many savage nations". In this early text, Crofts already refers to thriving fisheries for abalone in Japan, California and Gurnsey and, tellingly, makes mention of such a decline in the Gurnsey fishery that it necessitated the suspension of fishing activity. The human exploitation of abalone for commercial purposes has to a large degree shaped the nature of the research agenda around this genus. At the time of the first International Abalone Symposium in La Paz, Mexico, in 1989, approximately two thirds of the resulting published papers were directly related to some aspect of abalone utilisation by humans including abalone fisheries, fisheries biology, stock enhancement and aquaculture. The research undertaken during this period was very much centred on the 'how' line of enquiry, with methodological or descriptive studies and manipulative experiments designed to elucidate how abalone would respond to a defined stimulus. When Fleming

et al. (1996) published their extensive review on the development of formulated feeds in the mid 1990's, abalone culture was already well underway and there was a widespread appreciation of the need to develop formulated feeds to foster the long-term sustainability of this industry. An examination of the themes presented in that review highlights the classical reductionist paradigm of nutrition that shaped the research thrust at the time. Given that abalone were the first marine molluscs to be farmed intensively, this approach was likely informed by approaches adopted for other farmed animals including prawns, fish, terrestrial mammals and indeed humans. Under this paradigm, variably described as 'quantifying nutritionism' or 'balanced nutrition', the premise held that the biochemical composition and caloric content of a given feed should correspond to the nutritional requirements of the organism (Kuz'mina, 2008). A monogastric animal nutrition model was applied, assuming the simple enzymatic digestion of food substrates. The important components of nutrition (proteins, lipids, carbohydrates, vitamins and minerals) and their energy values were defined, their requirement determined, and suitably balanced diets formulated as a result (Fleming et al., 1996). This approach led to the development of a number of commercial abalone feed products that have remained, in many cases, largely unchanged since their initial development. While these diets have proved sufficient to establish commercially viable abalone farms in the absence of macroalgae, we have surprisingly little understanding of the unique nutritional physiology of these organisms.

It has become increasingly evident that some aspects of nutrient utilisation by abalone, such as the highly efficient utilisation of nutrients in macroalgae, do not necessarily align with the simple 'monogastric enzyme machine' model. Parallel to, and possibly inspired by the current era of "functional nutritionism" that emerged in human nutrition in the mid 1990's (Fardet & Rock, 2015), research effort has diversified to begin investigating similar concepts in abalone studies. From studies exploring topics as diverse as the role of gut microbiota in abalone digestion (Erasmus et al., 1997; Nel et al., 2017b; Sawabe et al., 2003), to the health benefits associated with feeds such as macroalgae (Dang et al., 2011), to the role of age and abiotic factors in nutrient utilisation (Bansemer et al., 2016c; Stone et al., 2013), there is a growing awareness of a complexity to abalone nutrition that extends beyond the simple biochemical composition of the diet. It is likely that modelling production outcomes such as growth using a linear cause-effect relationship between food compound 'x' and outcome 'y'

may be limiting, and that exploring multicausal, nonlinear relations could be of value (Fardet & Rock, 2014). This is in many ways the "why" period of investigation as researchers attempt to understand mechanism as much as outcome.

Central to this thesis is the simple observation that the formulated abalone diets that were developed under the "quantifying nutritionism" paradigm have resulted in an end-product that is noticeably divergent from the natural algal diets that abalone have evolved to utilise. Using a multidisciplinary approach, drawing on influences from both the quantifying and functional paradigms, the research presented here aims to elucidate the response of aspects of the nutritional physiology of abalone to these novel formulated feeds. Any research approach is shaped as much by the epistemological era it is developed in as by the technology available at the time to provide novel avenues of investigation, and this body of work is no different. For example, the availability of rapid and affordable estimates of stable isotope composition facilitate new methods for tracing nutrient utilization, while advances in oxygen sensor technology, software integration and mathematical modelling allow for previously unavailable resolution in and interpretation of metabolic data. Insights gathered here not only add to our understanding of the organisms at a fundamental level but can ultimately be applied to inform current abalone farming practices and nutrition approaches.

Any attempt to investigate the effect of modern diets on abalone is best anchored in an understanding of the evolutionary context and how the animal is uniquely adapted, both anatomically and physiologically, to function in its natural environment. In the following section, the nutritional anatomy and physiology of abalone is reviewed in order to motivate the study's research approach.

1.1 Abalone taxonomy and distribution

The term abalone is generally used to describe the marine gastropod molluscs belonging to the family Haliotidae, although the region-specific names of 'paua', 'awabi', 'perlemoen' and 'ormer' are employed in New Zealand, Japan, South Africa and Europe respectively (Behrens et al., 2002; Gaty & Wilson, 1986; Olaechea et al., 1993a; Sales & Britz, 2001b). The Haliotidae are situated in the clade Vetigastropoda, an ancient

lineage dating back to the Paleozoic comprised of an estimated 2000 - 3700 exclusively marine species (Geiger et al., 2008; Geiger & Thacker, 2003). The Vetigastropoda retain many basal characters, including paired organs in the mantle. Other common but non-diagnostic characters include slits / holes in the shell, which has a nacreous inner layer, paired gills and a heart with two auricles (Geiger et al., 2008). The family Haliotidae is composed of a single genus, *Haliotis*, with the most recent taxonomic review validating 56 species and an additional 18 subspecies (Geiger & Owen, 2012). Abalone exhibit a near-global distribution with representatives recorded on most rocky shore environments in both temperate and tropical waters, generally preferring the shallow subtidal zone to a depth of 30 metres (Figure 1) (Geiger, 2000). A noticeable exception is the west coast of South America where abalone are absent, despite suitable habitat and food sources, an anomaly attributed partially to the warm water dispersal barrier between hemispheric temperate zones (Geiger, 2000). Despite a widespread distribution at genus level, no species exhibits a global distribution. Although some Indo-Pacific taxa exhibit extensive distributions, the majority of species have restricted ranges, with regions of high endemism in South Africa (five species), New Zealand (three species), western North America (six species) and temperate Australia (nine species) (Geiger, 2000).

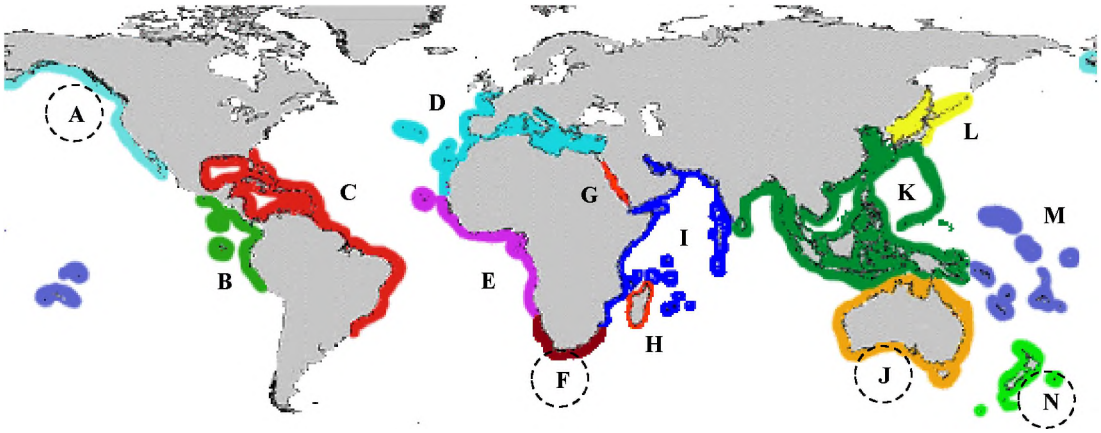


Figure 1. Global distribution of the family Haliotidae. Coloured zones depict distinct biogeographical regions as follows (with species richness in parenthesis): A – North Eastern Pacific (8), B – Panamaic (2), C – Caribbean and South America (2), D – Europe / Mediterranean (5), E – West Africa (1), F – South Africa (6), G – Red Sea (2), H - Madagascar (4), I – Indian Ocean (6), J – Australia (19), K – Indo-Malayan (14), L – Japan (6), M – central Pacific (11) and N – New Zealand (3). Circled region codes indicate areas of high endemism. Map adapted from Geiger (2017).

1.2 Digestive system morphology & physiology

As with all prosobranch molluscs, abalone undergoes torsion during the veliger larval stage (Page, 1997). Hence, although their digestive system closely conforms to the primitive molluscan arrangement, it exhibits a notable diversion from the anterior mouth / posterior anus arrangement (Bevelander, 1988; Owen, 1966). The resultant body plan has the gills, mantle cavity and anus anterior to the heart, and the nervous system twisted into a figure of 8 (Bevelander, 1988). Basic dissection and histological methods have been used to describe the anatomy of the alimentary canal and associated structures for abalone belonging to several species including *H. tuberculata* (Bevelander, 1988; Crofts, 1929), *H. cracherodii* (Campbell, 1965), *H. laevigata* (Harris et al., 1998a) *H. rufescens* (McLean, 1970), and *H. rubra* (Johnston et al., 2005). Given the consensus between these studies, a generalised description of the abalone digestive system (Figure 2) can be constructed as follows. The buccal region comprises the cylindrical mouth, vertical mouth cleft, chitinous radula activated by the muscular odontophore, buccal cavity and paired salivary glands. The oesophagus links the buccal cavity to the stomach region (SR) and is composed of upper (radula sac to oesophageal pouches (OPS)), middle (around OPS) and lower (OPS to start of SR) regions. The lower oesophagus opens into the crop, alternatively referred to as the post-oesophagus, via a semi-circular valve. Initially narrow and ciliated, the crop abruptly widens and is divided into two regions, a capacious anterior region and a smaller posterior region, separated by an oesophageal fold. The wall opposite the fold forms a partition between the stomach and the posterior region of the post-oesophagus. A stomach fold that runs parallel to and opposite the partition considerably narrows the passage between the post-oesophagus and stomach. The alimentary canal turns through 180 degrees at this point, and enters the stomach proper. The caecum, a pouch-like extension composed of a spiral tube of decreasing diameter, also occurs in this region.

The stomach floor consists of a series of highly ciliated ridges that form the posterior sorting area with the remaining anterodorsal surfaces covered by a fibrous sheet known as the gastric shield. These ridges, along with the primary typhlosole, oesophageal and stomach folds extend into the caecum to its tip. Anterodorsally, the stomach leads into the muscular style sac. At the entrance to the style sac the stomach ridges curve to run

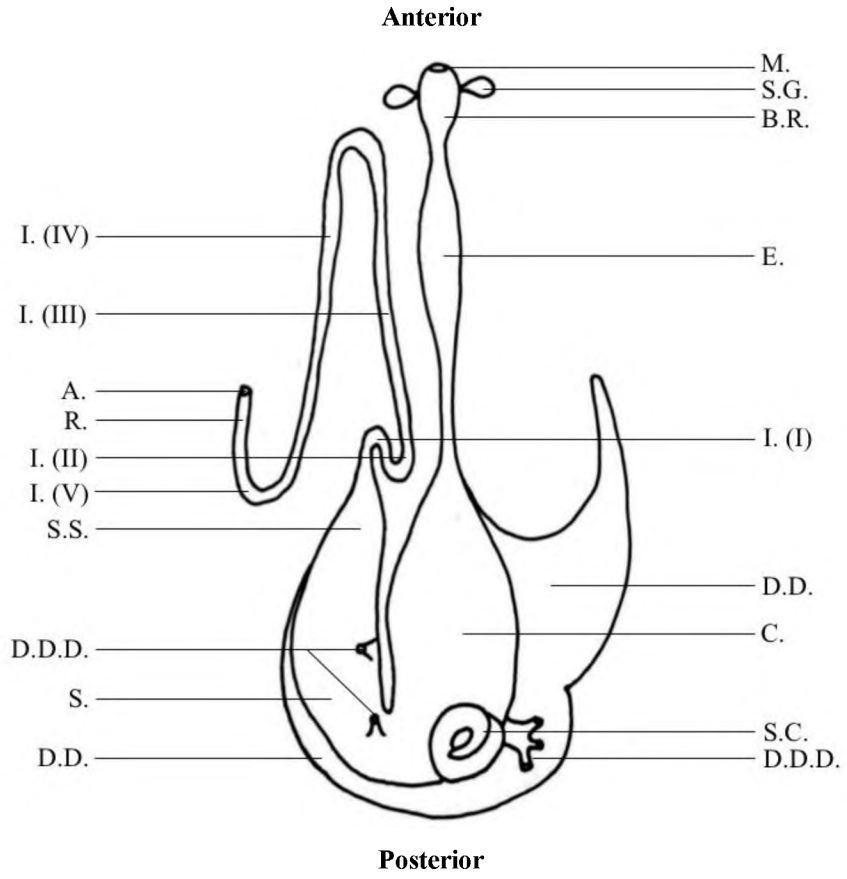


Figure 2. Generalised schematic diagram of the abalone digestive tract in dorsal view, intestine displaced to the left. A, anus; B. R., buccal region; C, crop; D. D., digestive diverticulum; D. D. D., duct of digestive diverticulum; E, eosophagus; I (I-V), intestine (regions 1 – V); M, mouth; S, stomach; S. C., spiral caecum; S. G., saliva gland; S. S., style sac; R. rectum. Image adapted from Campbell (1965), McLean (1970) and Harris et al. (1998a).

parallel with the major typhlosole, forming the intestinal groove that runs the length of the intestine. The intestine begins at the valve that opens out of the style sac. It undergoes a convolution before extending anteriorly as far as the oesophageal pouch, where it turns through 180° and returns posteriorly between the oesophagus and initial loop as far as the style sac. Here it undergoes a second 180° turn, passes through the ventricle and ends in the mantle cavity. The rectum lies predominantly within the mantle cavity. The digestive diverticulum (DD), a large gland occupying much of the visceral space, surrounds most of the alimentary tract posterior to the ventricle. It consists of multiple blind tubules ending in acini that open into branching ducts. The morphological left diverticulum communicates with the posterior region of the post-oesophagus, below the oesophageal fold, via multiple openings in its floor. By contrast,

the right diverticulum by contrast connects with the stomach via two openings, one overhung by the gastric shield associated with a ciliated channel that forms a tube to the style sac region, and the second near the style sac that opens directly into the stomach lumen.

A corrosion casting method was employed in the current study to produce three-dimensional acrylic resin models of the digestive system of *H. midae* (Figure 3; Figure 4; Figure 5; Appendix 1), as an alternative method to confirm anatomical structures and their relative spatial arrangement in this species. These models generally concur with descriptions from previous anatomical studies on abalone, however some minor variations were observed when examining the resin casts[1].

[1] Several studies refer to the caecum as "spiral" shaped in abalone, with supporting figures indicating multiple coils (Campbell, 1965; Crofts, 1929; Harris et al., 1998b). In *H. midae* the caecum better matches the "pouch-like extension" description of Bevelander (1988), with a single semi-circular curve from insertion at the base of the crop to termination, although the potential for deformation of the structure from pressure during the resin injection process may exist. Secondly, the 180° intestinal turn that passes near the oesophageal pouch does not match the simple curve previously described (Bevelander, 1988; Campbell, 1965; Crofts, 1929; Harris et al., 1998a), but rather resembles a simple curve that has been tucked and rolled back towards the arriving and departing intestinal tubes. Finally, the corrosion casting method provides for the first time a 3-dimensional visualisation of the layout, insertion and internal structure of the duct-tubule-acini lumen arrangement of the digestive diverticula. The location of two insertion points of the right diverticulum into the stomach described by Crofts (1929) and Campbell (1965) are confirmed, with the resin cast indicating that the primary ducts linking to these openings do not join and service separate regions of the right digestive gland. The location of the insertion of the left diverticulum at the base of the caecum is also confirmed (Campbell, 1965), although there appears to only be a single opening, in contrast to the dual or multiple openings previously reported (Campbell, 1965; Crofts, 1929). The multiple openings reported by Campbell (1965) may relate to the effect of viewing the first branches of the primary duct through the opening of the primary duct during dissection. The primary duct system of the left diverticulum forms a contiguous network throughout the tissue.

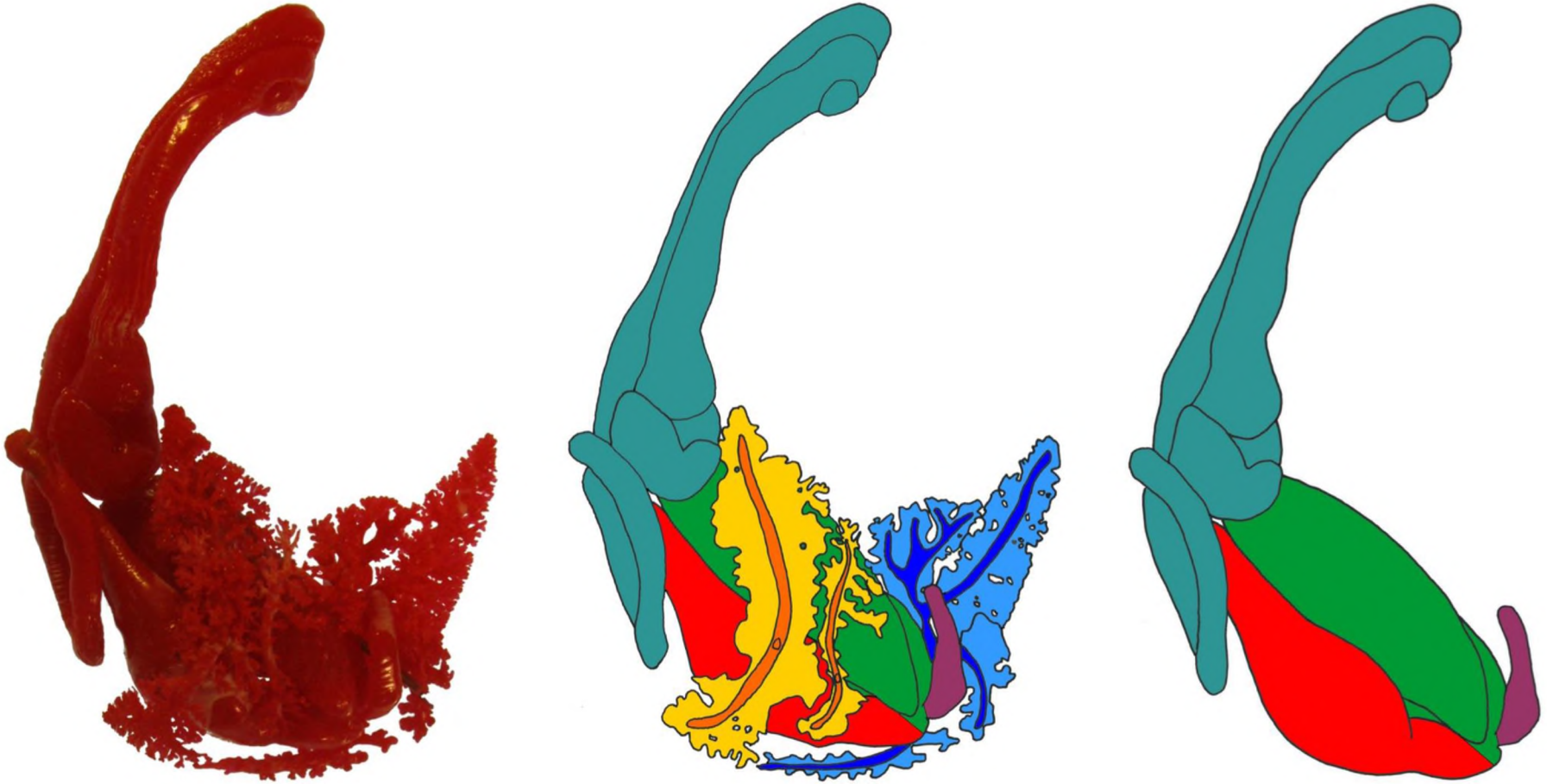
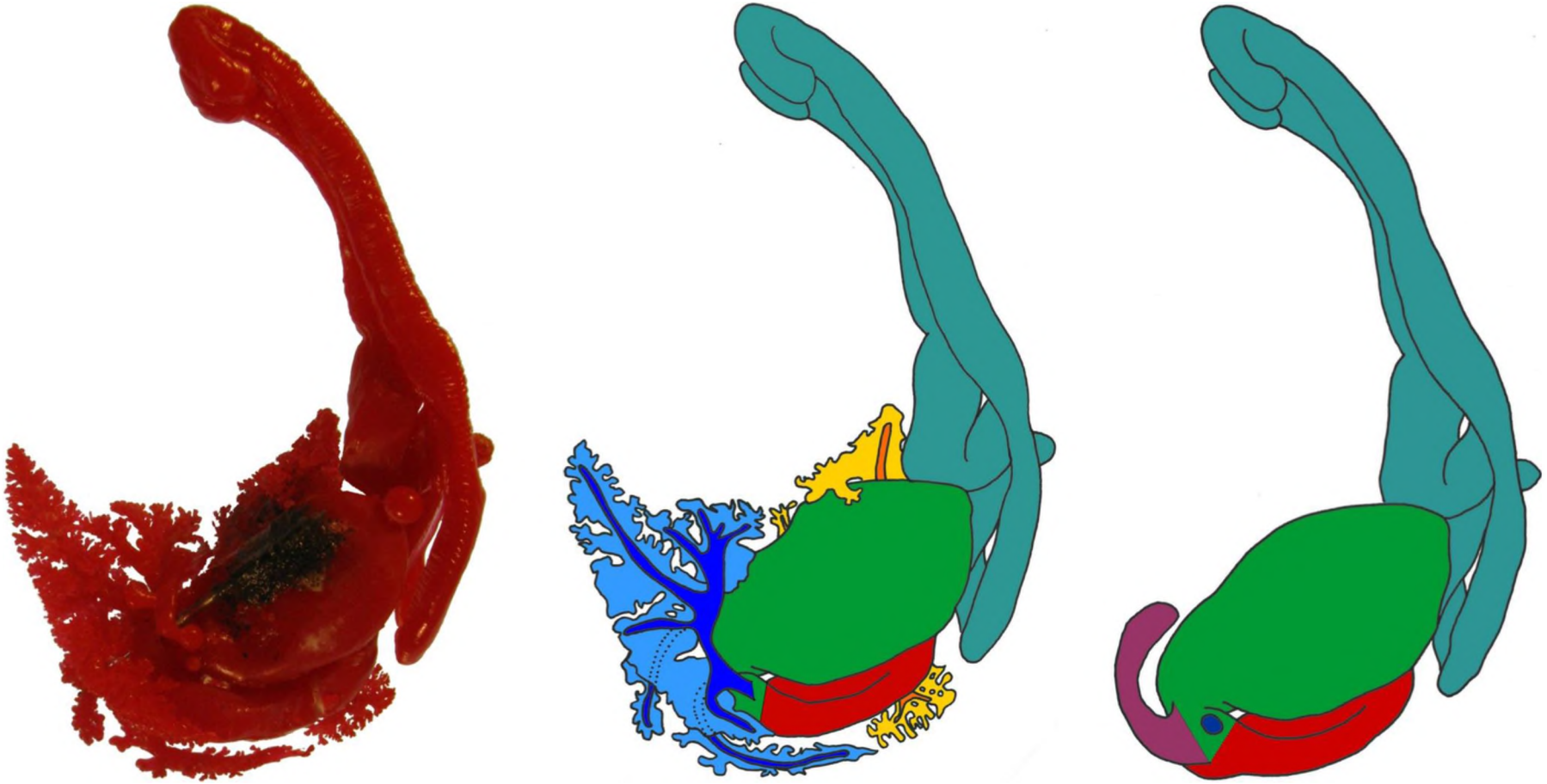


Figure 3. Ventral view of an acrylic resin cast and associated anatomical drawings of the digestive system of the abalone *H. midae*. The drawings are keyed as follows: - stomach, - crop / post-oesophagus, - intestine, - caecum, - left digestive diverticulum (DD) primary duct, - left DD branching secondary tubules and acini, - right DD primary duct, - right DD secondary branching tubules and acini.



ACRYLIC RESIN CAST

OUTLINE WITH DIGESTIVE DIVERTICULUM PRESENT

OUTLINE WITH DIGESTIVE DIVERTICULUM REMOVED

Figure 4. Dorsal view of an acrylic resin cast and associated anatomical drawings of the digestive system of the abalone *H. midae*. The drawings are keyed as follows: - stomach, - crop / post-oesophagus, - intestine, - caecum, - left digestive diverticulum (DD) primary duct, - left DD branching secondary tubules and acini, - right DD primary duct, - right DD secondary branching tubules and acini. Note: Dark colouration on resin cast is attachment point for mounting needle.

ACRYLIC RESIN CAST

OUTLINE WITH DIGESTIVE DIVERTICULUM PRESENT

OUTLINE WITH DIGESTIVE DIVERTICULUM REMOVED

Figure 5. Posterior view of an acrylic resin cast and associated anatomical drawings of the digestive system of the abalone *H. midae*. The drawings are keyed as follows: - stomach, - crop / post-oesophagus, - intestine, - caecum, - left digestive diverticulum (DD) primary duct, - left DD branching secondary tubules and acini, - right DD primary duct, - right DD secondary branching tubules and acini.

From a physiological perspective, the abalone gut appears to function in a similar way to other primitive gastropod microfeeders (Campbell, 1965; Graham, 1949; Morton, 1953; Owen, 1966), being primarily cilia-driven and relying on a combination of intracellular and extracellular digestion. Following food acquisition using the rhipidoglossan radula, a mixture of coarse and fine particles enters the digestive tract (Campbell, 1965). Abundant mucous cells in the salivary gland and oesophagus produce a mucous stream that entrains food particles and which is moved by ciliary action to the crop. Secretory cells, and their fragmentation spherules in the oesophagus and its pouches indicate the possible release of enzymes into the food stream at this point (Campbell, 1965; Harris et al., 1998b). The crop receives the food particles, mucous and secretions from the oesophagus. Given the lack of cilia, the arrangement of crop folds that regulate movement of its contents to the stomach and its large lumen size, it acts primarily as a storage organ for ingested food (Harris et al., 1998b), although evidence exists that some digestion does occur (Foale & Day, 1992), likely facilitated by enzymes secreted in the oesophagus (Campbell, 1965), from the numerous secretory cells in the crop's own epithelium (Harris et al., 1998b) and possibly by bacteria (Erasmus et al., 1997). The valve-like nature of the folds between the crop and stomach shunt much of the particulate food to the caecum for initial sorting (Campbell, 1965).

Material entering the stomach is composed of some coarse material directly from the crop, fine material issuing from the caecum and material from the digestive diverticula (Campbell, 1965). The muscular contraction of the style sac converts undigested material into a faecal rod known as the protostyle, which is constantly rotated by cilia (Morton, 1953). The action of the protostyle is indicated to serve multiple purposes including: (1) ensuring the constant mixing of stomach contents and the repeated sweeping of feed particles over the anterior ciliary sorting areas, (2) assisting in mechanical breakdown by its abrasive action against the digestive shield (3), regulating the rate of food passage through the digestive track and (4) assisting the movement of material into the stomach by it capstan-like action (Owen, 1966). Material from the digestive diverticulum and posterior sorting area move along the intestine in a separate channel bounded by the typhlosoles, before being bound in a mucous sleeve with the majority of intestinal contents in the rectum and ejected into the mantle cavity (Campbell, 1965; Graham, 1949). The digestive diverticulum is considered to be both a major source of digestive enzymes (Garcia-Esquivel & Felbeck, 2006) and an important

site of nutrient uptake, in the form of both soluble products and the intracellular digestion of small particles following phagocytosis/pinocytosis (Campbell, 1965; McLean, 1970; Owen, 1966). Owing to the general lack of diverticular cilia, the movement of material in and out of the diverticulum is most likely driven by the muscular contractions of the stomach (Campbell, 1965). Abundant secretory cells in the stomach epithelium are a potential further source of enzymes, introducing them into the food mass by "pinching-off" their cell tips into the gut lumen (Campbell, 1965; Harris et al., 1998b). Phagocytes have been observed in the crop, stomach and intestine of abalone (Campbell, 1965; Crofts, 1929; Harris et al., 1998b). These, combined with the permeability of the epithelium in these areas to products of digestion and granular inclusions in some cell types (Harris et al., 1998b; McLean, 1970), indicate that nutrient uptake also occurs outside of the digestive diverticulum. The described anatomy and digestive physiology has evolved to enable abalone to exploit successfully the most abundant food source, algae, available in the rocky marine environments in which they occur.

1.3 Natural diet

The preference of abalone for shallow subtidal habitats is probably driven by the availability of algae, whose presence and productivity will be regulated in part by light intensity. The form of algae utilised by abalone, however, follows a distinct ontogenetic progression. Following multiple planktonic, lecithotropic larval stages, and provided suitable settlement cues are present, larvae will settle on to the substratum and metamorphose into benthic post-larvae (Roberts & Lapworth, 2001; Searcy-Bernal, 1999; Takami, 2003). During metamorphosis, the larval velum is shed and development of the foot, gills, peristomal shell and mouthparts occur (Kawamura et al., 2001; Takami, 2003). Although particle feeding commences following metamorphosis, the presence of residual yolk indicates a gradual shift of the primary nutrition source from endogenous to exogenous sources over the initial post-larval period (Kawamura, 1998; Takami, 2003; Takami et al., 2002). Small post-larvae (< 0.8 mm) rely predominantly on biofilm components including the extracellular products from diatoms, mucus trails and possibly bacteria, with their dietary scope broadening gradually to include whole diatom cells, turf and crustose coralline algae as the animal grows (Johnston et al., 2005; Kawamura, 1998; Kawamura et al., 2001; Takami, 2003).

Studies investigating the stomach contents of wild juvenile and adult abalone across multiple global locations indicate a herbivorous diet based primarily on marine macroalgae (Barkai & Griffiths, 1986; Guzman del Próo et al., 2003; Poore, 1972; Sawatpeera et al., 1998; Shepherd, 1973; Shepherd & Steinberg, 1992; Wells & Keesing, 1989). It should be noted, however, that recent studies using stable isotopes and fatty acids as chemical tracers indicate that diatoms and detritus are also important contributors to the diet in some abalone species (Guest et al., 2008; Vega-García et al., 2015). The transition from microalgae to macroalgae is preceded by preparatory morphological changes in the radula and digestive system and physiological shifts in enzyme production (Johnston et al., 2005; Kawamura et al., 2001; Onitsuka et al., 2004; Roberts et al., 1999; Takami et al., 1998). The size at which abalone make the shift to a macroalgal diet varies between species, ranging from 5 – 10 mm in *H. discus hannai* (Kawamura, 1998; Kawamura et al., 2001) to 10 - 20 mm in *H. asinina* and *H. ovina* (Jarayabhand & Paphavasit, 1996). Even within species, the timing of the switch to macroalgae can be highly variable and may be governed by the type of macroalgae available in the environment (Takami, 2003). Following transition to a macroalgae-dominated diet, juvenile and adult abalone utilise all three major macroalgae groups, namely the Chlorophyta green algae, Rhodophyta red algae and Phaeophyta brown algae.

Investigating the preference of abalone for a specific algal type has the potential to improve understanding of their nutritional physiology, owing to the expected links between preference, algal composition and growth-performance indicators (Angell et al., 2012). Distinct patterns in geographic, and by association, species variation in algal preference is apparent (Angell et al., 2012; Shepherd & Steinberg, 1992). A pattern commonly reported in the literature (Guest et al., 2008; Harris et al., 1998b; McShane et al., 1994; Shepherd & Steinberg, 1992) suggests the following geographic distribution of macroalgal preference. Australasian and tropical Western Pacific abalone species, including *H. iris*, *H. australis*, *H. cyclobates*, *H. laevigata*, *H. roei*, *H. ruber*, *H. scalaris* and *H. asinina*, show a general preference for red macroalgae as determined from both gut contents analyses (Poore, 1972; Shepherd, 1973; Tahil & Juinio-Menez, 1999) and multi-choice experiments (Angell et al., 2012; Poore, 1972; Shepherd & Steinberg, 1992). Conversely, species from other global locations show a preference for brown macroalgae. In South Africa, *H. midae* feeds predominantly on the kelp *Ecklonia*

maxima (Barkai & Griffiths, 1986; Zeeman et al., 2012), in Central and North America *H. rufescens*, *H. fulgens* and *H. corrugata* prefer phaeophytes including *Macrocystis* and *Egregia* (Leighton, 1966; Vega-García et al., 2015) and in Japan, *H. discus hannai* and *H. diversicolor* preferentially consume *Laminaria*, *Undaria* and *Sargassum* (Sakai, 1962; Takami, 2003; Uki et al., 1986b).

Perhaps more interesting than these apparently general trends in macroalgal preference, are the examples that contradict these trends, as potentially they offer insight into the degree of digestive plasticity in abalone. Considerable variation in abalone diet at the species level, in both seasonality and geographic location, has been extensively reported. Although Shepherd (1973) reported a preference for red macroalgae in *H. cyclobates*, *H. laevigata*, *H. roei*, *H. ruber* and *H. scalaris* in South Australia, there was also considerable seasonality in their diet, with brown and green macroalgae included in months when red algae were less available. Towards the northern edge of its range in Western Australia, *H. roei* is reported to consume a wide variety of macroalgal species with extensive variation between reefs and seasons within a given locality (Wells & Keesing, 1989). In Eastern Tasmania, combined fatty acid and stable isotope analysis indicate a reliance on brown macroalgae and detritus for *H. rubra* (Guest et al., 2008). Contradictory reports on the diet of *H. iris* show alternative preferences for red (Marsden & Williams, 1996; Poore, 1972) or brown macroalgae (Cornwall et al., 2009), or no preference (Taylor & Steinberg, 2005). In Thailand, mature *H. asinina* inhabiting the coral reef environment exist almost entirely on benthic diatoms (80.3% of gut contents) rather than macroalgae (Sawatpeera et al., 1998). The diet of *H. midae* in South Africa shows strong geographical variation. Studies conducted in the Western Cape report the dominance of brown kelp *E. maxima* in the diet, although red and green algae were also consumed, and both site and seasonal differences were observed (Barkai & Griffiths, 1986; Zeeman et al., 2012). By contrast, the diet of adult abalone in the Eastern Cape, where *E. maxima* is absent, was dominated by red macroalgae (Wood & Buxton, 1996). In the southern part of its range in Baja Mexico, *H. fulgens* feed on a variety of red and brown macroalgae, as well as the Anthopthyte sea grass *Phyllospadix torreyi*, which can make up almost half its feed intake in some localities (Guzman del Próo et al., 2003; Serviere-Zaragoza et al., 1998), and distinct seasonal variation in its diet has been reported (Mazariegos-Villarreal et al., 2012; Vega-García et al., 2015), as well as the possible role of detritus and epiphytic diatoms (Vega-García et al., 2015).

Various models have been suggested to explain apparent dietary selectivity in abalone including: (1) the role chemical defences in macroalgae (Shepherd & Steinberg, 1992; Steinberg, 1988; Stepto & Cook, 1996; Winter & Estes, 1992), particularly polyphenols and their secondary metabolites in the Phaeophyta, (2) algal morphology in the form of 'toughness' (McShane et al., 1994; Steneck & Watling, 1982; Stepto & Cook, 1996), (3) the nutritional composition of macroalgae, particularly digestible protein (Angell et al., 2012; Fleming, 1995a, 1995b; Stepto & Cook, 1996), (4) functional feeding behaviour in the form of a preference for acquiring food using trapping or grazing (or a combination of both) and its response to environmental variables (water flow) and predation (Cornwall et al., 2009; Day & Branch, 2002; Poore, 1972; Shepherd, 1973; Wood & Buxton, 1996), and (5) the role of feeding attractants (Angell et al., 2012). However, it has been argued that perhaps the largest overriding factor in determining the dietary composition of abalone is the relative abundance of local algal flora (Mazariegos-Villarreal et al., 2012; Poore, 1972; Shepherd, 1973; Shepherd & Steinberg, 1992; Vega-García et al., 2015; Wood & Buxton, 1996). Abalone can therefore be characterised as bounded opportunistic herbivores, whose specific dietary intake directly represents available algae species, mediated by the need to balance sufficient nutrient intake against mechanical and chemical defences, and predation risk.

The green Chlorophyta, red Rhodophyta and brown Phaeophyta vary markedly in their nutritional composition, both between groups and by comparison to terrestrial plants. This is particularly so in terms of the types and abundance of their storage and structural carbohydrates (Jiménez-Escrig & Sánchez-Muniz, 2000). Green macroalgae, as with higher plants, store excess photosynthetically fixed C in their chloroplasts in the form of starch (a mixture of the α-1,4-glucan amylose and α-1,6-glucan amylopectin), while the reserve carbohydrates in brown macroalgae are laminarins (β-1,4 glucans with β-1,6 branches and some reducing ends replaced by mannitol) (Jiménez-Escrig & Sánchez-Muniz, 2000; Viola et al., 2001). Red macroalgae by comparison, store excess C outside their plastids in the cytosol (as with glycogen in fungal and animal cells) and in the form of floridean starch (α-1,4-glucan with α-1,6 branches, similar to amylose) (Viola et al., 2001; Yu et al., 2002). Typical structural polysaccharides in green macroalgae are cellulose, xylans, mannans and ionic polysaccharides with sulphated groups and uronic acid. In brown macroalgae, structural polysaccharides are alginates (Ca, Mg or Na salts

of alginic acid), fucans and cellulose while red macroalgae contain cellulose, xylan, mannan and the sulphated galactans agar and carrageen (Dawczynski et al., 2007; Jiménez-Escrig & Sánchez-Muniz, 2000; Lahaye, 1991). Macroalgae generally contain large amounts of these structural polysaccharides, which results in fibre content (calculated as non-starch polysaccharides and lignin), ranging from 25 – 75% of tissue on a dry weight basis. Furthermore, the proportion of soluble fibre is high compared to terrestrial plants, ranging from 51 – 85% of total fibre (Jiménez-Escrig & Sánchez-Muniz, 2000; Lahaye, 1991). Lipid content is low (< 6%) in macroalgae, with high concentrations of certain long-chain polyunsaturated fatty acids (Bocanegra et al., 2013; Dawczynski et al., 2007; Fleurence et al., 1994, 2012; Holdt & Kraan, 2011). Total protein content in macroalgae varies between species, with brown macroalgae generally exhibiting lower protein fractions (3 – 15%) compared to red and green macroalgae (10 – 47%), but with marked variation related to season and environmental conditions (Dawczynski et al., 2007; Fleurence, 1999). Macroalgal protein contains all essential amino acids (Dawczynski et al., 2007), although seasonal variability in concentration is reported (Galland-Irmouli et al., 1999). Mineral content in macroalgae (as determined by ashing) is generally high (8 – 40%) compared to land plants, with variation attributed to phylum, geographic region and seasonal and environmental factors (Mabeau & Fleurence, 1993; Rupérez, 2002). Given this considerable potential variability in the nutritional composition of macroalgae, both in terms of type and seasonality, and their resident nature, abalone can be expected to have evolved a degree of plasticity in their digestive capacity to remain successful in a dynamic nutritional environment.

There is little indication of coarse phenotypic plasticity in the general morphology of digestive tract features, as evidenced by the strong congruence in the descriptions provided for species from divergent global locations and nutritional preferences (Bevelander, 1988; Campbell, 1965; Crofts, 1929; Harris et al., 1998b; McLean, 1970). Information on the phenotypic plasticity of the digestive system of abalone at a cellular level in response to diet is limited. Serviere-Zaragoza et al. (2016) report that digestive gland structure and the abundance of proteoglycan granules is variable in relation to different macroalgal diets and may form a useful tool for determining nutritional status. Furthermore, Schaefer et al. (2013) have shown significant effects of protein level in a formulated feed on gut epithelial thickness in sub-adult *H. laevigata*. It is unclear from these early studies whether changes observed are adaptive mechanisms to compensate

for dietary shortcomings or whether they reflect the absence of specific diet-derived drivers of cell development or function. An alternative and perhaps more rapidly employed adaptive mechanism relates to physiological plasticity, particularly with regard to enzyme secretion. Abalone have been shown to produce a range of endogenous enzymes, capable of targeting the major substrate groups encountered in their natural diet including: (1) proteins (protease, aminopeptidases, trypsin, α-chymotrypsin), (2) storage and structural carbohydrates (α-amylase, cellulose, alginate lyase, carbomethylcellulase, laminarinase, agarose, carrageenase) and to a lesser extent, (3) lipids (lipase, esterase, phosphohydrolases) (Bansemer et al., 2016c; Britz et al., 1996; Cui et al., 2000; Edwards & Condon, 2001; Erasmus et al., 1997; García-Carreño et al., 2003; Garcia-Esquivel & Felbeck, 2006; Johnston et al., 2005; Knauer et al., 1996; Picos-García et al., 2000; Serviere-Zaragoza et al., 1997; Viana et al., 2007a). Digestive enzyme activity in abalone has also been shown to respond to changes in diet (Bansemer et al., 2016c; Erasmus et al., 1997; García-Carreño et al., 2003; Garcia-Esquivel & Felbeck, 2006; Knauer et al., 1996; Nel et al., 2017a), indicating a potentially adaptive response. Furthermore, several studies report the supportive role of extracellular enzymes, particularly polysaccharases, produced by the enteric gut bacteria in digestion in abalone, as well as shifts in the gut microbiome related to diet changes (Erasmus et al., 1997; Macey & Coyne, 2005; Nel et al., 2017b; ten Doeschate & Coyne, 2008; Zhao et al., 2012). The apparent plasticity in their digestive physiology could play a role in facilitating successful exploitation by abalone of the varied macroalgal food sources in their natural habitat. Furthermore, it is potentially a contributor to the successful domestication of many species globally, providing an inherent ability to utilise novel food types in the farming environment.

1.4 Domestication

Abalone has formed a component of the diet of subsistence fishers since prehistoric times (Barkai & Griffiths, 1988; Farlinger & Campbell, 1992; Schiel, 1992; Vellanoweth et al., 2006). The commercial exploitation of abalone was first initiated in a number of localities by Chinese immigrants towards the end of the 19th century (Braje et al., 2009; Prince & Shepherd, 1992), but it was only by the early to mid-20th century when the modern, large-scale commercial fisheries were established. Commercial fishing in California for *H. rufescens* began in 1916 and for *H. fulgens*, *H. corrugata*

and *H. cracherodii* in 1943 (Parker et al., 1992; Tegner et al., 1992). The first statistical records of commercial fishing for *H. fulgens* and *H. corrugata* in Mexico date back to 1923. Fishing was initially conducted by Japanese divers until 1939 when local fishermen's cooperatives were established by government (Guzmán del Próo, 1992). The fishery for *H. midae* in South Africa began in Gansbaai in 1949 (Tarr, 1992). Post Word-War II, *H. iris* was fished for its shell in New Zealand, although commercial fishing for flesh only began in the 1960s (Schiel, 1992). The modern Australian industry evolved from small scale collectors near urban settlements in New South Wales in the early 1960s. It spread rapidly to Tasmania, Victoria and South Australia with fishers targeting predominantly *H. rubra*, but also *H. laevigata* and *H. roei* (Prince & Shepherd, 1992). Smaller fisheries developed in the 1970s for *H. mariae*, *H. tuberculata* and *H. kamtschatkana* in Oman, Europe and the Pacific north-west respectively (Farlinger & Campbell, 1992; Johnson et al., 1992; Orstrom, 1992). The last 45 years has seen a continual decrease in landings, from 20,000 metric tons (mt) in 1970 to just 6500 mt in 2015, driven down by overexploitation, illegal harvest, disease, predation and habitat loss (Figure 6) (Cook, 2016). One of the outcomes of this decline was an increased interest in the culture of abalone, both as a means to produce seed for supplementation of wild stocks as well as to exploit the growing culinary demand for abalone. Despite early mention in the literature relating to abalone culture (Ino, 1952; Murayama, 1935), it was not until the 1970s when discoveries around the manipulation of spawning in abalone acted as a catalyst to the emerging industry (Kikuchi & Uki, 1974a, 1974b, Morse et al., 1977, 1979; Uki & Kikuchi, 1974). By the 1980s, emergent abalone aquaculture industries were emerging across multiple global locations including Korea (Park & Kim, 2013), China (Nie, 1992; Zhang et al., 2004a), the USA (Ebert & Houk, 1984), Japan, Taiwan (Chen, 1984), New Zealand (Tong & Moss, 1992), Chile (Flores-Aguilar et al., 2007) and Mexico (Salas-Garza et al., 1992). Over the next 20 years this list expanded to include South Africa, Australia, France, Ireland, the UK, Thailand and the Philippines. By 2015, estimated abalone production from aquaculture sources was 129,287 mt, a dramatic increase from only 50 mt in 1970 (Figure 6) (Cook, 2016).

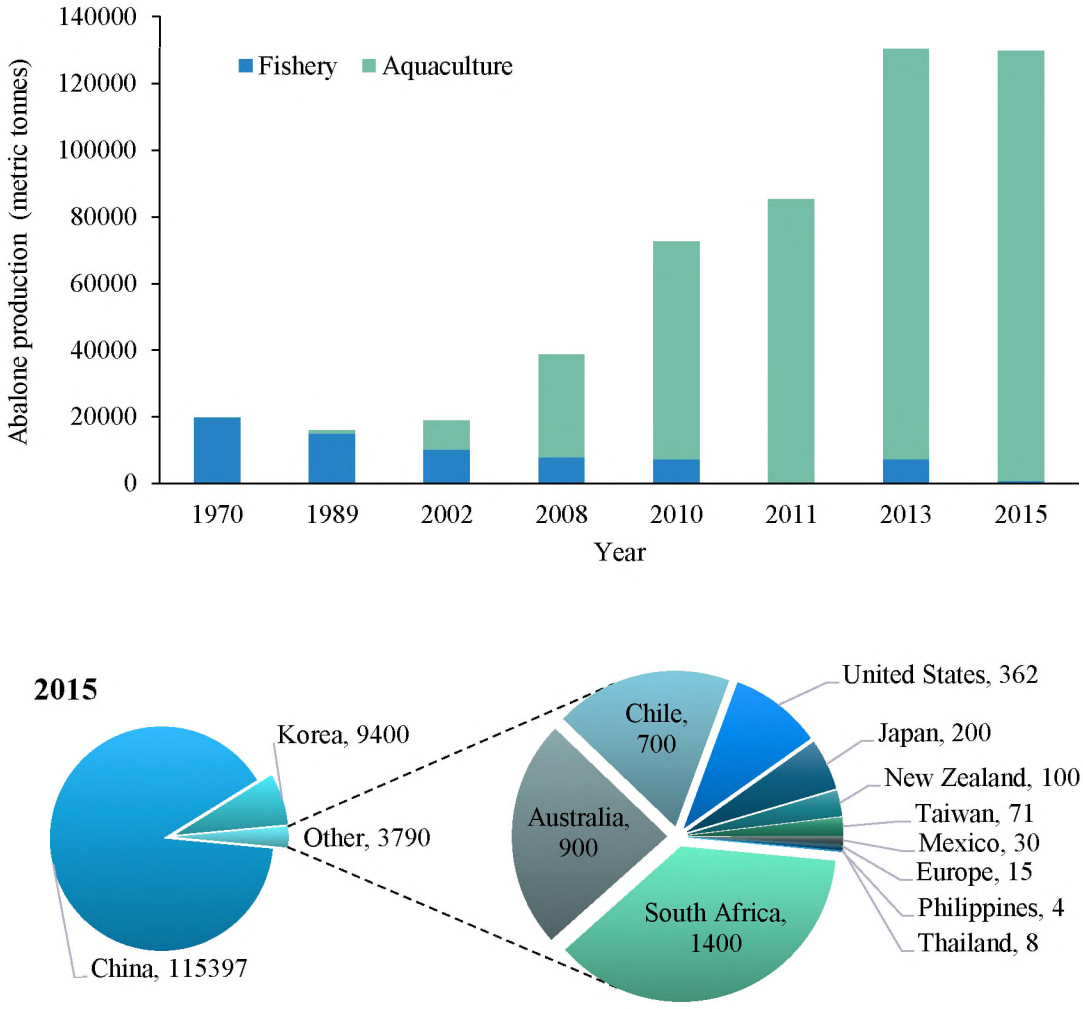


Figure 6. Global abalone production for the period 1970 – 2015 from legal-capture fisheries and aquaculture. Pie chart shows breakdown of abalone aquaculture production per region in 2015. Production data as reported by Cook (2016).

1.5 Nutrition under culture conditions

Abalone are farmed using a variety of aquaculture systems, including oceanic cages, longline barrels and cages, intertidal ponds, pump ashore raceway tanks and recirculating systems. Historically, farmers relied on feeding abalone with locally available fresh macroalgae as the culture diet (Kirkendale et al., 2010). This is still common practice, with wild-harvested and farmed macroalgae forming an important component of the diet of abalone for some of largest producers globally including China, Korea, Chile, South Africa and the USA (Cook, 2016; Flores-Aguilar et al., 2007; Langdon et al., 2004; Park & Kim, 2013; Troell et al., 2006). Although feeding macroalgae exclusively is logical, given the evolution of abalone to successfully exploit this diet in their natural environment, it does come with numerous drawbacks and

challenges in a commercial aquaculture setting. These include that: (1) it can be costly and labour-intensive to harvest, (2) it is difficult to store for extended periods as availability and nutritional composition can vary thereby complicating farm management, (3) it can have a low protein content, high water content, low nutrient density and unbalanced amino acid profiles, (4) the resource is limited and requires the farm location to be close to the resource, and (5) it can be associated with the introduction of pests and disease (Bansemer et al., 2014; Bautista-Teruel et al., 2011; Britz et al., 1994; Hahn, 1989; Robertson-Andersson et al., 2011; Troell et al., 2006).

It was recognised during the nascent stages of the abalone culture industry that the development of suitable formulated feeds for abalone was crucial for its long-term efficiency and growth (Britz et al., 1994; Fleming et al., 1996; Fleming & Hone, 1996; Sales & Britz, 2001b). A 'formulated feed' is defined here as a feed produced by combining defined dietary ingredients into a single product to meet the complete nutritional requirements of the target species. It was envisioned that formulated feeds would offer husbandry and cost advantages (Britz et al., 1994), promote commercially viable growth rates and allow the development of abalone farms where local macroalgal supplies were not suitable or available (Bansemer et al., 2014; Coote et al., 2000). Research into the development of formulated feeds for abalone had its origins in Japan as early as the 1960's (Ogino & Kato, 1964; Ogino & Ohta, 1963), with further extensive contributions in the 1980's (see Uki & Watanabe 1992 for a review). When Fleming et al. (1996) reviewed the state of abalone artificial feed development, there were 17 entities, including abalone farmers, feed companies, universities and government groups involved in research and development and only 10 involved in commercial production, all in China and Japan. By 2010, it was estimated that over 20 commercial diets were available globally, with production sites now extending to include South Africa, Australia, New Zealand, Europe, Chile and the USA (Kirkendale et al., 2010).

An extensive body of literature has emerged as multiple research groups have explored aspects of the diet formulation process as it relates to their local context and species. Key areas of investigation have included determining: (1) optimal inclusion levels of macronutrients such as proteins (Bansemer et al., 2016c; Britz, 1996a; Coote et al., 2000; Mai et al., 1995; Sales et al., 2003b; Stone et al., 2013; Uki et al., 1986a), lipids (Bautista-Teruel et al., 2011; Britz & Hecht, 1997; Green et al., 2011a; Mai et al., 1995,

1996; Mai et al., 1995) and carbohydrates (Ferreira et al., 2015; Lee et al., 2017), (2) ingredient digestibility (Fleming et al., 1998; Sales et al., 2003a; Sales & Britz, 2001a, 2002b, Shipton & Britz, 2001a, 2001b, 2002; Vandepeer et al., 1999), (3) protein:energy ratios (Bautista-Teruel & Millamena, 1999; Gómez-Montes et al., 2003; Green et al., 2011b), (4) vitamin and mineral inclusion levels (Chen et al., 2005; Mai, 1998; Tan & Mai, 2001; Zhu et al., 2002) and (5) suitable ingredient types (Bansemer et al., 2016a; Reyes & Fermin, 2003; Shipton & Britz, 2001a; Viana et al., 1996; Viera et al., 2015). Much of this work has occurred in collaboration with the abalone culture and feed industry and has, in many cases, resulted in the realisation of commercial feed products.

Interestingly, feeds that have reached commercial production are remarkably similar and exhibit a fairly narrow nutritional profile in that they are generally high in protein (mean 32.5%; range 26 – 53%) and carbohydrate (mean 50.5%; range 44 – 61%) and low in lipid (mean 4.7%; 2 – 11%), ash (mean 9.3%; range 4.8 – 19.7%) and fibre (mean 2.5%; range 1.4 – 4.5%) (Table 1). Commercial diet formulations are generally held as proprietary information by the producers, but anecdotal evidence provides some insight into the ingredients being used. Diets are formulated using predominantly terrestrial ingredients. A notable exception is fish meal, which, along with legumes (i.e. defatted soybean meal), cereal grains (i.e. gluten), oilseeds (i.e. lupins) and pulses, are used as protein sources (Bansemer et al., 2014; Bautista-Teruel et al., 2003; Fleming et al., 1996). Abalone have been shown to use lipids ineffectively as an energy source (Bautista-Teruel et al., 2011; Britz & Hecht, 1997; Green et al., 2011a; Mai et al., 1995), and as such lipid inclusion levels are low and may originate partially as secondary associates of protein sources such as fish meal and soybean. Carbohydrates therefore provide most of the energy source and are included in the form of inexpensive terrestrial cereal products such as the processed flours and starches of wheat, maize, rice and soya (Fleming et al., 1996). Gluten and starches are exploited for their binding properties as gel binding, although common in experimental diets, is considered economically unviable (Fleming et al., 1996).

Table 1. Proximate composition of commercial formulated feeds for abalone aquaculture.

Commercial diet	% Dry matter	% Crude protein	% Lipid	% Ash	% Carbohydrate / Nitrogen free extract (NFE)	% Fibre	Energy ($MJ.kg^{-1}$)	Study
Adam & Amos "a"	90	34.2	2.3	7.8	55.2	1.8		Dlaza et al. (2008)
Adam & Amos "b"	90	28.8	3.2	7.8	60.2	2.1		Dlaza et al. (2008)
Adam & Amos "c"	90	28.1	3.3	7.6	61	2.3		Dlaza et al. (2008)
Adam & Amos “biscuit”		>29	3.5	4.8		4.5		Mulvaney et al. (2013b)
Adams & Amos		27						Boarder & Shpigel (2001)
Aquafeeds Australia	90	34	5	6.9	54.1		16.89	Bansemer et al. (2016b)
Commercial feed 1 (Australia)	87.3	32.81	5.42	5.81	55.96		19.32	Mulvaney et al. (2013a)
Commercial feed 2 (Australia)	86.16	31.87	7.1	7.47	55.56		19.3	Mulvaney et al. (2013a)
Cosmo Ocean Pasture	87.2	32	5.8	19.7	19.7 (dissolved)	2.6		Kirkendale et al. (2010)
EPAquafeeds	92	30	4	15	43			www.epaquafeeds.com.au
EPAquafeeds	92.1	36.9	5	7.3	50.6		16.83	Bansemer et al. (2016b)
Gulf feed	98.8	33.1	11.5	13.5	41.9		18.4	Kirkendale et al. (2010)
Haliogro (E.N. Hutchinson)		30						Boarder & Shpigel (2001)
Japan Agriculture Industry	87.6	31.3	4.4	17.7	32 (dissolved)	2.2		Kirkendale et al. (2010)
NNKKK		30	1.5		46	3		Fleming et al. (1996)
Marifeed – Abfeed S34	90	34.7	2.4	5.6	57.3	1.6		Dlaza et al. (2008)
	90	34.6	5.3	5	43.3	1.2		Naidoo et al. (2006)
		38.4	5.8	6.3	48.1	1.3		Guzmán & Viana (1998)
Marifeed – Abfeed K26	90.1	26.2	3.4	4.8	55.7		17.1	Kemp et al. (2015)
Marifeed - Abfeed		34.6						Boarder & Shpigel (2001)
Promak Technology Ltd		53	5.3		32			Fleming et al. (1996)
Skretting Halo	91.6	32.6	5.7	9.1	44		15.1	Stone et al. (2016)
	91.1	36.7	6.7	8.3	48.3		17.01	Bansemer et al. (2016b)
		30	4	6		3		Mulvaney et al. (2013b)
Average		**32.5**	**4.7**	**9.3**	**50.5**	**2.5**		

The process of diet development and formulation process is aimed at creating diets that meet the full nutritional requirements of abalone, but it is apparent that these diets exhibit notable differences from the macroalgae that accomplishes the same outcomes in the abalone's natural environment. Levels of protein included in formulated feeds generally exceed the protein content of macroalgae, apart from some enriched and cultured rhodophytes and chlorophytes. Dietary protein is essential for soft-tissue growth in abalone and inadequate availability can limit growth (Coote et al., 2000; Fleming et al., 1996; Mai et al., 1995; Stone et al., 2013). A protein-dose effect has been reported for abalone fed both formulated feeds (Britz, 1996a; Coote et al., 2000; Mai et al., 1995; Sales et al., 2003b; Stone et al., 2013; Uki et al., 1986a) and macroalgae (Robertson-Andersson et al., 2011; Shpigel et al., 1999; Viera et al., 2011), indicating that up to an optimal level, increases in suitable dietary protein result in increased tissue growth. Studies that have compared the growth of abalone on formulated feeds against macroalgae are summarised in Table 2. There is little consensus amongst these studies as to which diet provides superior growth outcomes in the culture environment. This is likely influenced by a myriad of variables, including but not limited to interspecies differences, formulations of artificial feed, the species and condition of macroalgae, culture systems, dietary history or acclimation and experiment duration (Fleming et al., 1996; Mulvaney et al., 2013). However, from a digestive physiology perspective, it is possible to highlight several interesting observations from these studies. Firstly, in almost all cases, formulated feeds are compared to a single species of macroalgae, which is often included as a control to calibrate the performance of the formulated feeds. This practice potentially undermines the referencing role of the control, given the tendency of abalone to utilise multiple varieties of macroalgae in their natural environment and the indication that mixed-species macroalgal diets outperform single-species diets when tested under experimental conditions (Day & Fleming, 1992; Gordon et al., 2006; Qi et al., 2010; Simpson & Cook, 1998; Stuart & Brown, 1994; Viera et al., 2011). Even with the use of monospecific reference diets, macroalgal diets have regularly equalled or outperformed formulated diets, despite often exhibiting often markedly lower protein contents (Capinpin & Corre, 1996; Corazani & Illanes, 1998; Durazo-Beltrán et al., 2003b; Hernández & Uriarte, 2009; Jackson et al., 2001; Ju et al., 2016; Kim et al., 2013; Lee et al., 1997; Naidoo et al., 2006). It should be noted that in several cases when formulated feeds ranked first, the protein content of the reference macroalgae was very low (2 – 9%) compared to the formulated feed (26 - 44%) (Francis

et al., 2008b; Garcia-Esquivel & Felbeck, 2009; Viana et al., 1993). In addition, when combination diets (formulated feed fed in conjunction with macroalgae) were tested, they resulted in equal or superior growth performance compared to formulated feeds, despite that by offering a combination diet, the supplied dietary protein is reduced as the macroalgal component generally contains less protein than the formulated diet component (Dang et al., 2011; Dlaza et al., 2008; Durazo-Beltrán et al., 2003b; Hernández & Uriarte, 2009; Ju et al., 2016; Lee et al., 1997; Mulvaney et al., 2013a). There are therefore several indications that macroalgal protein is utilised more efficiently for growth than the protein in formulated feeds, and that the co-feeding of macroalgae with a formulated diet (combination diets) increases the efficiency of protein utilisation.

The ability of protein to support growth is related not only to its crude content, but also its apparent quality in terms of its amino acid profile, and its accessibility to the organism as measured by digestibility. Formulated feeds are often formulated to match the amino acid profile of abalone soft tissue in an attempt to provide an ideal protein (Coote et al., 2000; Sales et al., 2003b; Stone et al., 2013). This method is potentially misleading as it fails to take into consideration amino acid turnover or conversion rates, or identify limiting amino acids or account for the physiological roles of free amino acids (Mai et al., 1994; Sales & Janssens, 2004). Furthermore, as a whole animal soft tissue amino acid profile is used, it does not consider differential requirements of the viscera and muscle tissue, and is potentially biased towards the amino acid profile of foot muscle given its greater relative weight. Macroalgal protein is known to contain all essential amino acids (Dawczynski et al., 2007) and it is possible that improved growth with some single species macroalgal diets over formulated feeds, despite lower crude protein levels, could be a reflection of specific amino acid availability. The improvement in growth when diets are diversified (mixed macroalgal or combination diets) would support the protein quality hypothesis, as the amino acid profile of a diverse diet is likely to be superior to that of a single species.

Table 2. A summary of studies comparing the growth rate of abalone fed on formulated feeds (FD) to single macroalgal diets (MS), mixed macroalgal diets (MM) and combination diets (CD) consisting of formulated feed fed in conjunction with macroalgae.

			Formulated diet (FD)	Macroalgae - single sp. (MS)	Macroalgae - mixed sp. (MM)	Combination (MS/MM + FD)
Study count			25	20	5	8

Study	Starting size	Duration	Species	Diets	Rank 1	2	3	4	5	6	7	8
Capinpin & Corre (1996)	0.5mm	120 d	H. *asinina*	MS – [1]*Gacilaria*, [2]*Kappaphycus*; FD – NNKKK.	[1]MS[a] [17]	FD[a] [32]	[2]MS[b] [5]					
Kruatrachue et al. (2004)	45g	90 d		MS - [1]*Acanthophora*; FD - [1]casein, [2]fishmeal, [3]soya, [4]rice bran.	[1]FD[a]	MS[b]	[3]FD[c]	[2]FD[c]	[4]FD[d]			
Jackson et al. (2001)	0.4 - 2.7g	24 w		MS – *Gracilaria*; FD - [1]Commercial(a), [2]commercial(b), [3]commercial(c), [4]commercial(d).	MS[a] [16]	[1]FD[a] [40]	[2]FD[a] [35]	[3]FD[b] [32]	[4]FD[b] [22]			
Ju et al. (2016)	4g	7 m	*H. discus hannai*	MS – *Palmaria*; FD – non-commercial; CD – FD + Palmaria	MS[a] [20]	CD[a] [<20]	FD[b] [27]					
Corazani & Illanes (1998)	21mm	9 m		MS - [1]*Lessonia*, [2]*Macrocystis*, [3]*Ulva*; FD - non-commercial.	FD[a]	[3]MS[b]	[1]MS[c]	[2]MS[cd]				
Kim et al. (2015)	1.4 - 1.7g	10 m		MS - Seaweed X; FD - non-commercial.	FD[a] [68]	MS[a] [70]						
Kim et al. (2013)	51 g	90 d		MS - [1]*Laminaria*, [2]*Undaria*; FD - non-commercial.	[1]MS[a]	FD[a] [33]	[2]MS[a]					
Lee et al. (1997)	.3g	17 w		MS - *Undaria* (dry); FD - [1]commercial, [2]commercial, [3]commercial; CD - [1]FD + *Undaria* (dry).	CD[a] [<35]	[1,2]FD[a] [35-7]	[3]FD[a] [34]	MS[a] [13]				

Chen & Lee (1999)	2.1g	395 d	*H. diversicolor supertexta*	MS – *Gracilaria*; FD - non-commercial.
Viana et al. (1993)	13 mm	90 d	*H. fulgens*	MS – *Macrocystis*; FD - [1]fishmeal, [2]casein.
Durazo-Beltrán et al. (2003b)	0.02g	329 d		MS – *Macrocystis*; FD – non-commercial; CD – FD + *Macrocystis*.
Bansemer et al., (2016b)	0.8 g	93 d	*H. laevigata*	MS – [1]*Ulva*, [2]*Ulva* enriched, [3]*Gracilaria*, [4]*Gracilaria* enriched; MM - [1]*Ulva* + *Gracilaria*, [2]*Ulva* enriched + *Gracilaria* enriched; FD – Commercial.
Dang et al. (2011)	80 mm	12 w		MS – [1]*Ulva*, [2]*Spyridia*; FD - Adam & Amos; CD – FD + *Ulva* & *Spyridia*.
Daume et al. (2007)	7 mm	23 w		MM - *Ulva*, *Navicula* & *Ulvella*; FD - Adam & Amos.
Taylor & Tsvetnenko (2004)	1g	6 w		MS - [1]*Ulva*, [2]*Ulva*, [3]*Ulva*, [4]*Ulva*; FD - Adam & Amos.
Francis et al. (2008b)	46g	3 m	*H. midae*	MS – *Ecklonia*; FD - Abfeed K26.
Britz (1996b)	1.8g	124 d		MS – [1]*Ecklonia*, [2]*Plocamium*; FD – [1]spirulina, [2]casein, [3]fishmeal, [4]torula, [5]soya.
Dlaza et al. (2008)	0.3g	11 m		FD – [1]Abfeed, [2]Adam&Amos (a), [3]Adam&Amos (b), [4]Adam&Amos (c), [5]Feed X; CD – [1,2,3,4,5]FD + *Ecklonia* & *Ulva*.

Table 2 con.

FD[a] [30]	MS[b] [26]					
[1,2]FD[a] [35-44]	MS[b] [2]					
CD[a] [<31]	MS[b] [10]	FD[b] [31]				
FD[a] [34-37]	[2]MM[b] [33]	[4]MS[c] [38]	[1]MM[c] [9]	[3]MS[c] [13]	[2]MS[d] [28]	[1]MS[e] [5]
FD[a]	CD[a]	[1]MS[b]	[2]MS[b]			
MM[a]	FD[b]					
FD[a] [27]	[1]MS[b] [20]	[3]MS[bc] [26]	[4]MS[bc] [32]	[2]MS[c] [22]		
FD[a] [26]	MS[b] [9]					
[3]FD[a] [29]	[1]FD[a] [19]	[1]MS[b]	[2]FD[b] [31]	[4]FD[b] [29]	[5]FD[b] [32]	[2]MS[c]
CD[a] [<19-34]	FD[b] [19-34]					

Table 2 con.

Naidoo et al. (2006)	7.5 g	9 m		MS – [1]*Ecklonia*, [2]*Ecklonia* dry; MM - [1]*Ulva* & *Gracilaria*, [2]*Ecklonia* & macrophyte; FD – Abfeed; CD - FD + *Ecklonia.*	[1]MM [<36]	[2]MM [10+]	[1]MS [10]	CD [<36]	FD [34]	[2]MS [10]		
Boarder & Shpigel (2001)	6 g	3 m	*H. roei*	MS – Ulva; FD - [1]FRDC, [2]Adam & Amos, [3]Abfeed, [4]Haliogro, [5]Deakin diet, [6]non-commercial.	[2]FD[a] [27]	[3]FD[ab] [35]	MS[ab] [32]	[1]FD[ab] [35]	[6]FD[bc]	[4]FD[bc] [30]	[5]FD[c]	
Mulvaney et al. (2013a)	0.2g	12 w	*H. rubra* x *laevigata*	MM - [1]*Gracilaria* + *Ulva*(a) + *Ulva*(b), [2]*Ulva*(a) + *Ulva*(b), [3]*Gracilaria* + *Ulva*(a), [4]*Gracilaria* + *Ulva*(b), [5]*Gracilaria* + *Ulva*(b) enriched; FD – [1]Commercial (b), [2]Commercial (a); CD – [1]FD + [1]MM.	[5]MM[a] [24]	[4]MM[ab] [21]	[1]MM[abc] [22]	[3]MM[bc] [19]	[2]MM[bc] [21]	CD[cd] [27]	[2]FD[de] [32]	[1]FD[e] [33]
Garcia-Esquivel & Felbeck (2009)	42mm	190 d	*H. rufescens*	MS – *Macrocystis*; FD - [1]non-commercial, [2]non-commercial.	FD[a] [36]	FD[b] [23]	MS[c] [9]					
Corazani & Illanes (1998)	36mm		(*H. rufescens*)	MS - [1]*Lessonia*, [2]*Macrocystis*, [3]*Ulva*; FD - non-commercial.	[2]MS[ab]	FD[bc]	[1]MSc	[3]MS[c]				
Hernández & Uriarte (2009)	0.06g	90 d	*H. rufescens*	MS - [1]*Porphyra*, [2]*Macrocystis*; FD – non-commercial; CD – FD + *Porphyra*.	[1]MS[a] [27]	CD[ab] [<44]	[2]MS[bc] [12]	FD[c] [44]				
Viera et al. (2015)	4.7 g	6 m	*H. tuberculata coccinea*	MM – *Ulv*a + *Gracilaria*; FD – [1]non-commercial, [2]non-commercial, [3]non-commercial.	MM[a] [21]	[1]FD[bc] [35]	[2]FD[e] [35]	[3]FD[b] [35]				

Note: The performance of diets for each study were ranked according to specific growth rate (% / day) where available, alternatively final mass or length were used. Superscript number preceding dietary code indicate specific diet treatments within a diet category. Different superscript letters within a study row indicate significant differences in growth. Values in parenthesis are reported protein content.

Digestibility studies related to individual formulated feed ingredients indicate that many plant-based protein sources such as soybean meal, lupines, canola meal and sunflower meal exhibit high apparent protein digestibility (APD) coefficients (80 – 99%) (Fleming et al., 1998; Sales & Britz, 2003; Sales & Janssens, 2004; Shipton & Britz, 2002; Vandepeer & Van Barneveld, 2003). Fishmeal appears to be slightly less digestible, with APD coefficients of 46 – 83% and indications that the origin and prior processing can affect protein availability (Sales & Britz, 2003; Sales & Janssens, 2004; Shipton & Britz, 2002). Furthermore, digestibility coefficients for individual ingredients appear to be additive when included together in formulated feeds, indicating that whole-diet digestibility can reasonably be predicted from the digestibility of individual ingredients (Vandepeer et al., 1999). This is supported by the high whole-diet protein digestibility of 88.7 – 90.1% reported by Currie et al. (2015). Comparable information on the digestibility of seaweed protein in abalone is very limited. Fleming et al. (1996) reports protein digestibility coefficients of up to 81% in some seaweeds and total energy digestibility ranging from 60 – 83%. It would appear unlikely that protein digestibility is a limiting factor with commonly used ingredients in formulated diets.

Given that the efficiency at which protein is utilised in formulated feed does not appear to be directly limited by its digestibility, it can be hypothesised that efficiency of protein utilisation is being affected by other factors, such as post-absorption metabolic dynamics or the interaction with other dietary processes and ingredients. Firstly, the process of digestion itself incurs a metabolic cost to an organism and can influence the apparent efficiency of nutrient utilization as the energy yield of any diet must factor in the energy costs of its digestion (Secor, 2009). Secondly, the provision of protein without a sufficient level of alternative energy in the diet can result in the use of protein to meet energy requirements, rather than being channelled to somatic growth (Gómez-Montes et al., 2003; Montaño-vargas et al., 2005). Evidence that lipid is poorly used as an energy source in abalone and that elevated levels can negatively affect growth, combined with the high carbohydrate / low lipid composition of macroalgae, suggest that abalone are adapted to utilise carbohydrate as a primary energy source (Green et al., 2011a; Mai et al., 1995). The protein-sparing effect of energy supplied in carbohydrate form has been demonstrated in *H. midae* (Green et al., 2011b). Although carbohydrate levels are generally comparable between formulated feeds and macroalgae, carbohydrate structure varies markedly. Macroalgae-based carbohydrates are cell bound

and composed of a large proportion of structural and mucilaginous polysaccharides, which manifest in high dietary fibre values characterised by high soluble:insoluble fibre ratios. By contrast, carbohydrates incorporated into formulated feeds are refined flours/starches derived from terrestrial grains that exhibit high starch and low fibre content (Table 1). Furthermore, the application of heat (in the presence of water) associated with the processing of these starches either before inclusion or during extrusion, or to a lesser degree pelletisation, generally leads to a degree of gelatinisation of the starch present (Bhattacharya & Hanna, 1987; Kraugerud & Svihus, 2011; Sun et al., 2006; Svihus et al., 2005). Gelatinisation leads to initial swelling in the amorphous region, but not in crystalline regions, which places stress on the bonds between amylose (amorphous) and amylopectin (crystalline) (Donald, 2001; Svihus et al., 2005). Gelatinisation occurs when these bonds are irreversibly broken (50 – 70 °C), resulting in leaching of amylose from the starch granule and the formation of gels of solubilised amylose, which increases viscosity and assists in binding feed particles (Svihus et al., 2005). The presence of stable, well-bound abalone feeds in the presence of high-starch carbohydrates and the absence of alternative binding mechanisms would therefore indicate a degree of gelatinisation during processing. This has implications for the digestibility of carbohydrates, as the loss of crystalline structure increases susceptibility for enzymatic degradation (Holm et al., 1988; Peres & Oliva-Teles, 2002).

Abalone display high α-amylase activity in multiple regions of the digestive tract, including upper areas such as the salivary glands, oesophagus and especially the crop (Macey & Coyne, 2005; McLean, 1970). Given the presence of these enzymes, responsible for catalysing the endohydrolysis of α(1 – 4) glycosidic linkages in starch, it is not surprising then that gelatinised starches have been shown to be highly digestible (90 – 99%) by abalone (Sales & Britz, 2002b; Vandepeer & Van Barneveld, 2003). Furthermore, the transport of soluble food, including glucose, through the crop epithelium to the blood has been shown to occur rapidly, as early as 15 min post feeding (McLean, 1970). The potential therefore exists that abalone can rapidly digest gelatinised carbohydrates and experience a subsequent rapid elevation in haemolymph glucose post-feeding. It is likely that the dynamics of the postprandial haemolymph glucose response related to gelatinised starch would differ from one resulting from the digestion of the complex carbohydrates of macroalgae. Although studies investigating starvation and stress-induced changes in body chemistry in abalone have provided some

snap-shot views of circulating haemolymph glucose concentrations (Carefoot et al., 1993; Cheng et al., 2004; Viana et al., 2007b), there are no reported data of postprandial dynamics or the effect of diet composition. Extended diet-induced hyperglycaemia has been associated with reduced performance indicators, such as growth and protein efficiency in prawns (Radford et al., 2005), which have been attributed to perturbation of glycolysis and other cellular functions (Shiau, 1998). Furthermore, circulating haemolymph glucose concentration has been implicated in the rate of glycogen synthesis. Glycogen is the major energy storage mechanism in gastropod molluscs and can compromise up to 40% of foot tissue dry weight (Fluckiger et al., 2011) in abalone. It has been cited as a factor in the sensory perception of abalone (Chiou & Lai, 2002) and has been anecdotally linked with processing yield of abalone product (Jones & Britz, 2006). Assuming elevated postprandial haemolymph glucose levels in abalone fed formulated feeds were observed, it would also shed light on the capacity of abalone to regulate their circulating haemolymph glucose concentration.

There is a growing awareness of the potential benefits of incorporating macroalgae into the diet of abalone in a culture environment, including feeding stimulation, health improvements and product quality manipulation (see Bansemer et al. (2014) for a review). This, coupled with the practical considerations of feeding macroalgae including difficult feed management, limited availability and nutritional deficiencies of particularly single-species macroalgal diets, has led to an expanding body of literature exploring the potential of integrated feeding regimes to harness the advantages of macroalgae while maintaining some of the benefits of formulated feeds. These regimes occur in two forms, either as combination diets, consisting of formulated feed and macroalgae fed together (Dang et al., 2011; Dlaza et al., 2008; Ju et al., 2016; Mulvaney et al., 2013a; Naidoo et al., 2006), or by the inclusion of macroalgae as a dietary ingredient in formulated feed (Bansemer et al., 2016a; Nel et al., 2017b; O'Mahoney et al., 2014; Viera et al., 2015). This creates a complex nutritional situation where abalone are potentially receiving nutrients from multiple dietary components/ingredients at any given time. Understanding the dynamics of nutrient allocation within this complex food environment would provide insight into which dietary components are promoting growth. Data from traditional nutrients utilisation indices, such as food conversion ratio (FCR; feed consumed / abalone weight gain), protein efficiency ratio (PER; abalone weight gain / protein consumed) and energy efficiency ratio (EER; abalone weight gain

/ energy consumed), provide some insight into nutrient utilisation efficiency on a whole diet basis. However, little is understood of the contribution of individual dietary ingredients/components to abalone tissue deposition. This knowledge would provide valuable information to design more efficient feeds and feeding regimes that maximise growth and reduce potential nutrient waste.

1.6 Aims and Objectives

The research framework employed in the current study aimed to utilise a multi-disciplinary approach incorporating traditional and novel assessment methodologies to provide a holistic and integrated examination of the effect of diet on cultured abalone. Initial growth trials, conducted with *H. rufescens* and *H. midae,* served to compare the growth and processing outcomes related to feeding macroalgal, formulated and combination diets in two species of temperate abalone. The growth trial in *H. midae* further provided an extended and controlled dietary acclimation period for a suite of metabolic studies aimed at gaining insights into the post-absorption dynamics and utilisation of dietary nutrients, particularly protein and carbohydrates. The outcomes from this study can facilitate the development of more efficient feeding regime and diets for the culture of abalone.

To address this broad aim, research was focused on the following specific objectives:

1) To determine the effect of feeding formulated, mixed macroalgae and varying ratio combination diets on the growth performance and gross nutritional efficiency indices of two species of abalone, the red abalone *H. rufescens* and the perlemoen *H. midae*.
2) To determine the assimilation of carbon and nitrogen into the foot muscle of *H. midae* from dietary components for animals fed formulated, mixed macroalgae, and combination diets using N and C stable isotopes as natural biomarkers.
3) Determine the metabolic cost of the digestion of a formulated, mixed macroalgae and combination diet standard meal in *H. midae* using oxygen consumption determined by intermittent respirometry techniques. High resolution data on pre- and post-prandial oxygen consumption should allow for the robust estimation of the specific dynamic action (SDA) and SDA coefficient.

4) Investigate the degree of utilisation of protein as an energy substrate by assessing the postprandial dynamics of ammonia excretion in response to a formulated, mixed macroalgae and combination diet standard meal in *H. midae*.
5) Determine the postprandial dynamics of haemolymph glucose concentration in response to a formulated, mixed macroalgae and combination-diet standard meal in *H. midae*.

2 GROWTH, FEED UTILISATION AND PROCESSING YIELD IN THE RED ABALONE *HALIOTIS RUFESCENS* FED MACROALGAL, FORMULATED AND COMBINATION DIETS.

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2.1 Introduction

Fresh macroalgae is the primary feed source used on abalone farms in Chile. Farms in the North (Region III – V) generally use four wild-harvested species namely *Lessonia trabeculata*, *Lessonia berteroana*, *Lessonia spicata* and *Macrocystis pyrifera* while those in the South in the vicinity of Chiloé rely predominantly on wild-harvested *Macrocystis pyrifera* but utilise cultured *Gracilaria chilensis* during winter months (Flores-Aguilar et al., 2007). When surveyed in 2006, land-based farmers considered feed to be the dominant constraint to the development of the industry, expressing concerns over the medium to long-term supply of macroalgae (Flores-Aguilar et al., 2007). Following from this concern the majority of farmers, both land and sea-based, listed feed as the most pressing research requirement. Industry has been testing various formulated diets, both locally produced and imported, but concerns over poor growth rates and price had resulted in limited commercial adoption (Enríquez & Villagrán, 2008; Flores-Aguilar et al., 2007).

The experience of Chilean abalone farms in substituting macroalgal feeds with a formulated feed has been mixed, with the nutritional quality of the formulated diets,

water stability and culture-system design influencing performance. Culture systems designed for macroalgal diets are generally not suited to formulated diets, and require modifications to facilitate feed presentation and associated alterations to husbandry practices. As Chilean farmers were reluctant to convert to pelleted diets, the option of combination diets (i.e. macroalgae and formulated feed fed together) thus provided a pathway for abalone farmers to explore the use of formulated feeds without abandoning macroalgal dietary options entirely. Combination diets allow for an extended weaning period to facilitate dietary switch and there is a growing body of literature to suggest that combination diets provide superior production outcomes when compared to single diets, either macroalgal or formulated (Dlaza et al., 2008; Durazo-Beltrán et al., 2003b; Naidoo et al., 2006). The effect of diet on the growth of abalone in commercial culture must however always be balanced against its effect on the quality and yield of the final product. Diet has been shown to affect the taste, texture, chemical composition and colour of abalone meat (Allen et al., 2006; Bewick et al., 1997; Chiou & Lai, 2002; Smit et al., 2007, 2010). While some Chilean abalone farmers have been using combination diets since as early as 2006 (Flores-Aguilar et al., 2007), there is a paucity of information regarding the performance of these diets and their subsequent effect on yields of the dominant Chilean abalone export product, canned abalone.

Therefore, the aim of this study was to evaluate the effect of fresh macroalgal, formulated pellet and two combination diets (low and high macroalgal supplementation with formulated diet) on production performance indicators and canning yields of juvenile abalone *Haliotis rufescens* in a culture system modified for the use of formulated feeds.

2.2 Materials and Methods

2.2.1 Culture system and experimental animals

The experimental culture system consisted of a 5000-l fiberglass raceway (10 m x 1 m x 0.5 m) with a single inflow of seawater (2500 l hr^{-1}) and formed part of the production infrastructure at the AWABI Abalone Production Centre, Universidad Católica del Norte, Coquimbo, Chile (29°57'S; 71°21'W). Mean incoming water temperature at the facility over the experimental period was 13.3 ± 0.3 °C (range: 12.7 – 14.5 °C). Eight

plastic mesh baskets (800 mm x 300 mm x 450 mm) were suspended in the raceway, with each divided by a double mesh wall (40 mm gap) to form two mesh compartments (380 mm x 300 mm x 450 mm) and resulting in 16 experimental units. A double wall was used to prevent animals accessing food from an adjacent basket through the mesh spaces. Each compartment housed a vertical rack system of four high-density polyethylene (HDPE) plates (300 mm x 300 mm) separated at 70 mm intervals by PVC spacers. A corrugated fibre-cement feeding plate rested on the vertical rack system with a distance of 100 mm between the submerged feeder plate and water surface. Dual parallel airlines ensured direct aeration to each mesh compartment. The experimental animals were hatchery-reared juvenile abalone *H. rufescens* (20.55 ± 1.69 mm shell length (SL); 1.1 ± 0.3 g, 431 ± 23 days post-spawning) sourced from production stock at AWABI Abalone Production Centre. The nutritional history of these animals consisted of post-settlement rearing on diatom covered polycarbonate sheets followed by a diet of fresh macroalgae (*Macrocystis pyrifera*, *Lessonia berteroana* / *L. spicata* and *Gracilaria* spp.).

2.2.2 Diets

Four dietary treatments with four replicates were allocated to basket compartments according to a randomised block design that ensured the even distribution of treatments along the length of the raceway. Dietary treatments consisted of a formulated pelleted diet (FD – *ad libitum*), two combination diet regimes consisting of *ad libitum* pelleted feed with a fresh macroalgae supplement fed in conjunction (CMH – high macroalgae at 7.5% bw day^{-1}; CML – low macroalgae at 1.5% bw day^{-1}), and a fresh macroalgae-only diet (MD - macroalgae at 15% bw day^{-1}). The pelleted diet was formulated from two sources, steam-dried, formaldehyde-free, mackerel fishmeal (Oceana (Pty) Ltd., South Africa) and soya-oil cake. Starch carbohydrates and a vitamin/mineral mix were included according to a proprietary commercial formulation (Marifeed (Pty) Ltd., South Africa). Rehydrated, minced *M. pyrifera* was included at low levels to replace the macroalga normally included in the commercial formulation (*Ecklonia maxima*) with a member of the Laminariales commonly used in the commercial culture of abalone in Chile. Proximate analysis of the diet was conducted on triplicate samples taken from 100g of homogenized pellets. These analyses indicated a composition of 26.7 ± 1.7%

crude protein (semi-micro Kjeldahl; N x 6.25), 3.4 ± 0.4% total lipid (Soxhlet solvent extraction using chloroform and methanol at 2:1), 9.9 ± 0.1% moisture (oven drying at 95 °C), 4.9 ± 0.1 % ash (residue after combustion at 550 °C), 55.1 ± 0.3% non-nitrogenated extract (calculation by difference) and 17.1 kJ g^{-1} digestible energy (Protein: 23.6 kJ g^{-1}, lipid: 39.5 kJ g^{-1}, and carbohydrate: 17.2 kJ g^{-1} after Bureau et al. 2002).

The species of fresh macroalgae used was based on local availability and consisted of either *M. pyrifera* or *Lessonia berteroana*/*L. spicata*, with shore-collected *Lessonia berteroana*/*L. spicata* generally dominating during high sea events when the harvest of *M. pyrifera* by diving was not possible. The average ratio of *M. pyrifera* to *L. berteroana*/*L. spicata* across all treatments was 55:45, 17:83 and 80:20 for months 0-2, 2-4 and 4-6 respectively. The feeding regimes for the four dietary treatments (FD, CMH, CML and MD) are described in Table 3.

Table 3. Feeding regimes for the four dietary treatments consisting of a formulated pellet diet (FD), two combination diet regimes (CMH and CML) and a fresh macroalgae diet (MD). Days referred to in this table relate to the revolving 7-day feeding cycle employed in the experiment.

	FD	CMH	CML	MD
Formulated feed				
Pellets into baskets	days 1, 3, 5	days 1, 3, 5	days 1, 3, 5	n/a
Pellets topped up if required	days 2, 4, 6	days 2, 4, 6	days 2, 4, 6	n/a
Uneaten pellets removed	days 1, 3, 5	days 1, 3, 5	days 1, 3, 5	n/a
Fresh macroalgae				
Macroalgae ration (% BW day^{-1})	0	7.5%	1.5%	15%
Macroalgae into baskets	n/a	days 1 & 4	days 1 & 4	days 1 & 4
Uneaten macroalgae removed	n/a	days 1 & 4	days 1 & 4	days 1 & 4

[a] Fresh macroalgae ration was calculated weekly as a percentage of body weight based on the basket weight at the previous weighing and measurement and adjusted weekly for mortality. A full ration (100%) was set at 15% bodyweight per day.

2.2.3 Experimental procedure

Experimental animals (n = 420 per basket compartment) were moved from general production at AWABI to the experimental basket units over a three-day period, acclimated for two days on a diet of *M. pyrifera* and then purged for 48 h prior to anaesthetization using BZ – 20® (20% benzocaine; concentration 1:10000; Veterquímica, Chile). Abalone mass and long-axis shell length were determined on a subsample (n = 200) using a digital scale (0.1 g, Acculab V-1200) and from digital photographic images using calibrated on-screen digital calliper software (0.01 mm, Screen Callipers, Iconico Inc., New York) respectively. This photographic method was employed to reduce handling and emersion time. The weighing and measurement procedure was repeated on days 50 - 52, 106 - 108 and 162-164 of the trial.

Animals were fed according to the feeding regimes described in Table 1. Fresh macroalgae rations were calculated weekly for each basket and fed proportional to the number of days that macroalgae would be in the basket (day 1 - 3/7 ration; day 4 – 4/7 ration). Uneaten macroalgae was removed and weighed before new feed was introduced to the basket. Uneaten formulated feed was collected by hand on days 1, 3 and 5 from the feeder-plate surface and basket floor, pooled for each week and stored at -20 °C until oven dried to a constant mass at 95 °C. Dry weight conversion factors were determined for all species of macroalgae and the pelleted feed by oven drying pre-weighed samples in triplicate at 95 °C. Mortalities were recorded on days 1, 3 and 5 as the feeding plate could be removed to observe the basket floor.

Linear shell growth rate (LGR), specific growth rate (SGR), proportional feed intake (PFI), food conversion ratio (FCR), protein efficiency ratio (PER) and condition factor (CF) were calculated as follows:

(1) LGR = (final shell length in mm ÷ initial shell length in mm) ÷ time in days

(2) SGR = [(Ln final weight – Ln initial weight) ÷ time in days] × 100

(3) PFI = [(wet weight dietary component consumed × dry weight conversion factor) ÷ total dry weight of all feed consumed)] × 100

(4) FCR = grams dry feed consumed ÷ grams wet weight gained

(5) PER = grams wet weight gained ÷ grams dry protein consumed a

(6) CF = 5575 × (grams abalone weight ÷ mm shell length 2.99) (Britz, 1996a)

The protein content used in the PER calculations for the formulated diet were based on the proximate analyses conducted. The protein content range of macroalgae (10 – 15%) was based on the results of proximate analyses conducted on the same macroalgal species collected from localities along the Pacific coastline stretching from Southern Chile to Baja, California (Chavez, 2011; Demes et al., 2009; Duarte et al., 2014; Gómez & Westermeier, 1995; González et al., 2012; Gonzalez et al., 2008; Ortiz et al., 2009; Rodríguez-Montesinos & Hernández-Carmona, 1991; Serviere-Zaragoza et al., 2002; Westermeier et al., 2012).

Furthermore, if it is assumed that the PER for macroalgae remained constant when fed as macroalgae only or in a combination diet, then the PER of the formulated component of combination diets can be calculated as follows:

$$PER^{f} = ((Bm^{f} - Bm^{i}) - ((M^{d} \times P^{m}) \times PER^{m})) \div (FP^{d} \times P^{f})$$

Where: PER^{f} = Protein efficiency ratio (formulated component); Bm^{i} = initial basket mass (g); Bm^{f} = final basket mass (g); M^{d} = macroalgae consumed (g; dry weight); Pm = Proportional crude protein content of macroalgae; PER^{m} = Protein efficiency ratio of macroalgae calculated from macroalgae only diet; FP^{d} = Formulated pellet consumed (g; dry weight); P^{f} = Proportional crude protein content of formulated diet.

2.2.4 Simulated canning

A simulated canning trial was conducted following the conclusion of the growth trial to determine the effect of dietary treatment on the abalone meat weight yield. Ten abalone were randomly selected from each experimental basket and blotted dry using paper towelling. Animals were weighed as a group to the nearest 0.01g (Acculab V-200) and individual long-axis shell lengths measured using digital hand-held callipers (Mitutoyo CD-8, 200 mm). They were then processed for canning according to the specifications

provided by APROA (Association of Abalone Producers, Chile). Abalone were shucked using a flat spatula and the visceral mass, meat and shell separated. The meat from each replicate was placed in individual plastic containers and drained overnight in a refrigerator (5 °C). The meat was then placed in an 18% NaCl solution for 25 min and agitated sporadically by hand before being transferred to potable water for 15 min. Any remaining pigment and mucus was brushed from the meat and frill using a soft bristle brush and water, and the radula was notched out with a sharp blade. The meat from each replicate was placed in a standard can and topped with a 2% NaCl solution. The can temperature was then raised to 70 °C before the lids were sealed and then autoclaved for 65 min at 112 °C. After shucking the abalone, the meat, shell and visceral mass for each replicate was recorded. Mucus mass was assumed to be the difference between initial whole abalone mass before shucking and the combined mass of the meat, shell and viscera following shucking. Meat mass was then recorded following washing and notching (can in), after canning following draining (commercial yield) and following drying with paper towelling (true yield).

2.2.5 Statistical analysis

Specific growth rate (SGR, % body weight day^{-1}) values per replicate were determined from the slopes of natural log transformed weight data plotted against time × 100. Mean log transformed weight data from each replicate was then plotted for each treatment and the slopes of the single regressions per treatment assessed for significant differences using a homogeneity of slopes model ($P < 0.05$). Initial and final abalone weight and length, SGR, mortality, FCR, PER, final CF and all canning trial data were tested for homogeneity of variance and normal distribution of residuals using Levene's test and the Shapiro–Wilk W test respectively. When these assumptions were met, parametric one- way ANOVA and Tukey's HSD post hoc tests were employed to test for differences in dependent variables between dietary treatments (significance level $P < 0.05$). A non-parametric Kruskal–Wallis ANOVA by ranks tests ($P < 0.05$) was used when variances were heterogeneous. A linear regression was employed to define the relationship between true yield and macroalgae consumption data. All statistical tests were performed using Statistica 12.0 for Windows (StatSoft, Inc., 2013).

2.3 Results

2.3.1 Growth and nutritional indices

Abalone weight increased significantly for all treatments over the 162 d period of the growth trial with growth rates described by the exponential curves as follows: (PD) y = 1.1497exp(0.0049x), $R^2 = 0.98$, $P < 0.05$; (MD) y = 1.1163exp(0.0061x), $R^2 = 0.97$, $P < 0.05$; (CMH) y = 1.1165exp(0.0064x), $R^2 = 0.99$, $P < 0.05$ and (CML) y = 1.1492exp(0.0053x), $R^2 = 0.97$, $P < 0.05$ (Figure 7). The homogeneity of slopes model showed significant differences between treatments in the slopes of natural log transformed weight data plotted against time ($F_3 = 9.0$, $P < 0.005$). Furthermore, there was a statistically significant difference between treatments in terms of final abalone length ($F_{3,12} = 18.95$, $P < 0.005$), final abalone weight ($F_{3,12} = 30.194$, $P < 0.005$), SGR ($F_{3,12} = 15.77$, $P < 0.001$) and LGR ($F_{3,12} = 22.30$, $P < 0.001$) as determined by one-way ANOVA. The Tukey's HSD test revealed that final abalone weight and length as well as SGR were significantly higher for the MD and CMH diets compared to the PD and CML diets (Table 4). Mortality rates were not significantly different ($F_{3,12} = 2.54$, $P = 0.11$) between treatments. There was no significant difference in final CF values between dietary treatment ($F_{3,12} = 1.92$, $P = 0.18$) (Table 4).

The total dry feed intake per basket ranged from 590 ± 50 g for diet FD to 1279 ± 41 g for diet MD (Table 5). The proportional feed intake ratio on a dry weight basis of formulated feed to macroalgae for diets CMH and CML were 76:24% (1:0.32) and 32:68% (0.47:1) respectively (Table 3). The distribution of *Lessonia berteroana* / *L. spicata* and *Macrocystis pyrifera* within the macroalgal component of diet MD, CMH and CML was relatively even, with a small bias towards *Lessonia berteroana* / *L. spicata*. FCR was significantly lower for diet CML compared to diets FD, MD and CMH ($H_{3,16} = 8.91$, $P = 0.03$). There was a positive relationship between the proportional intake of macroalgae in the diet and PER. PER values ranged from 1.6 ± 0.5 for diet FD to 4.5 ± 0.2 for diet MD (macroalgal protein: 10%), with all treatments significantly different from each other ($F_{3,12} = 69.34$, $P < 0.001$) (Table 5).

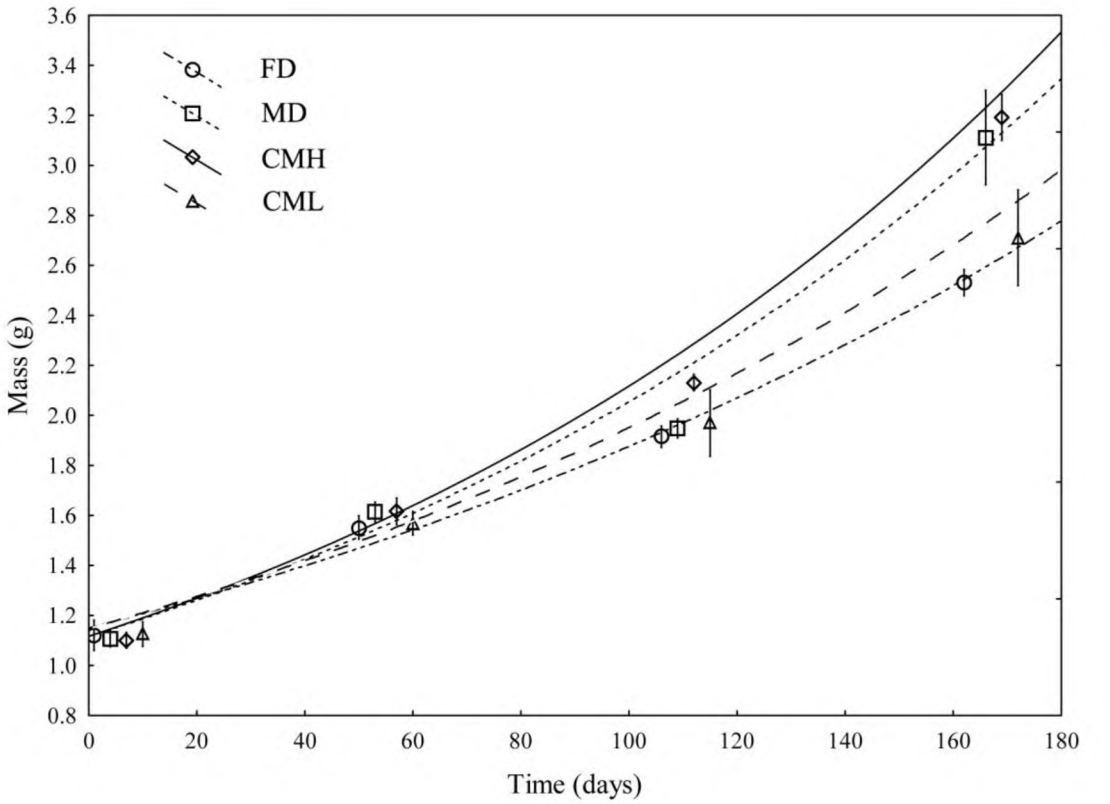


Figure 7. Regressions of mean weight data per replicate of juvenile *H. rufescens* reared for 162 days on formulated pellet (FD), fresh macroalgae (MD) and two combination diet regimes (CMH & CML). Means of weight data for each treatment at each measurement interval (0, 50, 106 & 162 days) are presented. Symbols denoting treatments are spread at each measurement interval for clarity. Error bars indicate standard deviation.

Table 4. Growth performance parameters and mortality for juvenile *H. rufescens* reared for 162 days on a formulated pellet (FD), fresh macroalgae (MD) and two combination diet regimes (CMH & CML).

	Diet			
	FD	MD	CMH	CML
Initial weight (g)	1.12±0.06 [a]	1.11±0.03 [a]	1.10±0.03 [a]	1.13±0.05 [a]
Initial length (mm)	20.47±0.58 [a]	20.49±0.28 [a]	20.57±0.56 [a]	20.64±0.30 [a]
Final weight (g)	2.53±0.05 [a]	3.11±0.19 [b]	3.19±0.09 [b]	2.71±0.19 [a]
Final length (mm)	26.19±0.15 [a]	28.69±0.47 [b]	28.86±0.57 [b]	27.04±0.57 [a]
SGR (% bw.day^{-1})	0.50±0.05 [a]	0.64±0.04 [b]	0.66±0.01 [b]	0.54±0.04 [a]
LGR (μm.day^{-1})	34 ± 3 [a]	49 ± 2 [b]	49 ± 1 [b]	38 ± 5 [a]
Final CF	0.81±0.02 [a]	0.76±0.05 [a]	0.77±0.03 [a]	0.79±0.02 [a]
Mortality (%)	21.7±13.4[a]	13.4±3.8 [a]	9.6±1.0 [a]	10.0±1.5 [a]

Values reported are treatment means of four replicates and standard deviation. Values with the same superscript in each row are not significantly different ($P < 0.05$)

This pattern was less marked at a macroalgal protein content of 15%, although significant differences were still observed ($F_{3,12} = 23.63$, $P < 0.001$). PER values ranged from 3.0 ± 0.1 for diet MD to 1.6 ± 0.5 for diet FD (Table 5). A significant positive relationship was observed between macroalgae consumption and the protein efficiency ratio of the formulated component of the diet (PER^f) (Fig 5). PER^f was significantly lower for diet FD than diet CMH, while diet CML was not significantly different from diets FD and CMH ($F_{2,9} = 6.72$, P = 0.0164) (Table 5).

Table 5. Feed intake and nutritional performance indices for juvenile *H. rufescens* reared for 162 days on a formulated pellet (FD), fresh macroalgae (MD) and two mixed-diet regimes (CMH & CML).

	Diet			
	FD	MD	CMH	CML
Dry feed intake (g)				
Formulated pellet	580 ± 50		292 ± 51	485 ± 56
Fresh macroalgae as:		1279 ± 41	929 ± 30	223 ± 9
Macrocystis pyrifera		615 ± 16	402 ± 3	94 ± 3
Lessonia berteroana / L. spicata		664 ± 25	527 ± 28	130 ± 6
Total	580 ± 50	1279 ± 41	1221 ± 45	709 ± 63
Proportional feed intake (%)				
Formulated pellet	100		23.9 ± 3.5	68.4 ± 1.7
Fresh macroalgae as:		100	76.1 ± 3.5	31.6 ± 1.8
Macrocystis pyrifera		48.1 ± 0.3	32.9 ± 1.0	13.3 ± 0.8
Lessonia berteroana / L. spicata		51.9 ± 0.3	43.2 ± 2.7	18.4 ± 1.0
Nutritional indices				
FCR	2.0 ± 0.7 [a]	2.2 ± 0.1 [a]	1.8 ± 0.1 [a]	1.5 ± 0.1 [b]
PER (10% macroalgal protein)	1.6 ± 0.5 [a]	4.5 ± 0.2 [b]	3.6 ± 0.3 [c]	2.5 ± 0.2 [d]
PER (15% macroalgal protein)	1.6 ± 0.5 [a]	3.0 ± 0.1 [b]	2.9 ± 0.2 [b]	2.3 ± 0.2 [c]
PER^f (formulated component)	1.6 ± 0.5 [a]		2.9 ± 0.8 [b]	2.2 ± 0.2 [ab]

Values reported are treatment means of four replicates and standard deviation. Values with the same superscript in each row are not significantly different (P < 0.05)

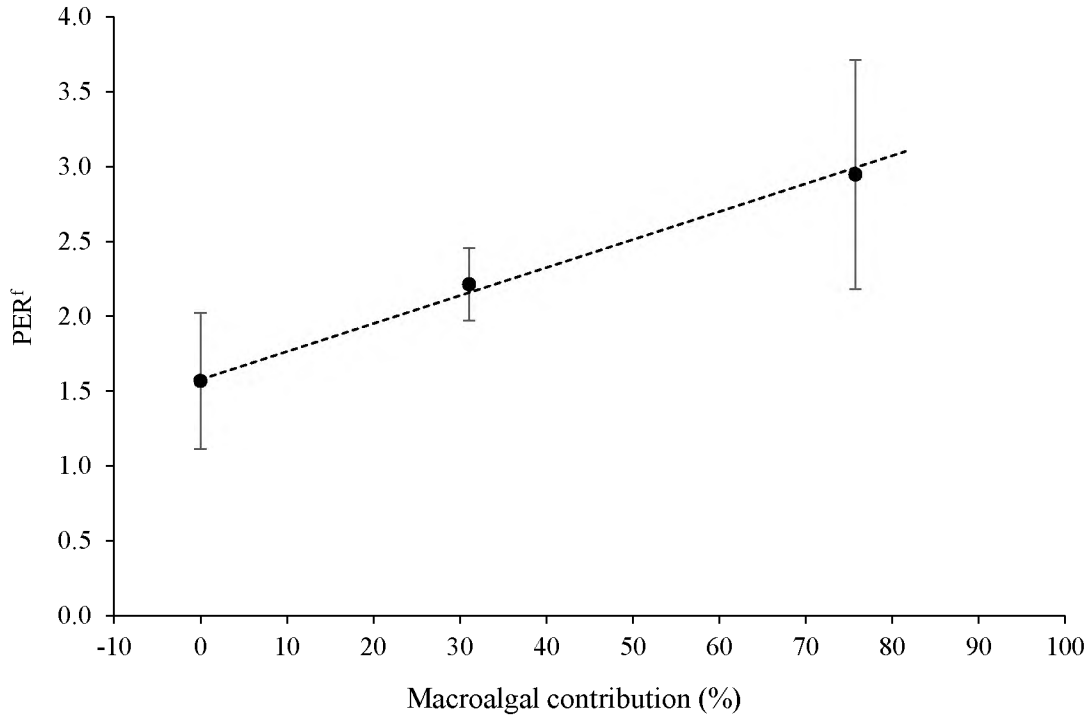


Figure 8. A liner regression (y = 0.0187x + 1.5819, $R^2 = 0.64$, $P = 0.0018$) of the protein efficiency ratio of the formulated component of the diet (PER^f) against macroalgae consumption (% of dry weight of total feed consumed) from a growth trial of juvenile abalone *H. rufescens* reared for 162 days on a formulated pellet (FD), fresh macroalgae (MD) and two combination diet regimes (CMH & CML).

2.3.2 Canning

There was a significant difference between treatments for all components of body composition including shell ($F_{3,12} = 9.20$, $P = 0.002$), mucus ($F_{3,12} = 11.736$, $P = 0.001$), meat ($F_{3,12} = 14.06$, $P < 0.001$) and viscera ($F_{3,12} = 30.69$, $P < 0.001$). The Tukey's HSD test revealed shell yield (%) to be significantly higher and mucus yield (%) significantly lower for diet PD than the remaining dietary treatments, which were not significantly different from each other (Figure 10, a-b). Meat yield (%) was significantly lower for diet MD than treatments CMH, CML and FD, which were not significantly different from each other (Figure 10, c). Viscera yield (%) was significantly higher for diet MD than diet CMH, CML and FD. Viscera yield was significantly higher for diet CMH compared to diet CML, but not significantly different from diet FD (Figure 10, d).

In terms of yield outcomes, there was a significant difference between treatments for meat processing loss ($F0_{3,12} = 37.55$, $P = 0.001$), commercial yield ($F_{3,12} = 35.16$, $P < 0.001$) and true yield ($F_{3,12} = 34.07$, $P < 0.001$). The Tukey's HSD test revealed that

meat processing loss was significantly lower for diet FD compared to diets CMH, CML and MD, which were not significantly different from each other (Figure 10, e). Commercial and true yield were higher for diet FD than diets CMH and CML, which were in turn significantly higher than diet MD (Figure 10, f-g). There was a significant positive linear relationship ($y = 0.0003x2 + 0.0119x + 14.859$, $R^2 = 0.7762$, $P < 0.001$) between final true meat yield and the percentage contribution of formulated pelleted feed to the total dry feed intake over the 162-day growth trial (Figure 9).

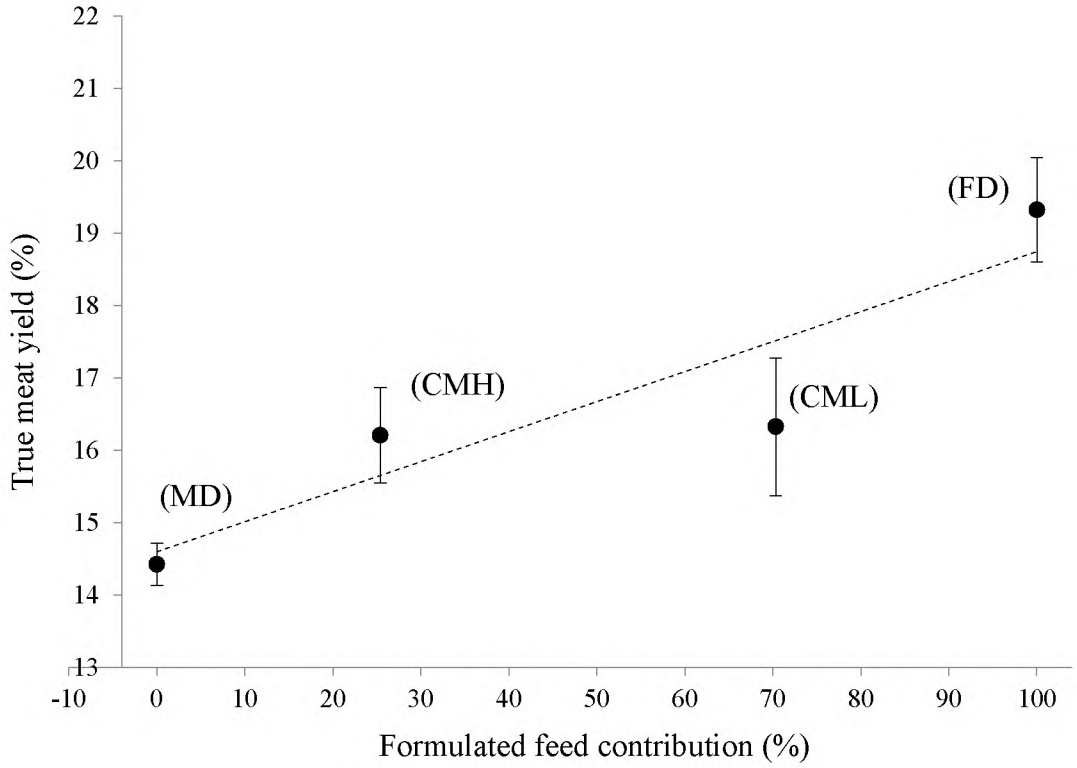


Figure 9. A linear regression ($y = 0.0414x + 14.5996$, $R^2 = 0.75$, $P < 0.001$) of true yield (% of total abalone wet weight at harvest) against formulated feed contribution (% of dry weight of total feed consumed) from a growth trial and subsequent simulated canning trial of juvenile abalone *H. rufescens* reared for 162 days on a formulated pellet (FD), fresh macroalgae (MD) and two combination diet regimes (CMH & CML).

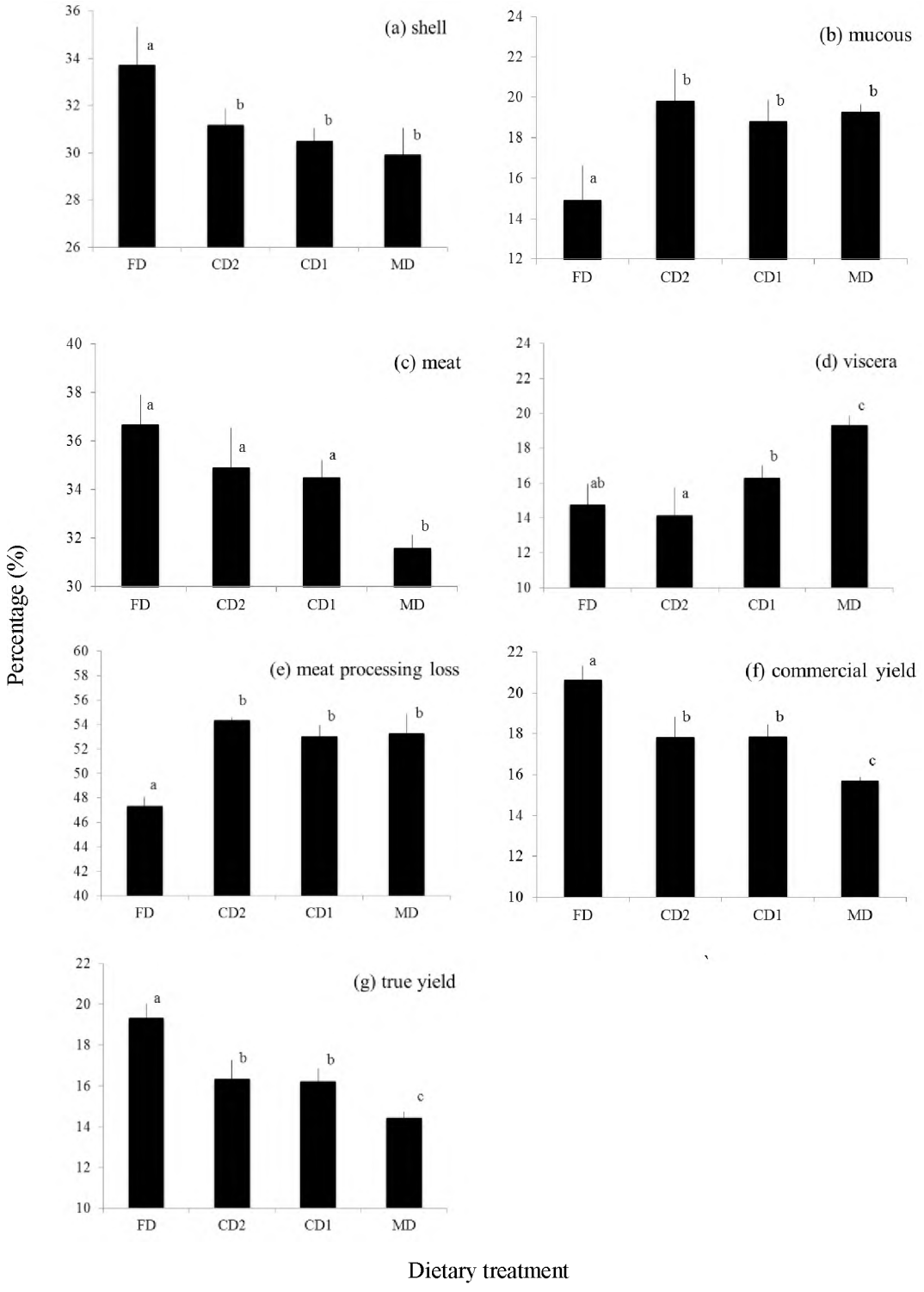


Figure 10. Body component breakdown and yields from a simulated canning trial of juvenile abalone *H. rufescens* reared for 162 days on a formulated pellet (FD), fresh macroalgae (MD) and two combination diet regimes (CMH & CML). Solid bars represent means for each treatment (n = 4). Error bars indicate standard deviation.

2.4 Discussion

The study demonstrated that for juvenile abalone *Haliotis rufescens* fed macroalgal diets, up to 24% of the total feed intake on a dry weight basis could be substituted with formulated feed without negatively affecting growth rates. In addition, the inclusion of formulated feed into the diet of the abalone positively influenced canning yields. The growth rates of 34 – 49 μm day^{-1} (initial shell length 20.6 mm) for abalone in this study fall within the range (15 – 198 μm day^{-1}; initial shell length 5.9 – 46.7 mm) previously reported for juvenile *H. rufescens* fed macroalgal, formulated and combination diets (Table 6). The relatively low experimental growth rates were probably due primarily to the low average water temperature recorded over the experimental period (13.3 ± 0.3 °C), which was below the estimated optimum range for *H. rufescens* of 16.7 – 17.2 °C using the polynomial equation ($T_{opt.G}$ = 12.91 + 0.26L – 0.01L2 + 0.0001L3) of Steinarsson & Imsland (2003). Nutritional factors cannot entirely be discounted, as rapid growth rates (> 80 μm day^{-1}) have generally been associated with feeding red macroalgal diets such as *Porphyra columbina* and *Palmaria mollis*, either in their wild harvested form or cultured in nutrient rich environments (Demetropoulos & Langdon, 2004; Evans & Langdon, 2000; Garcia-Esquivel & Felbeck, 2006). Previous studies where juvenile *H. rufescens* were fed a single species of brown macroalgae (*Macrocystis pyrifera*, *Macrocystis integrifolia* or *Laminaria digitata*) have reported growth rates in the range of 15 – 123 μm day^{-1} (Corazani & Illanes, 1998; Garcia-Esquivel & Felbeck, 2006; Hernández & Uriarte, 2009; Silva-Aciares et al., 2011; Trevelyan et al., 1998; Vivanco-Aranda et al., 2011). The growth rate of 49 μm day^{-1} for the MD treatment in this study is comparable with these previous studies, although towards the lower edge of the reported range. In terms of formulated feeds, growth rates of 37 – 75 μm day^{-1} have previously been reported at dietary crude protein levels of 25 – 47% (Canales-Gómez et al., 2010; Corazani & Illanes, 1998; Garcia-Esquivel & Felbeck, 2009; Hernández & Uriarte, 2009). The growth rate of 34 μm day^{-1} for the formulated feed treatment (FD) in the current study falls just below the lower end of this range.

Table 6. Growth data for cultured juvenile abalone *Haliotis rufescens* from studies testing macroalgal, formulated and combination diet feeding regimes.

	Culture system type	Growth period (days) [1]	Water temperature (°C)	Initial length (mm) [2]	Growth rate (μm day^{-1}) [3]	Study
Macroalgal feed						
Macrocystis pyrifera	flow-through	192	17	46.5	15	Garcia-Esquivel and Felbeck (2006)
Porphyra columbina	flow-through	90	15	6.6	110	
Macrocystis pyrifera					70	Hernandez *et al.* (2009)
Macrocystis integrifolia	flow-through	210	16-19	18.9	114	
Macrocystis integrifolia (with probiotic)	flow-through	210	16-19	18.9	123	Silva-Aciares *et al.* (2011)
Laminaria digitata & *Palmaria palmata* (9:1)	flow-through	120	13.7	17.2	50	
				26.6	60	Steinarsson and Imsland (2003)
Macrocystis pyrifera	flow-through	126	16.8	6.2	26	
	recirculating		16.1	5.9	22	Vivanco-Aranda *et al.* (2011)
Lessonia trabeculata	flow-through	240	not reported	34.5	46	
Macrocystis integrifolia				33.7	63	
Ulva rigida				34.9	26	Corazani and Illanes (1998)
Palmaria mollis	flow-through	360	8.7 - 13	10	80	Buchal *et al.* (1998)
Palmaria mollis	flow through	139	14.6 - 15.7	10	112 - 132	Evans and Langdon (2000)
Macrocystis pyrifera	flow-through	124	not reported	8.26	34	
Macrocystis pyrifera with *Microcladia coulteri* supplement					60	Trevelyan *et al.* (1998)
Nutrified Palmaria mollis	flow through	100	18	25	169 - 198	Demetropoulos and Langdon (2004)
Macrocystis pyrifera & *Lessonia berteroana* / *L. spicata*	flow-through	162	13.3	20.5	49	*This study*
Formulated feed						
Formulated pellet (38% protein)	flow through	190	15	46.2	51	
Formulated pellet (25% protein)				46.7	37	Garcia-Esquivel and Felbeck (2006)
Formulated pellet (43% protein)	flow through	90	15	6.6	50	Hernandez *et al.* (2009)
Formulated pellet (36% protein)	flow through	240	not reported	35.9	50	Corazani and Illanes (1998)
Formulated pellet (47% protein)	flow through	90	17	5.46	75	
Formulated pellets with carotenoids (28-32% protein)					53	Canales-Gomez *et al.* (2010)
Formulated pellet (26% protein)	flow through	162	13.3	20.5	34	*This study*
Mixed diets						
Porphyra columbina & formulated pellet (44% protein)	flow through	93	15	6.6	87	Hernandez *et al.* (2009)
Macrocystis pyrifera / *Lessonia berteroana* / *L. spicata* &	flow through	162	13.3	20.6	49	
As above at a ratio of 1:9	flow through	162	13.3	20.6	38	*This study*

[1]Where necessary, growth period in days was calculated from month values assuming 30 days per month. [2]Initial length was abalone shell length (mm) at the start of the growth experiment.
[3]When not directly reported in the study, growth rate as daily increment in shell weight (μm day^{-1}) was calculated as follows: final length (μm) - initial length (μm) ÷ growth period (days).

Data on the growth rates of juvenile *H. rufescens* fed macroalgal / formulated feed combination diets are limited to a study by Hernández & Uriarte (2009). The authors reported that a combination diet of *Porphyra columbina* and formulated feed (44% protein) outperformed both a formulated feed diet and single species brown macroalgal diet (*Macrocystis pyrifera*), but exhibited significantly lower growth rates than a single species red macroalgal diet of *P. columbina*. A study on the South African abalone *Haliotis midae* has also shown that combination diets consisting of a fresh macroalgae fed in conjunction with a formulated feed outperform the formulated feed fed alone (Dlaza et al., 2008). Durazo-Beltrán et al. (2003b) reported significantly improved growth rates for *Haliotis fulgens* on a combination diet (*M. pyrifera* and 31% protein formulated pellet) compared to either of its components fed individually. Dang et al. (2011) showed similar growth for *Haliotis laevigata* on formulated and a combination diet, both of which outperformed monospecific algal diets.

Feeding formulated feed with fresh macroalgae in a combination diet resulted in improved growth rates in abalone over a formulated feed only diet in the present study. A dose effect was evident, however, with 31% macroalgal inclusion insufficient to significantly improve growth rates, which only occurred at a macroalgal inclusion level of 75%. In situations where multispecific algal diets have outperformed monospecific diets (Day & Fleming, 1992; Qi et al., 2010; Stuart & Brown, 1994), it has been suggested that mixed diets are able to compensate for possible shortcomings in the chemical composition and subsequent nutrient availability of monospecific diets, and thereby better meet the nutritional requirements of abalone (Day & Fleming, 1992; Qi et al., 2010; Stuart & Brown, 1994). Combination diets of formulated feed and macroalgae may function in a similar way to multispecific algal diets, whereby the algal component is able to provide micronutrients not available in the formulated feed, which are generally formulated from a small body of terrestrial ingredients to meet crude protein and energy requirements. In addition, marine macroalgae are rich in bioactive compounds and have shown biological effects including anti-bacterial and anti-viral activity, anti-oxidant potential and anti-inflammatory properties in mammals and fish (O'Sullivan et al., 2010; Trichet, 2010). For example, Dang et al. (2011) showed enhanced antiviral activity in digestive gland extracts for the abalone *H. laevigata* fed *Ulva lactuca* and *Spyridia filamentosa* and have suggested macroalgal supplementation as a means to boost the antimicrobial defences of abalone. Given these potential health

benefits, feeding abalone macroalgae may improve their resilience to on farm stressors such as handling and anaesthesia (Bansemer et al., 2014) thereby improving scope for growth.

Abalone in the current study also exhibited an improved protein efficiency ratio (PER) with increasing proportional contribution of fresh macroalgae in their diet. Furthermore PER^f, calculated assuming that the PER for macroalgae remained constant when fed as macroalgae-only or in the combination diets, revealed a significant positive correlation between the macroalgal supplementation level in the diet and the PER of the formulated component of the diet (Figure 8). Therefore, the increase in total PER is likely driven by the increased availability of protein in the formulated component of combination diets associated with increasing levels of fresh macroalgal supplementation. Marine macroalgae contain high levels of dietary fibre other than cellulose, particularly soluble sulphated polysaccharides such as the galactans (agar and carrageenans) in the Rhodophytae and alginates, fucans and laminarins in the Phaeophyceae (Rupérez et al., 2002). Some of these polysaccharides have shown promising prebiotic activity in mammal models in that they are: (1) resistant to digestion in the upper gastrointestinal tract and (2) are selectively fermented by beneficial bacteria and consequently modulate the intestinal microflora and (3) induce a systemic effect that is beneficial to organism health (O'Sullivan et al., 2010). While abalone exhibit enzymes capable of digesting complex macroalgal polysaccharides (Erasmus et al., 1997; Garcia-Esquivel & Felbeck, 2006; Johnston et al., 2005) undigested feed particles can still provide substrates for bacterial colonisation. Furthermore, hydrolysis products from enzymatic polysaccharide digestion, such as mannooligosaccharides from manna, have shown prebiotic microflora modulation in humans (Dhawan & Kaur, 2007). Probiotic manipulation of the gut microflora in abalone has shown promising results for animal health, nutritional physiology and growth (Iehata et al., 2009, 2010; Macey & Coyne, 2005; Silva-Aciares et al., 2011; ten Doeschate & Coyne, 2008). Of particular interest in these studies are the elevated digestive enzyme activities associated with the administration of probiotics. If similar enhanced protease enzyme activity can be expected from intestinal microflora modulation through the prebiotic effect of macroalgal polysaccharides, it may provide a mechanism for the positive relationship between the proportional contribution of macroalgae in the diet of abalone and the PER reported in the current study (Nel et al., 2017a, 2017b). It is likely that the enhanced growth of abalone associated with the

supplementation of formulated feeds with macroalgae is the result of a complex interaction of drivers including micronutrient supplementation, direct effects on animal health from bioactive compounds and intestinal flora modulation with its associated benefits.

The current results indicate a strong positive relationship between proportional ingestion of formulated feed and final meat-mass yield of a canned product. Absolute meat yield was 34% greater for the formulated pellet diet compared to the macroalgal diet, potentially offering significant economic advantages for producers. This finding warrants further investigation at commercial scales and over full production cycles where the effect of the interrelated factors of growth rate and meat yield on commercial outcomes can be quantified. Elevated meat yields associated with increasing levels formulated feed in the diet appear to be determined by a combination of increased initial meat yield and the subsequent reduced processing losses. Brown et al. (2008) suggest weight loss associated with the canning of abalone is the result of the loss of water and water-soluble components. Glycogen, which is the major form of energy storage, can contribute up to 40% of the foot muscle on a dry weight basis (Braid et al., 2005; Fluckiger et al., 2011; Uki & Watanabe, 1992) and is known to bind water at a proportion of 2 – 4 g of water per gram of glycogen dependent on molecule size (Przybylski et al., 2006). It is feasible that the increased inclusion of the energy-dense formulated feed into the diet led to increased glycogen stores resulting in elevated water retention during processing. Furthermore, the extended thermal processing during the canning process leads to the conversion of collagen to water-soluble gelatin (Brown et al., 2008; Gao et al., 2001). A recent study by Øiseth et al. (2013) showed differences in the distribution and occurrence of collagen in the foot tissue related to diet, with wild abalone (macroalgal diet) having markedly larger collagen-rich areas than their farmed counterparts (formulated feed). Collagen content has also been shown to correlate with muscle toughness (Øiseth et al., 2013; Olaechea et al., 1993), with formulated feed fed abalone often described as having a crisp rather than chewy texture. Although not measured directly in this study, the reduced canning weight loss for abalone on formulated feed and associated increased yield may also be related to reduced collagen content of the meat and subsequent reduced loss of gelatinised collagen to the cooking water.

In conclusion, this study indicates that it is possible to substitute a proportion of an exclusively macroalgal diet with formulated feed without compromising growth rates in juvenile *H. rufescens*. Furthermore, by employing a combination diet approach, abalone farmers are potentially poised to harness both the health and nutritional benefits of macroalgae and the husbandry and processing yield advantages of formulated feeds. It is important to note that a positive relationship has been reported between water temperature and both optimum protein inclusion levels and leeching in formulated feeds fed to *H. laevigata* (Stone et al. 2013). Given that the current study was conducted at temperatures substantially lower than the thermal optimum for *H. rufescens*, it is possible that the observations in this trial may vary with culture temperature. Further research is required to optimise the inclusion level of each dietary component in combination diets and explore the complex interactions between macroalgal polysaccharides, gut microflora and nutrient digestion and assimilation.

3 GROWTH AND NUTRIENT UTILISATION DYNAMICS IN THE PERLEMOEN *HALIOTIS MIDAE* FED MACROALGAL, FORMULATED AND COMBINATION DIETS.

3.1 Introduction

The South African abalone (*Haliotis midae* L.), colloquially referred to as 'perlemoen', is the largest of the six indigenous species (*H. midae*, *H. spadicea*, *H. parvum*, *H. speciose*, *H. pustulata* and *H. queketti*) and the only one to be exploited commercially (Sales & Britz, 2001b). The commercial industry, based on an open-access fishery, was initiated in 1949, with rapid growth over the next 15 years resulting in record landings of 2800 tons in 1965 (Raemaekers et al., 2011; Tarr, 1992). In response to plummeting catches, formal catch regulations were instituted in 1968. Thereafter followed a period of relatively stable catches from the early 1970s through to the mid-1990s (Tarr et al., 1996), when a combination of ecological (distributional shift of the predatory West Coast rock lobster *Jasus lalandii*) and socio-economic (illegal fishing) factors resulted in an 88% decrease in catches from 615 tons in 1995/7 to 75 tons in 2007, and the ultimate closure of the fishery in 2008 (Raemaekers et al., 2011). The fishery has recently been reopened to accommodate a small experimental quota (150 mt), however the long-term sustainability of a wild fishery remains uncertain under current levels of illegal exploitation (Cook, 2016). While production from the legal wild fishery was declining, a parallel industry centred on the culture of *H. midae* in land-based aquaculture systems was emerging (Figure 11).

Commercial fishing companies, driven by supply/demand disparities in the South African abalone market, and inspired by developments around abalone culture

(principally for restocking wild fisheries) in other countries (Japan, Australia, UK, Canada, Chile, France, Mexico, Taiwan and the USA), began exploring the possibilities of controlled abalone culture in the late 1980's (Genade et al., 1988). The first successful spawning of captured adults occurred naturally (without induction with hormones or chemicals stimulating ovulation) in the laboratory in 1981; and was repeated in 1985 following induction stimuli (UV irradiated water exposure and emersion) (Chen, 1984) (Genade et al., 1988). Formal research programs investigating abalone culture were established in 1989/90, involving collaborations between research institutions (University of Cape Town, Rhodes University and the CSIR National Research Institute for Oceanology, Stellenbosch) and commercial fishing companies (Irvin & Johnson, Sea Plant Products and Oceana) (Hecht & Britz, 1990). Development followed rapidly, facilitated by both technology transfer from abroad as well as industry and research-institution driven innovation, with the FAO listing the first ton of commercial production in 1993 (FAO, 2016; Sales & Britz, 2001b). By 2013, abalone culture formed the majority component of non-plant marine aquaculture in South Africa, contributing 49% of total production by weight (DAFF, 2014a). A total of 20 farms were involved in the sector, with the majority (65%) located in the Western Cape, RSA. Annual production had reached 1470 tons, up from 181 tons in 2000; an effective average annual growth rate of 20% (DAFF, 2014a).

The model currently employed for abalone culture in South Africa is generally ubiquitous across commercial operations, with farmers using flow-through, land-based culture tanks and on-site hatcheries for independent seed production. There are however some variations on this approach. In 2012, four farms were focussed entirely on grow-out and there was a single operator each involved in both sea-cage farming and ranching respectively (DAFF, 2013). Furthermore, within a local context of rising electricity costs, some flow-through farms are experimentally investigating the serial-use of pumped water as a means of expanding production (Naylor et al., 2011, 2013, 2014) while others are exploring partial recirculation using macroalgal remediation (Bolton et al., 2009; Robertson-Andersson, 2003, 2006; Robertson-Andersson et al., 2008; Troell et al., 2006). The latter also offers the potential for on-site feed production and in some cases, makes a substantial contribution to feed production, providing as much as 30 - 40% in the case of the Wild Coast Abalone farm (P. Britz, Rhodes University, pers. comm., July 2017).

H. midae is an opportunistic herbivore in its natural environment, feeding on a wide range of acceptable algae species and guided mostly by their abundance within the surrounding habitat (Barkai & Griffiths, 1986). They employ both trapping (drift algae) and grazing (attached algae) as food acquisition behaviours, and may adapt their feeding mode to adapt to food availability in their immediate environment, as has been suggested for *H. iris* (Poore, 1972; Wood & Buxton, 1996). The brown kelp *E. maxima* is often reported as the dominant food source for *H. midae* (Barkai & Griffiths, 1986; Day & Branch, 2002; Zeeman et al., 2012). The apparently high contribution of kelp over other seaweeds may, however, be an artefact of study site location, where drift kelp dominates available algal food (Zeeman et al., 2012), ecological interactions that facilitate preferential access to kelp (Day & Branch, 2002) and a bias resulting from the tendency of gut-content studies to overemphasise the importance of brown algae with their thick cellulose walls and polyphenolics (Day & Cook, 1995; Foale & Day, 1992). In diet-composition studies where abalone fed on a diversity of species, they exhibited a preference for non-kelp species, such as *Ulva*, *Hypnae*, *Ralfsia* and *Plocamium*, as evidenced by proportional disparities between the availability of these macroalgae in the habitat and their representation in gut samples (Wood and Buxton, 1996). Despite this apparently broad natural diet encompassing representatives from all three major macroalgae groups and likely due to accessibility and biomass considerations, a single species of kelp dominates the utilisation of macroalgae in abalone culture, both as a feed and as a control for studies investigating alternative feeds.

The early development of the abalone culture industry in South Africa relied heavily on the wild kelp *E. maxima* as the primary feed source. Harvested kelp destined for use on abalone farms increased from 7 tons in 1993 to a peak of 5924 tons in 2001, while over the same period abalone production increased from 1 to 373 tons (Figure 11). Research aimed at developing a viable formulated feed for *H. midae* was initiated at the commencement of the abalone culture industry in the late 1980's, driven by the realisation that reliable supplies of wild-harvested kelp would ultimately become limiting to industry growth and the desire to harness the husbandry and operational benefits that formulated feeds could offer (Britz et al., 1994). Pilot-scale commercial production of the first iterations of a practical formulated feed where available from as early as 1991 (Anon, 1991). The ongoing development of these initial formulations was

driven by an extensive research program over a 20-year period (Britz, 1996b; Britz & Hecht, 1997; Green et al., 2011a, 2011b; Ismail et al., 2009; Knauer et al., 1993, 1996, Sales et al., 2003a, 2003b, Sales & Britz, 2002a, 2002b, 2003, Shipton & Britz, 2001a, 2001b, 2002), which has informed the current product range.

Uptake of formulated feed within the industry was initially limited to a small group of farms who adopted the technology from their inception, with the majority opting for a diet of fresh kelp. By 2003, the localised pressure on kelp resources resulting from the clustered distribution of abalone farms (NW of the Cape Agulhas and Cape Columbine regions) meant that kelp harvests were approaching the maximum sustainable yield (MSY) in these areas (Troell et al., 2006). Rapidly plateauing kelp harvesting, coupled with a growing awareness in the industry about the potential yield advantages, particularly in terms of canned product associated with feeding formulated feed resulted in a fundamental shift in the use of feed, from wild-harvested kelp to formulated pelleted feed. This shift, with kelp use by abalone farmers reaching a ceiling at 5924 tons in 2001 and the rapid growth in abalone production from 429 tons in 2002 to 1111 in 2012 (14% annual growth rate) was supported by a parallel rise in formulated feed use (14% annual growth from 2005 – 2012) (Figure 11). In addition, the use of cultured macroalgae, mainly in the form of *Ulva*, is becoming more widespread in the industry. Investigations into macroalgal cultivation were initiated in the 1990's at the Marine Growers farm at Port Elizabeth (Fourie, 1994; Hampson, 1998; Smit, 1997; Steyn, 2000), and *Ulva* production only became more widespread with the establishment a commercial scale integrated multitrophic aquaculture (IMTA) system at Wild Coast Abalone (WCA) in 2003, where macroalgae grown in 32 paddle raceways provided with abalone effluent water contribute a large portion of feed to the farm (Bolton et al., 2013). WCA also currently produce *Gracilaria* using rolling culture in circular tanks. Further commercial adoption of paddle raceways by I & J West Coast Abalone occurred in 2006, predominantly for water remediation in a partial recirculating system (Bolton et al., 2009). Three other farms also produced small amounts solely for feed supplementation (Bolton et al., 2009, 2013).

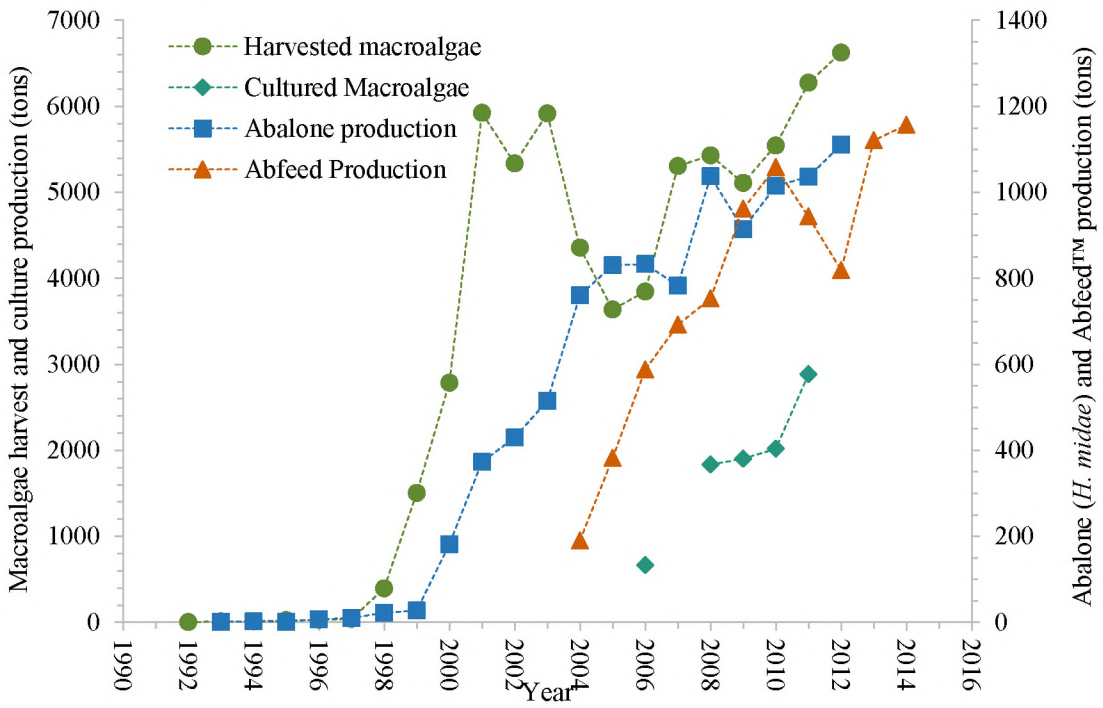


Figure 11. Annual production figures for farmed abalone *Haliotis midae*[1,4] in South Africa and the three major feed sources used in their culture: (1) formulated feed[2] (Abfeed ™), (2) kelp[2,3] (harvested whole fronds and fresh beachcast) and; (3) cultured macroalgae[4] (*Ulva* and *Gracilaria* species). (Data sourced from: [1] FAO (2016); [2] 2004: Troell et al. (2006); 2005 – 2014: Production figures for domestic distribution (*pers. comm.* Kurt Matchke, General Manager, Marifeed (Pty) Ltd.; [3](DAFF, 2014b), Troell et al. (2006); [4] (DAFF, 2014a)

The diet of abalone on commercial farms in South Africa has been varied as farmers constantly strive to balance the multiple considerations of growth outcomes, animal health, processing yields, availability, price and husbandry requirements in their quest to find the optimal dietary regime within their given context. Abalone are now likely to be exposed to feed as varied as wild-harvested kelp, a formulated pellet (34 or 26% protein), cultured macroalgae (both *Ulva* and *Gracilaria*) and increasingly frequently combinations of these feeds, particularly macroalgal/formulated combinations. Despite the use of combination diets for feeding *H. midae* under commercial culture conditions, often with a long history of implementation, there is a paucity of published literature on the subject. Naidoo et al. (2006) investigated the effect of a variety of macroalgal, formulated and combination diets on abalone growth in a flow-through culture system. Treatments included different forms of kelp (*E. maxima* as fresh blades, fresh blades with a red algal epiphyte, dried stipes and dried pellets), formulated pellets (Abfeed ™ S34), formulated pellets + kelp, a rotation diet and a mixed macroalgal diet (Ulva, Gracilaria and kelp). Although significant differences in growth were reported between

the dietary treatments (ANOVA), the lack of *post hoc* analyses in this study makes it problematic to ascertain where the cut-off point for these differences occurred. It should be noted, however, that fresh macroalgae generally exhibited better growth than the formulated feed and dried macroalgal products, macroalgae diets with some form of mixing outperformed the single-species treatments and combining formulated feed with fresh macroalgae improved growth over a formulated-feed-only diet. Further work by Dlaza et al. (2008) investigated the effect of five commercially available formulated abalone feeds, and combination diets consisting of the selected formulated feeds supplemented with fresh *Ulva* and kelp, on post-weaning abalone growth parameters. In all instances, the inclusion of a fresh macroalgal supplements improved growth over the formulated-feed-only diets. Studies on a variety of other abalone species have yielded similar results. Hernández & Uriarte (2009) reported that a mixed diet consisting of the red macroalgae *Porphyra columbina* and a formulated feed (44% protein) outperformed a formulated-feed-only diet in *H. rufescens*. Durazo-Beltrán et al. (2003a) showed significantly improved growth for *H. fulgens* when fed a combination diet of a formulated pellet (31% protein) and the brown macroalga *Macrocystis pyrifera* when compared to either of the dietary components fed independently. Growth rates of juvenile *H. laevigata* were not significantly different between formulated feed or a combination diet of formulated feed and macroalgae (*Ulva lactuca* and *Spyridia filamentosa*), although the mean growth rate was higher for the combination diet. Both these diets outperformed single-species macroalgae diets (Dang et al., 2011). While these studies provide initial indications of the potential value of combining formulated and fresh macroalgal feeds in combination diets, their design unfortunately precludes them providing insight into nutrient utilisation in combined diet regimes. The lack of specific consumption and nutritional composition data for individual dietary components means key nutritional indices, such as protein efficiency ratio (PER), cannot be calculated. Furthermore, without the ability to trace individual dietary components from ingestion to deposition, their role in the nutrition of the animal cannot be elucidated.

The use of stable isotopes as chemical tracers to investigate food web structure has expanded greatly in the last 20 years (Bond & Diamond, 2011). Despite this growth, abalone have received little attention, with only a handful of ecological studies investigating diet composition in wild populations (Guest et al., 2008; Pinnegar &

Polunin, 2000; Vega-García et al., 2015; Won et al., 2010, 2007). Increasingly, isotopic studies are also being used to understand nutrient flows and utilization within aquaculture systems and contexts (Beltrán, 2009; Gamboa-Delgado et al., 2008, 2013; Gamboa-Delgado & Vay, 2009; Gamboa-Delgado, 2014; Le Vay & Gamboa-Delgado, 2011). Biologically important elements (i.e. C, N, S & O) generally have two or more stable isotopes that differ only in the number of neutrons present in the atom (Gamboa-Delgado, 2014). Bioaccumulation of the heavier isotopes due to physiological processes, including fractionation during metabolic transformations and isotopic routing, results in a difference in the isotopic composition between an organism and its diet (Martínez Del Rio et al., 2009). This difference has been referred to under various terms, including fractionation factor, enrichment, trophic enrichment and apparent fractionation, although for clarity the term discrimination factor (as denoted by Δ) suggested by Martínez Del Rio et al. (2009) is best used as "*using fractionation confuses a pattern with only one of the processes that create it*". The discrimination factor ($\Delta^{13}C$) between diet and consumer is usually low (~ 1 ‰) in carbon (DeNiro & Epstein, 1978a, 1978b) and has been applied extensively to trace the source of carbon in food webs. For nitrogen, the discrimination factors ($\Delta^{15}N$) is generally higher at 3-4 ‰ (DeNiro & Epstein, 1981; Minagawa & Wada, 1984; Peterson & Fry, 1987), thereby allowing the estimation of trophic positions of organisms within an ecosystem (Estrada & Rice, 2003; Layman et al., 2007; Post, 2002). Naturally abundant stable isotopes therefore provide an important tool to determine elemental flux and cycling in systems (Phillips & Gregg, 2003). Furthermore, the recent advent of isotopic mixing models that provide quantitative estimates of proportional contribution of sources (feed items) to a mixture (consumer tissue) based on their isotopic signature (Caut et al., 2009; Parnell et al., 2010), has in turn facilitated a variety of diet reconstruction studies in natural ecosystems (Moreno et al., 2010; Newsome & Phillips, 2004; Polito et al., 2011; Votier et al., 2010).

Isotope mixing models have evolved greatly since their introduction. Early linear models provided unique solutions assuming no variation within sources (Phillips, 2001). Later developments integrated isotopic signature variability in the sources as well as the mixture (IsoError; (Phillips & Gregg, 2001), better representing natural systems (Parnell et al., 2010) although these models were limited by the number of sources (*n* isotopes +1) that they could incorporate. Further models (IsoSource, (Phillips & Gregg, 2003)

allowed for multiples sources, but at the expense of variation and uncertainty (Parnell et al., 2010). A shift to Bayesian inference has led to new models such as SIAR (Parnell et al., 2010) and MixSIR (Moore & Semmens, 2008) that are advantageous for a number of reasons including: (1) the ability to incorporate variability in the sources, consumer and discrimination factors (per sources), (2) the ability to handle multiple dietary sources, (3) the ability to incorporate prior information and, (4) being able to produce solutions as probability distributions (Bond & Diamond, 2011; Parnell et al., 2010). These models currently provide the most robust estimates of dietary contribution to tissue in complex nutritional systems with inherent variability in both source and consumer isotopic composition.

The current study aimed to expand on the experimental design employed in Chapter 2 by increasing the resolution of the graded combination diets treatments, using a representative species mixture of the three major macroalgae groups for treatments incorporating macroalgae, estimating the nutritional composition for all dietary components with increased accuracy and temporal resolution and employing tracers to track nutrient utilisation. This was approached by determining the effect of formulated, macroalgal and three graded combination diets on growth, processing (canning) parameters and nutrient allocation in *H. midae* using a controlled growth trial. Naturally occurring stable isotopes were modelled as nutrient tracers using a Bayesian mixing model (SIAR) to estimate the proportional contribution of sources (food types) to a mixture (consumer).

3.2 Materials and Methods

3.2.1 Culture system & experimental animals

Experimental abalone were reared in a recirculating aquaculture system (RAS) housed in a controlled-environment (CE) room at the Port Alfred Marine Research Laboratory, Eastern Cape, South Africa (33° 35' 49.9" S 26° 53' 32.9" E). The RAS consisted of 22 fiberglass tanks (500 mm × 600 mm × 300 mm, 1.98 m^3 total volume) linked via 110 mm PVC drainage lines to, in order of flow, a swirl separator (800 mm ∅ x 900 mm deep; 0.45 m^3 volume), fluidised media biological filtration unit (800 mm ∅ × 900 mm; 0.45 m^3; filter media – comparable to Kaldness K1) and pumping sump (500 mm ∅ × 700 mm; 0.14 m^3). A submersible pump (Resun SP 9600S) located in the sump pumped water through an ultraviolet steriliser (Ultrazap CC.; 55 W; quartz sleeve) to an overhead ring supply manifold (110 mm PVC pipe), which distributed water through sub-manifolds to the culture tanks. Water flow to each tank was adjusted to 270 l h^{-1} to allow for 3 exchanges per hour. A side loop of flow (500 l hr^{-1}) from the pumping sump to the biological filter passed through a 45-l foam fractionator (Ultrazap CC) with bubble generation via a venturi injector (250 W circulation pump).

Total system volume was calculated to be 3.19 m^3. Water exchange in the system occurred through an independent 260 l top-up tank mounted in the CE room. Seawater was pumped into the top-up twice daily from the Kowie River estuary mouth for a 2-hour period over high tide. Transfer from the top-up tank to the RAS was set at a rate of 60 l h^{-1}, thereby exchanging 360 l over a 6-hour period (2 h during, and 4 h post pumping). Total daily system water exchange was therefore 720 l or ~ 23% of system volume. This arrangement allowed a prolonged period for the introduction of new water into the system, thereby preventing system temperature fluctuations. Air temperature was maintained at a set point of 18 °C using a twin-fan chiller unit (Recoil 2.4 kW). Supplemental heat was supplied in winter months via an electrical heater. Light:dark period was maintained at 12L:12D (6am:6pm) using a timer switch and two 35 W fluorescent tubes. Opaque plates mounted below the light fittings prevented direct light from reaching the culture baskets, with an ambient light intensity of ~ 14 lux recorded at the water surface.

System tanks (n = 15) housing abalone contained an oyster mesh basket (400 mm × 550 mm × 300 mm) suspended from a 20 mm PVC pipe frame. Each basket contained a vertical rack system of five HDPE plates (180 mm × 500 mm) separated at 70 mm intervals by spacer bars (n = 5 per rack). A corrugated fibre-cement feeder plate (320 mm × 400 mm) rested horizontally on the vertical rack system with a distance of 50 mm between the top of the plate and the water surface. Mild aeration to each tank was provided via a PVC aerator bar (20 mm ∅, 5 x 1mm drilled holes). A framed oyster-mesh lid clipped onto the basket frame to prevent animals leaving their allocated baskets.

Culture tanks were cleaned weekly. A standard protocol was followed: (1) water flow to the tank was shut off and culture baskets housing abalone (n = 4 per batch) were lifted from the experimental tanks and moved to temporary holding tanks in the RAS, (2) the majority of solid waste on the base of the tanks was rapidly siphoned to waste, (3) the sides and base of culture tanks were lightly scrubbed using an abrasive sponge and allowed to stand for 15 min, (4) a second siphoning removed the remainder of fine solid waste that had settled, and (5) the water flow was reopened allowing the tanks to flush before the abalone baskets were then returned to their original position in the system. Approximately half the tank volume was lost during the siphoning process. This was replaced by trickle flow from the top-up tank, itself receiving constant flow from the main supply pump over the cleaning process. Abalone baskets were exposed to air for a maximum of 15 sec, split over two periods, during the cleaning process.

Water quality parameters were assessed weekly on day 1 prior to the weekly tank cleaning protocol. Values obtained can therefore be considered indicative of the upper limits of what abalone were likely to be exposed to over a weekly cycle. Water temperature was visually monitored daily (PT-100 probe linked to Zenith Instruments 301 PJK temperature control unit) to ensure correct chiller functioning and recorded weekly (18.2 ± 0.4 °C; range 17.3 – 19.0). Oxygen saturation (YSI 85; 94 ± 1.6%; range 91 – 96) and pH (Hanna HI 98128; 7.83 ± 0.06; range 7.70 – 7.96) were measured in-situ in the culture tank. System water samples were analysed for total ammonia nitrogen (TAN; week 1 – 26: < 0.25 mg l^{-1}; weeks 27 – 54: 0.10 ± 0.04; range 0 – 0.17), nitrite (NO_2 < 0.1 mg l^{-1}) and nitrate (NO_3 < 0.25 mg l^{-1}). A side trial indicated no significant

difference between experimental tanks and whole system TAN levels, therefore system samples were analysed weekly. Tests were conducted using Red Sea™ test kits for all parameters. However, by week 27 the procurement of a spectrophotometer (Spectroquant Pharo 100) allowed for more accurate determination of TAN using the Merck™ ammonium reagent set (test 1.14752.0001; range 0.01 – 3.00 mg l^{-1}).

3.2.2 Diets

Five dietary treatments with three replicates were allocated to tanks according to a randomised block design. Dietary treatments consisted of a formulated pelleted diet (FD), three combination diet regimes consisting of the pelleted feed and a fresh macroalgae supplement fed in conjunction (CML – low macroalgae at 25% target ration; CMM – medium macroalgae at 50% target ration and CMH - high macroalgae at 75% target ration), and a fresh macroalgae-only diet (MD).

3.2.2.1 Macroalgal feed

Three species of fresh macroalgae were used, *E. maxima*, *Gracilaria* and *Ulva*. Fresh *E. maxima* was sampled from kelp delivered to HIK Abalone Farm (Hermanus, Western Cape, RSA; 34° 26' 03.3" S 19° 13' 11.0" E), although fresh beach-cast kelp was substituted when high sea events interrupted deliveries. Blades were separated from the stipe, sealed in a plastic bag with a small amount of seawater and packaged in an insulated polystyrene box together with ice packs. This was couriered overnight to Port Alfred with transit time generally in the order of 24 – 28 hours. Upon arrival, the fronds were transferred to a tank of seawater to remove excess slime. Prior to weighing for feeding, fronds were placed on an oyster mesh surface for 10 min to allow excess moisture to drain off. *Ulva* and *Gracilaria* were cultured on site at the PAMRL from seed cultures obtained from Wild Coast Abalone (Haga Haga, Eastern Cape, RSA; 32° 45' 02.7" S 28° 16' 29.0" E). Classification of cultured *Ulva* to species level is difficult owing to the complicated taxonomy of the genus and limited morphological characteristics compounded by the lack of identifiable holdfast tissue in free-floating cultured stocks (Bolton et al., 2009). *Ulva* grown at Wild Coast Abalone has been described as initially being South African *U. rigida* but with more recent samples taken

in 2008 fitting the morphological description of *U. linza* (Bolton et al., 2009; Stegenga et al., 1997). The *Gracilaria* species used was understood to be *G. gracilis* (Amosu et al., 2013).

Based on observations of culture techniques employed at Wild Coast Abalone farm (WCA), *Ulva* and *Gracilaria* were cultured in two structurally different systems. *Ulva* was grown in two shallow D-ended raceway tanks (2000 mm x 1000 mm x 300 mm, 450 L) constructed from plywood and fiberglass (Figure 12). A paddle wheel driven by a geared motor (Bircraft TL 1145, 12 RPM, torque 370 nm, 38 W) circulated the water at a speed of approximately 0.2 m s-1. *Gracilaria* was grown in two rectangular HDPE tanks (1150 mm x 950 mm x 600 mm, Figure 13) as a rolling culture. A further single tank with a rolling culture of *Ulva* was used to provide seed stock to the raceway cultures. The rolling culture tended to fragment the fronds thereby providing seed stock with a high frond density per unit weight for further grow-out in the raceways. Seawater was pumped directly from the Kowie River estuary and trickled into the culture tanks at a rate of 250 l hr^{-1}. Fertilisation of the cultures was undertaken if visually observed paling of the macroalgal tissue occurred, from a baseline healthy colour (*Ulva* – dark green; Gracilaria – deep purple red). Water flow was shut off to the tank for 8 h during daylight hours (*Ulva*) and 16 h during overnight (*Gracilaria*), and limestone ammonia nitrate (LAN; Efecto brand – 28% N) applied. *Gracilaria* was fertilized overnight to reduce nutrient absorption by epiphytes. Cultures were completely replaced with fresh seed stock from WCA in weeks 17, 25, 37 and 50. Prior to weighing for feeding, *Gracilaria* and *Ulva* were collected in PVC baskets from their respective cultures and allowed to drain for 10 min. The material was then placed in a fine mesh cotton bag and compressed at 0.45 kg cm^{-2} until water stopped draining from the bag.

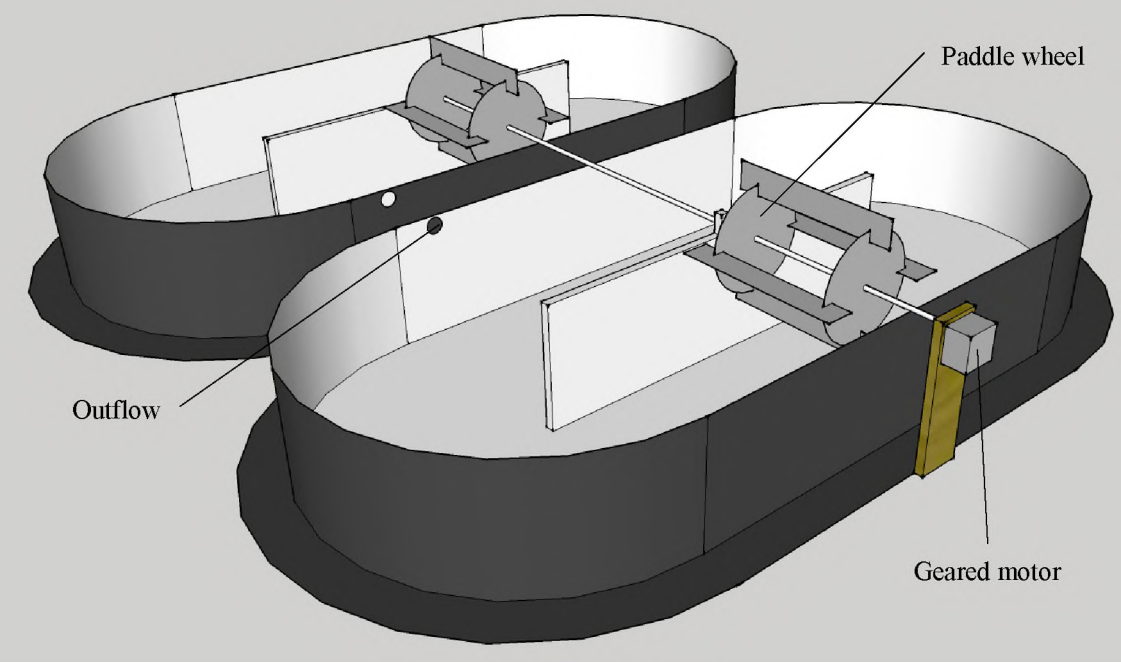


Figure 12. Schematic diagram of paddle-driven, de-ended raceway tanks used to culture *Ulva* at the Port Alfred Marine Research Laboratory.

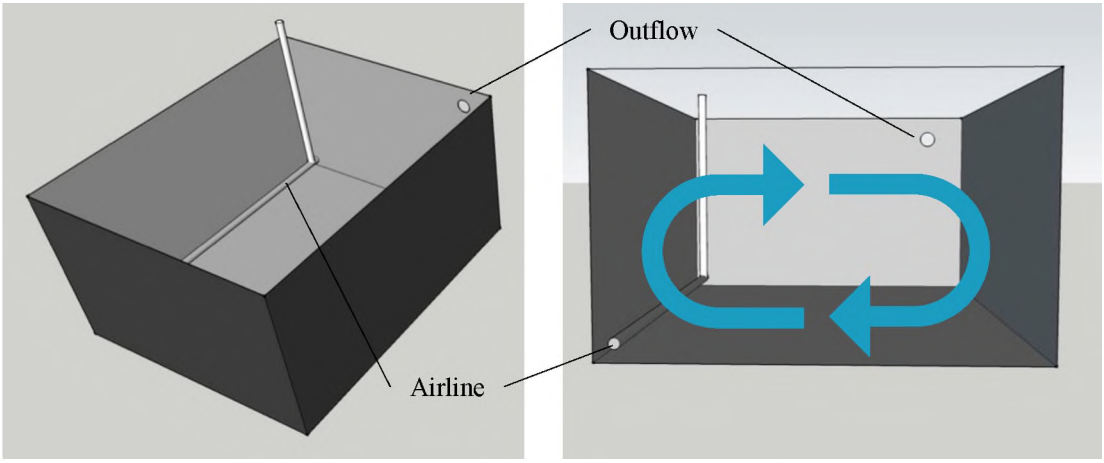


Figure 13. Schematic diagram of LDPE tanks, depicting rolling water motion, used to culture *Gracilaria* at the Port Alfred Marine Research Laboratory. Arrows indicate water circulation.

3.2.2.2 Formulated feed

The pelleted diet was formulated to contain 3% lipid and 34% protein from two sources, steam-dried, formaldehyde-free, mackerel fishmeal (Oceana (Pty) Ltd., South Africa) and soya-oil cake. Starch carbohydrates and a vitamin/mineral mix were included according to a proprietary commercial formulation (Marifeed (Pty) Ltd., South Africa). This feed is sold commercially under the name Abfeed™ S34.

3.2.2.3 Proximate analyses

Samples of the three macroalgal species and the formulated feed were analysed for their proximal composition (Table 7). Macroalgal samples were taken from healthy cultures at the PAMRL (*Ulva* and *Gracilaria*) and freshly shipped *E. maxima*, oven dried at 38 ºC (Scientific Series 9000) and milled prior to analysis. In addition, a sample of each macroalgal species was taken at every feeding event to calculate a dry weight conversion factor (oven dried to a constant weight at 100 ºC) for each macroalgal batch. Furthermore, a weekly sample of *E. maxima* (day 3) as well as *Ulva* and *Gracilaria* (day 5) were oven dried at 40 ºC for 36 hours in open Eppendorf tubes. The tubes were sealed and stored at -20 ºC prior to analysis for nitrogen (N) content using mass spectrometry (Europa Scientific 20-20 IRMS – refer to section 3.2.4.1 Sample preparation, for sample preparation protocol prior to analysis). A conversion factor of 6.25 is commonly applied to tissue N values to estimate tissue protein content (crude protein content (%) = N × 6.25). This assumes that the tissue being analysed is comprised of protein with 16% nitrogen and that there is minimal non-protein nitrogen (NPN) present (Diniz et al., 2011; Lourenço & Barbarino, 2002). However, plant tissues commonly exhibit total protein with less than 16% nitrogen (Yeoh & Wee, 1994) and high levels (> 30%) of NPN have been reported in a variety of macroalgal species (Diniz et al., 2011; Lourenço & Barbarino, 2002). Therefore, use of the factor 6.25 would likely lead to overestimation of the protein content of macroalgae. For the purposes of this study, the average N-Prot factors derived by (Lourenço & Barbarino, 2002) for the taxonomic groupings of red (n = 9, N-Prot 4.59), green (n = 6, N-Prot 5.13) and brown (n = 4, N-Prot 5.38) macroalgae were used to estimate protein content in *Gracilaria*, *Ulva* and *E. maxima* respectively from tissue N values.

Table 7. Nutritional proximate composition (g / 100 g) of the formulated pellet and three species of macroalgae (*E. maxima*, *Gracilaria* and *Ulva*) used in the formulated (FD), macroalgal (MD) and combination diets (CML, CMM & CMH).

	Formulated pellet	*E. maxima*	*Gracilaria*	*Ulva*
Nitrogen	5.4	1.7	3.3	3.2
Crude Protein	33.8	9.1	15.1	16.4
Total Lipids	5.0	0.8	0.7	1.2
Saturated fats	1.8	0.3	0.6	0.6
Mono -Unsaturated fats	1.5	0.2	0.1	0.3
Poly-Unsaturated fats	1.7	0.3	-	0.3
Omega 3 Fatty acids	1.1	0.1	-	0.2
ALA	0.0	0.2	-	-
EPA	0.4	0.0	-	-
DHA	0.6	0.0	-	-
DPA	0.1	0.0	-	-
Omega 6 Fatty acids	0.6	0.2	-	0.0
Moisture	9.7	10.3	3.6	8.4
Ash	7.6	27.2	44.0	27.3
NNE	44.0	52.6	36.6	46.7
Digestible energy (kJ/g) [c]	17.5	11.5	10.1	12.4
Total Dietary Fibre	10.3	44.1	35.1	34.5
Glycaemic carbohydrates	36.5	-	0.6	0.9
Fructose	-	-	-	-
Galactose	-	-	-	-
Glucose	-	-	-	-
Sucrose	1.3	-	-	0.1
Maltose	-	-	-	-
Trehalose	-	-	-	-
Sugar x	0.1	-	-	-
Starch	35.0	-	0.4	0.8

[a] Proximate analyses were conducted using standard methods - Crude protein: Dumas combustion method (N-Prot factor 6.25 for formulated feed, N-Prot factor for macroalgae as per Lourenço & Barbarino (2002) for the taxonomic groupings of red (n = 9, N-Prot 4.59), green (n = 6, N-Prot 5.13) and brown (n = 4, N-Prot 5.38), lipid: AOAC 996.06 (gas chromatography), moisture: AOAC 950.46, and ash: AOAC 923.03 (ashing by furnace), Total dietary fibre: AOAC 991.43, Sugars by gas chromatography (proprietary methodology – Michrochem Laboratory Services, RSA).

[b] Non nitrogenated extract calculated by difference.

[c] Estimated from mean digestible energy values: crude protein: 23.6 kJ g^{-1}, lipid: 39.5 kJ g^{-1}, and carbohydrate (as NNE): 17.2 kJ/g (Bureau et al., 2002).

3.2.3 Experimental procedure

3.2.3.1 Experimental animals

Experimental animals (n = 85 per replicate, 3 replicates per treatment) were collected from general production tanks at WCA. Farm production records indicate that the abalone were spawned in July 2011, settled onto polycarbonate sheets and fed a diet of micro and filamentous algae. Weaning onto formulated feed (Abfeed S34®) and macroalgae (*Ulva* and *Gracilaria*) was initiated in October 2011. Four months later (Feb 2011), abalone were transferred from the hatchery to post-weaning grow-out tanks and grown on a diet of *Ulva*, *Gracilaria* and Abfeed S34®. Experimental animals were separated from production stock on 23 August 2011, placed in sealed LDPE baskets and allowed to purge overnight. The following morning, they were removed from the culture tanks and placed in plastic bags with a sponge floor. The bags were pumped full of oxygen and flushed three times before being sealed with a cable tie and placed in a lidded polystyrene box housing two icepacks. Sponge dividers prevented the icepacks from touching the plastic bag. The container was sealed with packing tape and transported via road (205 km, approximately 3-hrs travelling time) to the PAMRL. Animals were unpacked upon arrival and counted into experimental baskets (n=85). The contents of each packing basket were evenly distributed between all treatments to counter any bias that may have occurred during animal selection on the farm. Animals were acclimated to the experimental system on a diet of *Ulva*, *Gracilaria* and formulated feed (*ad libitum*) for 29 days prior to tagging.

Individual abalone were double-tagged using 2 differently coloured and numbered bee tags (2 mm ∅; www.beeworks.com). Colour combinations were treatment specific (i.e. MD = tag 1 - yellow, tag 2 – blue). Abalone were anaesthetised with a 10% magnesium sulphate solution and excess water removed from the shell surface using a paper towel. A small circle of gel-type cyanoacrylate adhesive (Bostik Blits Stik Gel) was placed at the base of the shell spire on the side of the respiratory pores. Tag 1 was placed closest to the spire using forceps, with Tag 2 along-side in the direction of the respiratory pores. A dissecting needle was used to push the tag firmly onto the shell surface before a final coat of glue was applied over the surface of both tags. Abalone were placed on damp sponge in a partially covered container to prevent desiccation while the glue cured to

touch dry (5 - 10 min). Abalone were then returned to their baskets to recover for 10 days before recording the baseline weight and measurements for the growth experiment.

Weights and measurements (WM) were undertaken on days 1-2, days 123 – 124, days 249-250 and days 386-387. All food was removed 48 hours prior to the WM event to allow animals to purge. Baskets were first placed individually into a 10% magnesium sulphate solution for 10 min. Anaesthetised abalone were transferred to a container lined with damp foam and covered with a sheet of damp foam to minimise desiccation. Batches of abalone (WM 1 and 2: n = 9; WM 3 and 4: n = 4) were removed from the holding container, placed onto sponge and the excess water removed using a paper towel. Tag numbers were recorded before abalone were weighed to 0.01g (Denver Instruments MXX 612) and then transferred to a plastic laminated grid sheet (3 x 3 or 2 x 2) located 40 cm below a horizontally mounted digital camera. Once the grid was full, a digital image was taken and the abalone returned to the water of their experimental basket to recover. The time between being removed from the anaesthetic solution and the last abalone being returned to the water was ~ 25 min.

Length measurements were determined to 0.01 mm from digital photographic images, using calibrated on-screen digital calliper software (Screen Callipers, Iconico Inc., New York). This photographic method was employed to reduce handling and emersion time during WM events. Anecdotal observation suggested that diet may be having an effect on the incidences of shell damage. This hypothesis was investigated using digital images, as they could be used to visually assessed to determine shell damage in individual animals. Shell damage, characterized as missing shell material, was observed to occur in three regions: (1) along the shell edge parallel to the row of developed respiratory pores (Type 1), (2) on the growing edge in the region of new respiratory pore formation (Type 2), and (3) along the growing edge towards the spire (Type 3). All abalone at each WM event were assessed for shell damage and categorized according to type (Figure 14).

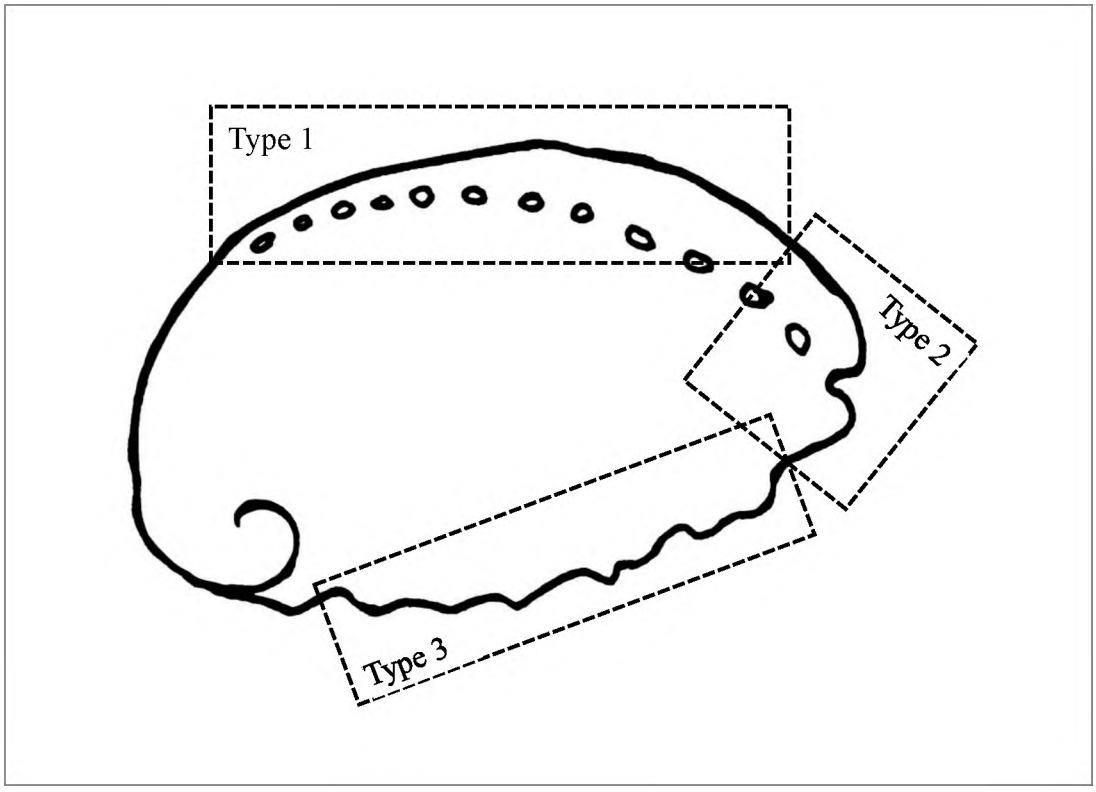


Figure 14. Schematic diagram of the dorsal surface of an abalone shell depicting the location of zoning for shell damage categories. Shell damage is defined as missing shell material along the shell edge parallel to the row of developed respiratory pores (Type 1), on the growing edge in the region of new respiratory pore formation (Type 2) and along the growing edge towards the spire (Type 3).

3.2.3.2 Biological samples

Five abalone were randomly selected at WM events 2, 3 and 4 for biological samples. Abalone were euthanised in 25% $MgS0_4$ solution (20 min soak time) before being shucked while taking care not to damage the viscera. The rear section of the pedal muscle was removed by scalpel and divided to provide muscle material for glycogen content and stable isotope analyses (Figure 15). Tissue for glycogen content analysis was immediately snap-frozen in liquid nitrogen and then stored at -20 ºC. Abductor muscle glycogen content was determined using a chemical assay method adapted from Woodcock & Benkendorff (2008) (Appendix 2). Slivers of muscle tissue for stable isotope analysis were oven dried at 40 ºC for 36 hours in open Eppendorf tubes. The tubes were then sealed and stored at -20 ºC prior to analysis (refer section 3.2.4.1)

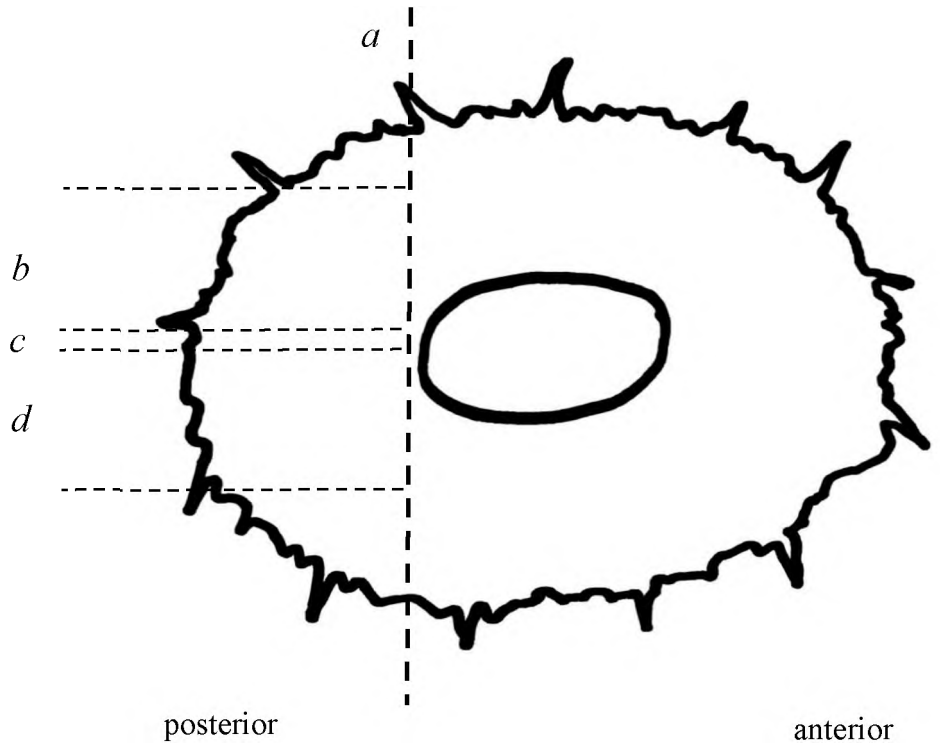


Figure 15. Dorsal view of abalone abductor muscle tissue post shucking (viscera not shown) depicting incision lines (dotted) for biological sample preparation. The initial incision occurred along line *a*, with tissue samples for glycogen analysis coming from regions *b* and *d* and stable isotope samples from zone *c*.

2.3.2 Feeding

There were five dietary treatments (FD, CML, CMM, CMH and MD; Table 8). A dynamic method was employed to calculate macroalgal ration for the combination diets on a weekly basis, anchored by the mean feed consumption data of the replicates of the MD treatment from the previous week. Macroalgae were fed in excess for the MD treatment. Macroalgae ration for the combination diets was calculated as follows:

$$CDr = \frac{((Ni - Mort) \times (0.1531 \times ((Li + (LGR \times Wg)) \div 10)^{3.0899})) \times MDc \times R)) \times 7}{100}$$

Where: *CDr* = Weekly combination diet ration; Ni = number of abalone (n) at previous WM; *Mort* = mortality (n) since previous WM; Li = mean abalone weight at initial WM

event; *LGR* = linear growth rate (mm/week) calculated from previous 4-month growth period (assumed to be 0.5 mm week^{-1} for first 4-month growth period across all treatments); *W* = period of growth since previous WM event (weeks); *MDc* = mean macroalgae consumed by replicates of treatment *MD* (% bw day^{-1}) and *R* = allocated ration for combination diet as proportion (i.e. 75% ration = 0.75).

The allocated weekly macroalgal ration was divided evenly by wet weight between the three macroalgal types. Exceptions occurred in weeks 1 and 29 when kelp was not available (macroalgal ration divided evenly between *Ulva* and *Gracilaria*) and in week 49 when *Gracilaria* was not available (*Ulva* fed at 2/3 weekly ration). The *E. maxima* ration was fed in its entirety on day three, while the *Ulva* and *Gracilaria* ration was fed proportional to the number of days that macroalgae would be in the basket before the addition of fresh macroalgae (day 1 – 2/5 weekly ration; day 5 – 3/5 weekly ration). Logistical constraints, including sea condition, public holidays and public service strikes, meant that in some weeks (11, 12, 17, 20, 21, 23, 32, 38 and 39) kelp only entered the tank on day 4 or 5. Day 5 *Ulva* and *Gracilaria* rations were fed early (day 3) in these weeks if kelp was only expected to be available on day 5. Uneaten macroalgae was collected at the end of each week and prepared for weighing as fresh macroalgae for feeding. Uneaten macroalgae from each replicate were manually separated into their constituent groups (*Ulva, Gracilaria* and *E. maxima*) and weighed. Formulated feed was scattered evenly onto the feeding plates in the FD, CML, CMM and CMH treatment baskets daily between 16h00 and 17h00. Uneaten formulated feed was collected by hand before 09H00 on the following day from the feeder plate surface, pooled for each week and stored at -20 °C until oven dried to a constant mass at 100 °C. Daily mortalities were recorded when the feeder plate could be lifted for observation of the basket floor after removal of uneaten formulated feed.

Table 8. Feeding regimes for five dietary treatments consisting of a formulated pellet diet (FD), three combination diet regimes (CML, CMM and CMH) and a fresh macroalgal diet (MD). Days referred to in this table relate to the revolving 7-day feeding cycle employed in the experiment

	FD	CML	CMM	CMH	MD
Formulated feed					
Pellets into baskets	days 1-7	days 1-7	days 1-7	days 1-7	n/a
Uneaten pellets removed	days 1-7	days 1-7	days 1-7	days 1-7	n/a
Fresh macroalgae					
Macroalgae ration [a]	0	25%	50%	75%	100%
Ulva / *Gracilaria* fed	n/a	days 1 & 5	days 1 & 5	days 1 & 5	days 1 & 5
E. maxima fed	n/a	day 3	day 3	day 3	day 3
Uneaten macroalgae removed	n/a	days 1	days 1	days 1	days 1

[a] Fresh seaweed ration was calculated weekly as a percentage of body weight based on the basket weight at the previous WM and adjusted weekly for expected growth and recorded mortality. A full ration (100%) was calculated weekly based on the mean feed consumption (% bw day^{-1}) of the MD treatment in the previous week.

Specific growth rate (SGR), linear shell growth rate (LGR), proportional feed intake (PFI), food conversion ratio (FCR), condition factor (CF), protein efficiency ratio (PER) and protein efficiency ratio of the formulated component (PER^f) were calculated as follows:

(1) $SGR = ((\ln(Wf)) - (\ln(Wi) \div t) \times 100$

(2) LGR = (final shell length in mm ÷ initial shell length in mm) ÷ time in days

(3) PFI = [(wet weight dietary component consumed × dry weight conversion factor) ÷ total dry weight of all feed consumed)] × 100

(4) FCR = grams dry feed consumed ÷ grams wet weight gained

(5) $CF = 5575 \times (\text{abalone weight in grams} \div \text{shell length in mm}^{2.99})$ (Britz, 1996a)

(6) PER = grams wet weight gained ÷ grams dry protein consumed [a]

(7) $PER^f = ((Bm^f - Bm^i) - ((M^d \times P^m) \times PER^m)) \div (FP^d \times P^f)$

Where: t = time (days); W_i = initial abalone mass (g); W_f = final abalone mass (g); PERf = Protein efficiency ratio (formulated component); Bm^i = initial basket mass (g); Bm^f = final basket mass (g); M^d = macroalgae consumed (g; dry weight); P^m = Proportional crude protein content of macroalgae; PER^m = Protein efficiency ratio of macroalgae calculated from macroalgae-only diet; FP^d = Formulated pellet consumed (g; dry weight); P^f = Proportional crude protein content of formulated diet.

3.2.4 Stable isotopes

3.2.4.1 Sample preparation

Macroalgae and abalone abductor muscle tissues were analysed for stable isotope ($\delta^{13}C$ and $\delta^{15}N$) and elemental composition (% C and % N) using a Europa Scientific 20-20 IRMS linked to an ANCA SL Elemental Analyser following preparation as follows. Oven dried abductor muscle tissue samples (refer section 3.2.3.2) were ground to a fine homogenous powder in 2 ml round-bottom Eppendorf tubes (Retsch™ Mixer Mill). Samples were weighed to the nearest 0.01 mg (Sartorius™ analytical balance) into a tin capsule which was crimped and folded into a small ball for loading into the auto-sampler. Target sample weight was 1 mg for abalone and *Gracilaria* tissue and 2 mg for *Ulva* and *E. maxima* tissue.

3.2.4.2 Analysis using isotopic mixing models

The proportional contribution of dietary sources to abalone abductor muscle tissue was evaluated using the Bayesian mixing model implemented in the software package SIAR (Stable Isotope Analysis in R) (Parnell et al., 2010). The model requires inputs in the form of: (1) stable isotope concentration values (and variation) from all feed sources (*Ulva, Gracilaria, E. maxima* and formulated feed) and the consumer (abalone), (2) concentration values of N and C in the feed sources, and (3) trophic enrichment factors of C and N in the consumer. Values for $\delta^{13}C$ and $\delta^{15}N$ used for macroalgae were the mean and SD of all samples collected weekly over the 386-day growth period. The formulated feed used in the trial was from a single batch: therefore, $\delta^{13}C$ and $\delta^{15}N$ values for the feed and its constituent ingredients were as analysed from a single sample

of each (200g homogenized) and an absolute value. Concentration values for dietary sources were estimated from samples as above. Significant variations in $\delta^{15}N$ of abductor muscle tissue over time were observed for animals in treatments FD, CML & CMM (Table 12). This indicated that these treatments were still moving towards an equilibrium state of diet-tissue fractionation. Therefore, only final (day 386) values for $\delta^{13}C$ and $\delta^{15}N$ were used in the SIAR model to give the most likely estimate for abductor muscle tissue in an equilibrium state. In the absence of laboratory defined figures, discrimination factors for each dietary component were calculated according to the diet dependent discrimination factor (DDDF) approach as described by Caut et al. (2008, 2009). In short, within taxonomic groups, the isotopic signature of the dietary component has been shown to be significantly correlated to the resulting discrimination factors reported. Specific DDDFs could, therefore, be calculated for each dietary component in the current study using the regression equations for invertebrates as reported in Caut et al. (2009). The SIAR model was instructed to run 500 000 iterations (50 000 initial discard) and the raw output data extracted to Excel. The model-estimated contribution of dietary components to abalone abductor muscle tissue deposition was depicted as box plots (box limits on 25th and 75th percentile, bars represent range of 90% credibility).

3.2.5 Simulated canning

A simulated canning trial was conducted 10 days following the final WM event of the growth trial on day 396. On day 394, ten abalone were randomly selected from each experimental basket and placed into submerged LDPE baskets to purge. The following day abalone were packed in polystyrene boxes for transport (refer section 3.2.3.1 Experimental animals) and driven to SPP Canning Pty. Ltd. (Hermanus, Western Cape, RSA; 821 km; ~ 11 hours travel time). Upon arrival, the baskets were unpacked and submerged in a raceway tank with continuous seawater flow overnight. On day 396, whole abalone were patted dry using paper towel, individually weighed to 0.01 g and shucked. The abductor muscle (hereafter referred to as 'meat'), viscera and shell were separated and weighed individually. The ten abalone per replicate were then combined and prepared for canning which involved salting, scrubbing to remove pigment, a period in refrigeration, notching of the mouthparts and soaking in freshwater according to a proprietary method. Abalone meats were patted dry and weighed after each stage of the

preparation process. The prepared meat from each replicate was then placed in a standard can and filled with freshwater. Can lids were placed on (not sealed) and the cans then passed through a steam box for 16 min raising the internal temperature to ~ 62 ºC. Upon exiting the steam box, the lids were sealed, and the cans placed in a retort to cook at 114 ºC for 45 min.

Meat mass was recorded following preparation (can in), after canning following draining (commercial yield) and following oven drying at 100 ºC to a constant mass (dry yield). Loss values were calculated as the percentage loss between two stages of preparation as follows:

1. Meat processing loss = (can-in meat mass ÷ meat mass post shucking) × 100
2. Canning loss = (can-out meat mass ÷ can-in meat mass) × 100
3. Water content = (meat mass post drying ÷ can-out meat mass) × 100

Yield values were calculated as the percentage of starting whole abalone wet mass as follows:

1. Can-in yield = (can-in meat mass ÷ whole abalone wet mass) × 100
2. Commercial yield = (can-out meat mass ÷ whole abalone wet mass) × 100
3. Dry yield = (dry meat mass ÷ whole abalone wet mass) × 100

To provide a reference point for comparison, 3 replicates of 10 abalone each (43.1 ± 4.1 g abalone^{-1}) were sourced from commercially produced abalone fed on formulated feed (Abfeed ® S34) in outdoor raceways (HIK Abalone Farm, Hermanus, RSA) and processed and canned together with abalone from the experimental dietary treatments.

3.2.6 Statistical analysis

Specific growth rate (SGR, % BW day^{-1}) values per replicate were determined from the slopes of natural log-transformed weight data plotted against time x 100. Mean log-transformed weight data from each replicate were then plotted for each treatment and the slopes of the single regression models per treatment assessed for significant

differences using a homogeneity-of-slopes model ($P < 0.05$). Replicate mean data for initial and final abalone weight and length, SGR, mortality, FCR, PER, final CF, all canning trial data and stable isotope values (C & N) were tested for homogeneity of variance and normal distribution of residuals using Levene's test and the Shapiro–Wilk W test, respectively. When these assumptions were met, parametric one- way ANOVA and Tukey's HSD *post hoc* tests were employed to test for differences in dependent variable means between dietary treatments or between growth periods within dietary treatments (significance level $P < 0.05$). A non-parametric Kruskal–Wallis ANOVA by ranks test ($P < 0.05$) was used when variances were heterogeneous. Linear regression modelling was employed to define the relationship between variables. All statistical tests were performed using Statistica 12.0 for Windows (StatSoft, Inc., 2013).

3.3 Results

3.3.1 Feed consumption and composition

Total dry feed consumption increased as the contribution of macroalgae increased in the diet, with all treatments significantly different from each other except for CMH and MD that were not significantly different from each other ($F_{4,10} = 112.14$, $P < 0.001$). The intake of feed in abalone fed exclusively on macroalgae (MD) was three-fold that of those fed exclusively on formulated feed (FD) (Table 9) The proportional feed intake ratio on a dry weight basis of formulated feed to macroalgae for combination diets CML, CMM and CMH was 56:44, 32:68 and 15:85 respectively. The intake of macroalgal type within the macroalgal component of the diet was relatively even across diets MD, CMH, CMM and CML, with proportional consumption of *E. maxima*, *Ulva* and *Gracilaria* in the range of 0.36 – 0.43, 0.35 – 0.38 and 0.21 – 0.25 respectively (Table 9, Figure 16).

Table 9. Feed intake and nutritional utilisation indices for juvenile *H. midae* reared for 386 days on a formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CML, CMM & CMH).

	Diet				
	FD	CML	CMM	CMH	MD
Total dry feed intake (g basket^{-1})	1317 ± 37^{a}	2106 ± 41^{b}	2806 ± 104^{c}	3669 ± 172^{d}	3889 ± 344^{d}
Proportional feed intake (%)					
Formulated pellet	100	56.3 ± 1.5	31.5 ± 1.0	14.5 ± 1.8	
Fresh macroalgae as:		43.7 ± 3.9	68.5 ± 1.0	85.5 ± 1.8	100
E. maxima		16.0 ± 1.1	24.7 ± 0.3	31.1 ± 0.6	43.1 ± 1.5
Ulva		16.8 ± 0.4	26.8 ± 0.3	33.3 ± 0.7	35.3 ± 0.8
Gracilaria		10.9 ± 0.4	17.1 ± 0.4	21.1 ± 0.5	21.6 ± 0.7
FCR	1.23 ± 0.15^{ab}	1.02 ± 0.04^{bc}	1.06 ± 0.01^{bc}	1.20 ± 0.02^{abc}	1.4 ± 0.08^{a}
PER (Total protein)	2.42 ± 0.28^{a}	3.6 ± 0.09^{b}	3.99 ± 0.04^{b}	3.98 ± 0.11^{b}	4.04 ± 0.22^{b}
PER^{f} (Formulated diet protein component)	2.42 ± 0.28^{a}	3.41 ± 0.14^{b}	3.94 ± 0.1^{b}	3.8 ± 0.42^{b}	

Values reported are the treatment means of three replicates and standard deviation. Values with the same superscript character in each row are not significantly different ($P < 0.05$)

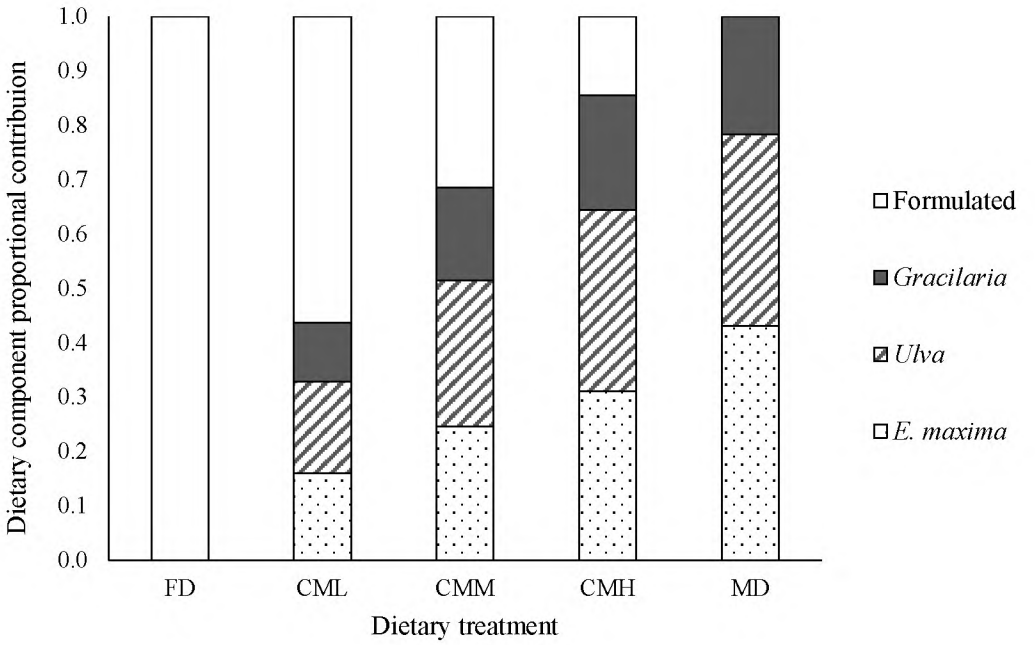


Figure 16. Proportional contribution (dry weight) of dietary components derived from feed consumption data in five dietary treatments consisting of a formulated pellet diet (FD), three combination diet regimes (CML, CMM and CMH) and a fresh macroalgae diet (MD) fed to the abalone over a 386-day growth trial.

Proximate analyses of representative samples of dietary components showed distinct differences between formulated feed and macroalgae (Table 7). Formulated feed could be characterised as a high protein (34%) feed with high levels of glycaemic carbohydrates (36.5%), mainly in the form of starch (35%), and low levels of dietary fibre (10%), ash (7%) and lipids (5%). By contrast, the macroalgae were characterized by low protein (9 – 16%), glycaemic carbohydrate (0 – 0.9%) and lipid (0.8 – 1.2%) levels, but high levels of dietary fibre and (35 – 44%) and ash (27 – 44%) (Figure 17). Although the proximate analysis results described above provided a generalized snapshot of macroalgal composition, the values can fluctuate based on the nutritional state of the organism. Macroalgal nitrogen levels and, by proxy, protein content varied noticeably over the 386-day culture period, particularly for the cultured species *Ulva* and *Gracilaria* (Figure 18). Mean ± SD and range values for protein composition (% dry weight) for *E. maxima, Ulva* and *Gracilaria* were 8.4 ± 1.8 (5.2 – 15.8), 18.3 ± 3.9 (11.3 – 28.4) and 18.9 ± 4.8 (9.0 – 30.0).

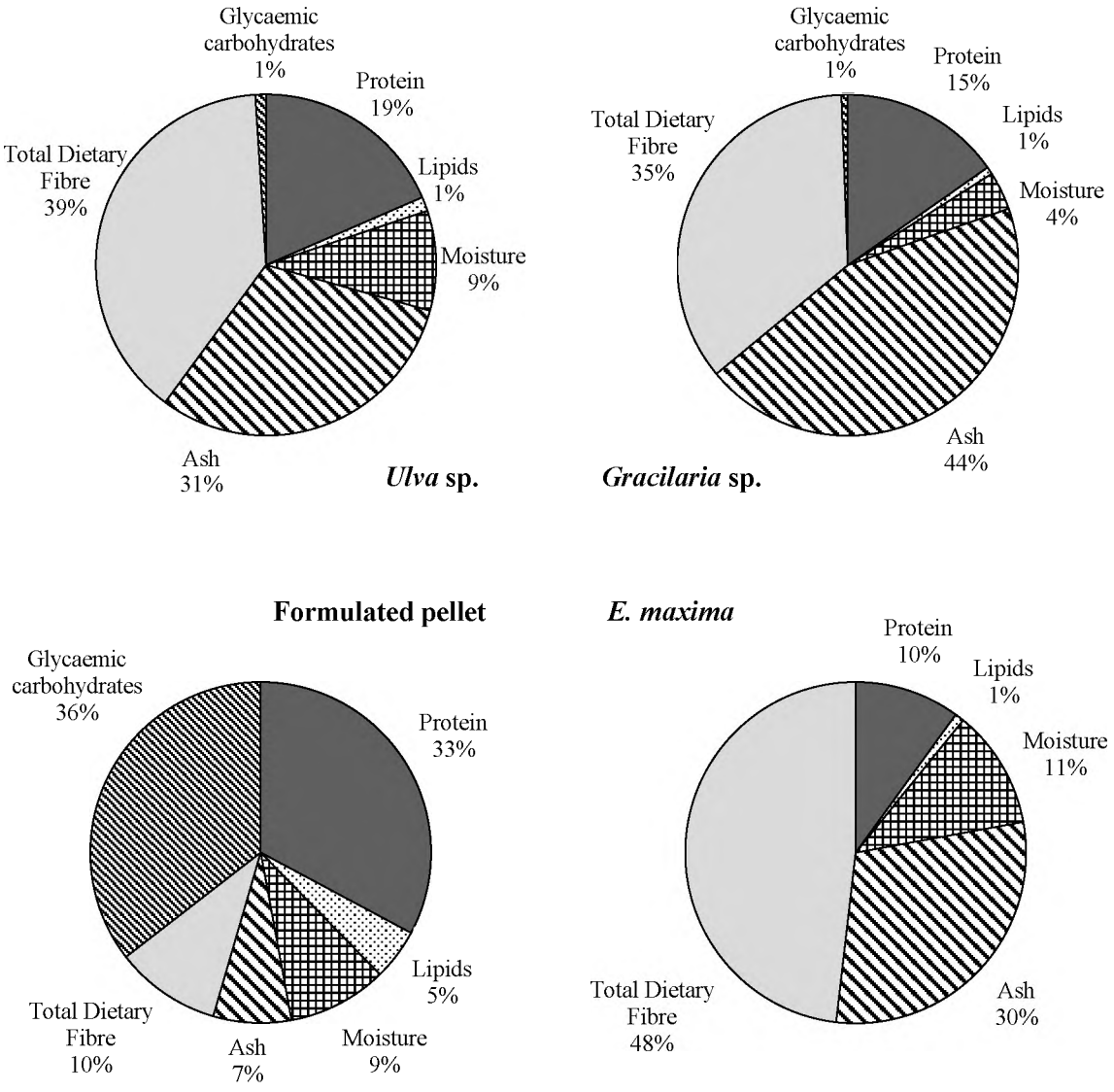


Figure 17. Proportional contribution of major nutritional groups based on proximate analyses conducted on three species of macroalgae (*E. maxima*, *Gracilaria* and *Ulva*) and a formulated pellet (refer Table 7).

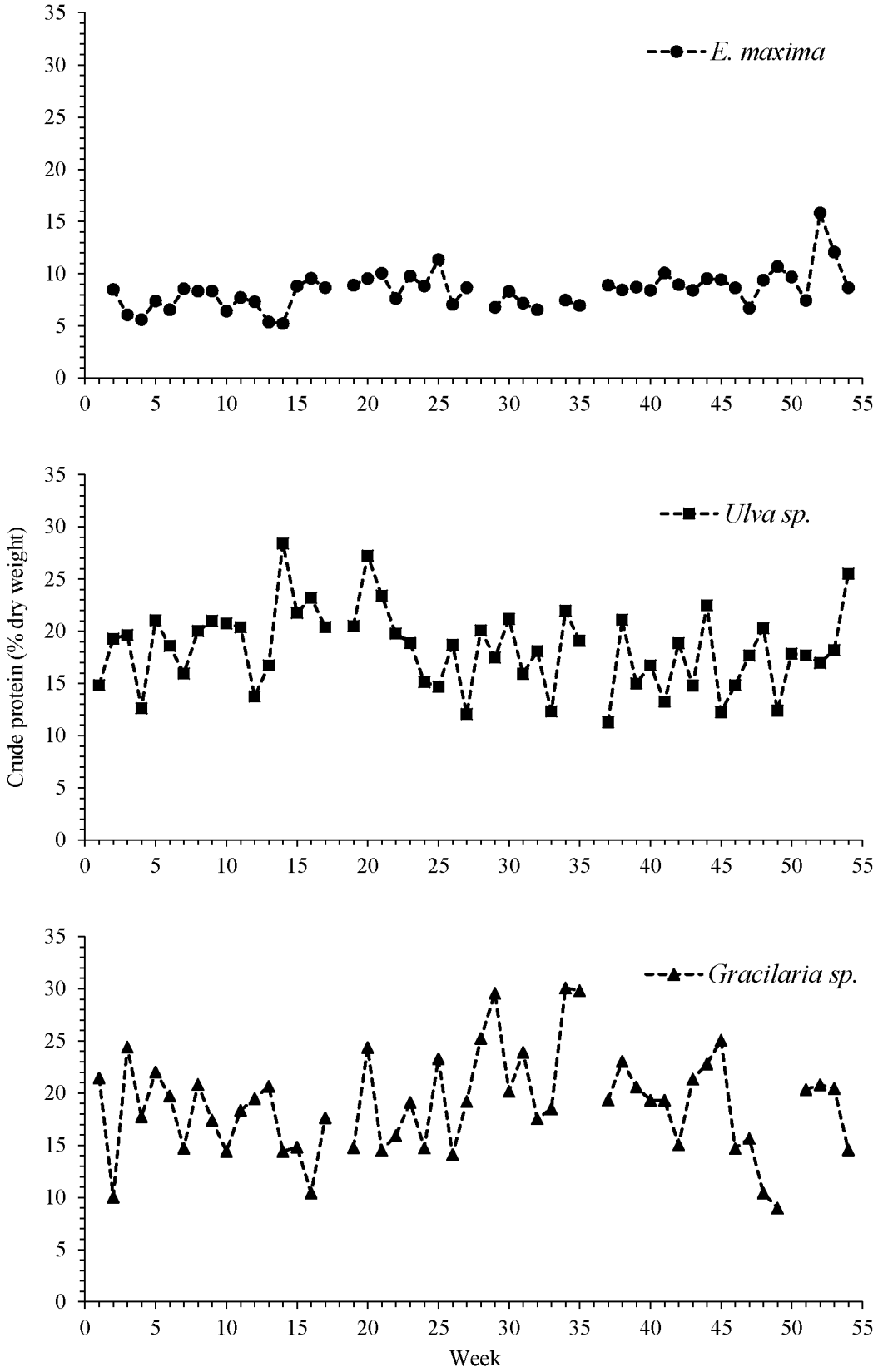


Figure 18. Crude protein content (% dry weight) of three species of macroalgae either harvested in the Hermanus region (*E. maxima*; Southern Cape, RSA) or cultured at the Port Alfred Marine Research Laboratory (*Ulva* and *Gracilaria*; Eastern Cape, RSA) over a 54-week period from Oct 2011 – Oct 2012. Samples were not collected for weeks 18 and 36 during WM events.

3.3.2 Shell condition

A significant positive linear relationship was evident between the proportion of shell damage and proportional contribution of macroalgae to the diet (Figure 24). Significant positive relationships were also observed between the proportion of shell damage and both SGR and LGR (Figure 24). Instances of shell damage (proportion of individuals in a culture basket) for all treatments generally increased over time during the growth period, peaking at day 249 before declining to the final observation on day 386. Damage occurred predominantly on the growing edge in the region of new respiratory pore formation (type 2) (Figure 19 – Figure 23). At 386 days, damage occurred in 45 – 55% of individuals in the CMM, CMH and MD dietary treatments, 12% in CML and only 4% in FD. The capacity for shell regeneration was evident given that the proportion of animals with no shell damage at day 386, which had previously experienced damage ranged from 17.6 – 32.6%. Individual growth rates (SGR and LGR) in diets CMM, CMH and MD were significantly lower for animals who exhibited shell damage at day 386 compared to those with no shell damage, either previously recovered or with no history of shell damage (Table 10).

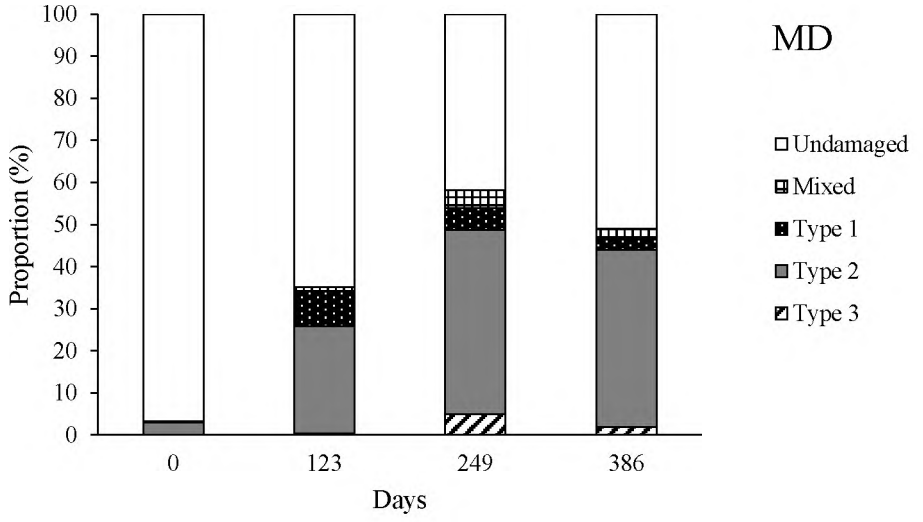


Figure 19. Shell condition of abalone at 0, 123, 249 and 386 days fed a mixed macroalgal (MD).

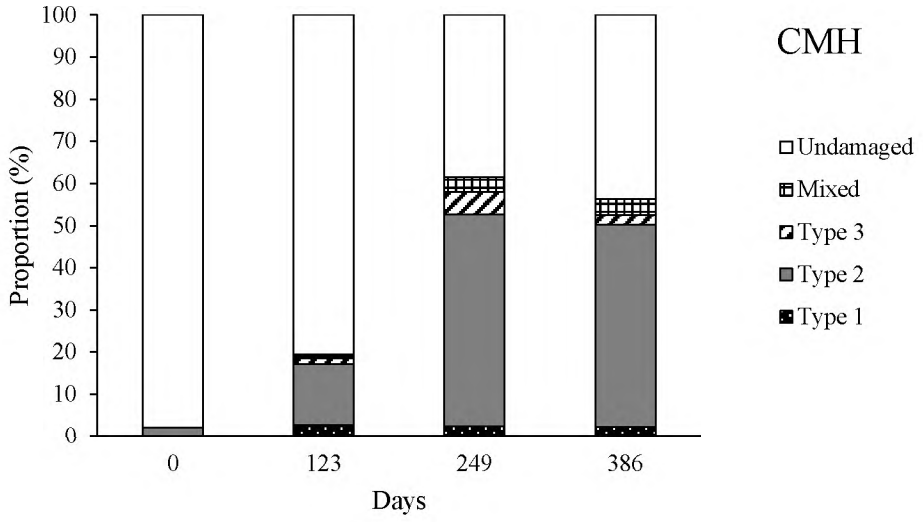


Figure 20. Shell condition of abalone at 0, 123, 249 and 386 days fed a high (85.5%) macroalgae / formulated feed combination diet (CMH).

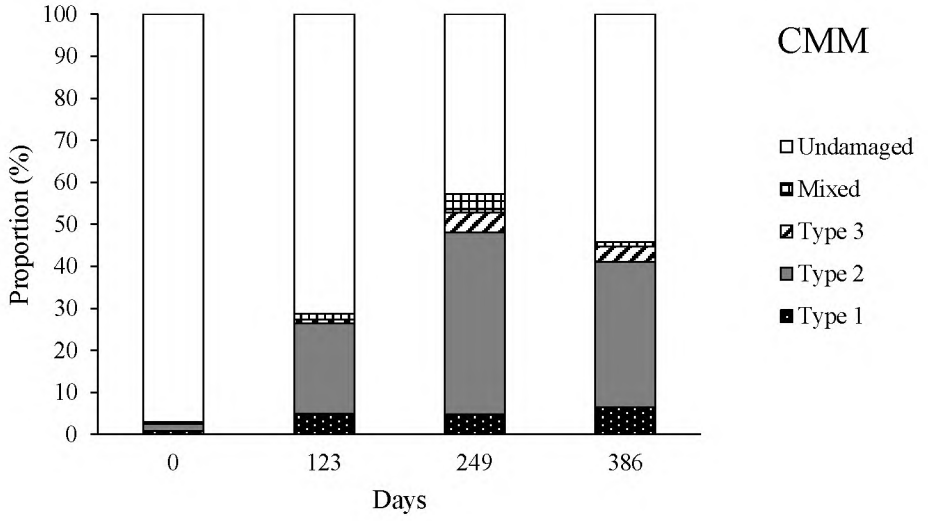


Figure 21. Shell condition of abalone at 0, 123, 249 and 386 days fed a medium (68.5%) macroalgae / formulated feed combination diet (CMM).

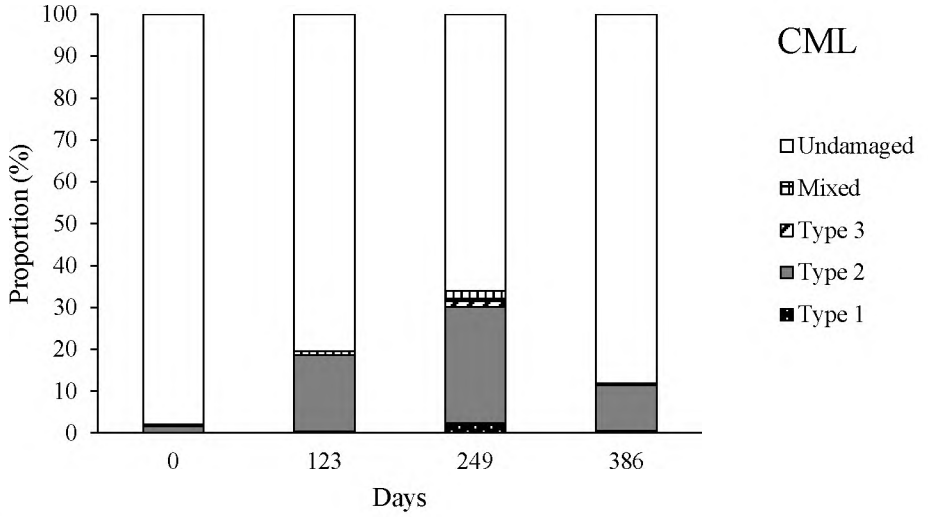


Figure 22. Shell condition of abalone at 0, 123, 249 and 386 days fed a low (43.7%) macroalgae / formulated feed combination diet (CML).

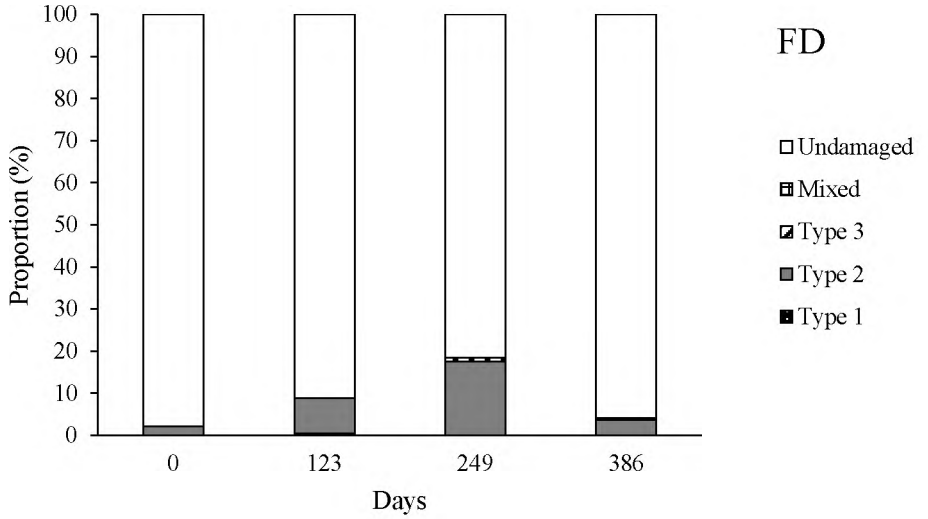


Figure 23. Shell condition of abalone at 0, 123, 249 and 386 days fed a formulated diet (FD).

Table 10. Shell condition at 386 days and shell condition related growth rates (SGR and LGR) of abalone fed five dietary treatments consisting of a formulated pellet (FD), three combination diet regimes (CML, CMM and CMH) and a fresh macroalgae diet (MD).

	FD	CML	CMM	CMH	MD
Shell condition (at 386 days)[1]					
Shell damage present (%)	4.1 ± 2.3	12 ± 8.4	45.8 ± 5.1	56.3 ± 6.2	49.1 ± 7.7
Recovered shell damage (%)	23.4 ± 4.5	32.6 ± 10.6	26 ± 7.4	18 ± 2.8	17.6 ± 11.1
No previous shell damage (%)	72.4 ± 5.5	55.4 ± 15.7	28.2 ± 3.1	25.7 ± 3.4	33.3 ± 11.9
SGR (% bw.day^{-1})[2]					
Shell damage present	0.29 ± 0.03 ^{a}	0.37 ± 0.02 ^{a}	0.43 ± 0.01 ^{a}	0.46 ± 0.01 ^{a}	0.45 ± 0.00 ^{a}
Shell damage absent	0.28 ± 0.02^{a}	0.40 ± 0.02^{a}	0.47 ± 0.01^{b}	0.51 ± 0.01^{b}	0.51 ± 0.01^{b}
Recovered shell damage	0.27 ± 0.04 ^{a}	0.39 ± 0.01 ^{a}	0.46 ± 0.02 ^{b}	0.50 ± 0.01 ^{b}	0.49 ± 0.02 ^{b}
No previous shell damage	0.29 ± 0.02 ^{a}	0.40 ± 0.02 ^{a}	0.48 ± 0.01 ^{b}	0.52 ± 0.01 ^{b}	0.51 ± 0.01 ^{b}
LGR (µm day^{-1})[3]					
Shell damage present	45.0 ± 3.9^{a}	58.8 ± 9.2^{a}	64.4 ± 3.50^{a}	68.8 ± 1.4^{a}	63.8 ± 1.0^{a}
Shell damage absent	47.6 ± 3.3^{a}	69.6 ± 0.1^{a}	81.3 ± 4.4^{b}	90.6 ± 5.3^{b}	85.5 ± 2.9^{bc}
Recovered shell damage	43.8 ± 5.8^{a}	66.3 ± 2.3^{a}	79.2 ± 5.7^{b}	85.5 ± 6.5^{b}	78.9 ± 4.0 ^{b}
No previous shell damage	48.7 ± 2.9^{a}	71.9 ± 2.7^{b}	83.0 ± 3.5^{b}	94.3 ± 4.3^{b}	88.5 ± 1.8^{c}

Different superscript letters in the same column and group denotes significant differences ($P < 0.05$). [1]Shell condition of abalone present at day 386; [2]SGR [ln W^{f} – ln W^{i} ÷ time in days x 100] was determined from individual abalone with a traceable history (no tag distortion) from day 1 – 386; [3]LGR [L(mm) ^{f} – L (mm)^{i} ÷ time in days] was determined from individual abalone as for SGR. All values presented are the mean and SD of replicate means.

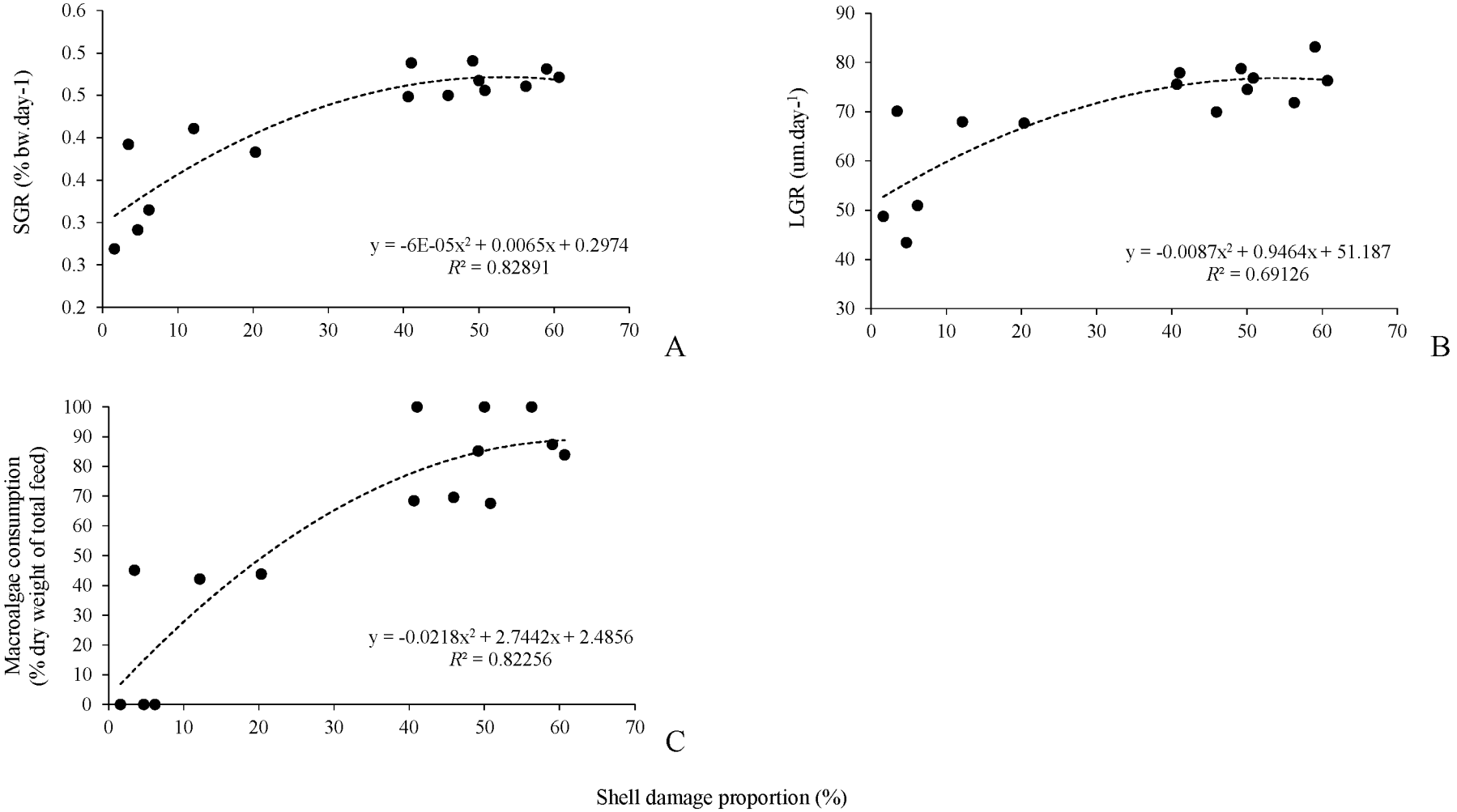


Figure 24. Polynomial regressions of abalone specific growth rate (A; SGR; % BW day^{-1}; y = -6E-05x2 + 0.0065x + 0.2974; $R^2 = 0.83$, $P < 0.001$), abalone linear growth rate (B; LGR; um day^{-1}; y = -0.0087x2 + 0.9464x + 51.187; $R^2 = 0.69$, $P < 0.001$) and the proportion of macroalgae consumed (C; % dry weight of total feed consumed; y = -6E-05x2 + 0.0065x + 0.2974; R1`^{2} = 0.83, $P < 0.001$) against abalone shell damage (% of animals in basket) at the end of a 386 day growth period.

3.3.3 Growth & nutrition indices

Abalone weight increased significantly for all treatments over the 386-day period of the growth trial with growth rates described by the linear equations as follows: (FD y = 0.0385x + 7.0468; $R^2 = 0.96$, $P < 0.05$; (CML) y = 0.0734x + 7.5374; $R^2 = 0.99$, $P < 0.05$; (CMM) y = 0.092x + 7.0154; $R^2 = 0.99$, $P < 0.05$; (CMH) y = 0.1074x + 6.9753; $R^2 = 0.99$, $P < 0.05$ and (MD) y = 0.1061x + 6.5818; $R^2 = 0.99$, $P < 0.05$ (Figure 25). The homogeneity-of-slopes model showed significant differences between treatments in the slopes of natural log transformed weight data plotted against time ($F_4 = 6.015$, $P < 0.001$). In addition, there was a statistically significant difference between treatments in terms of all growth parameters measured in the experiment including final abalone length ($F_{4,10} = 109.77$, $P < 0.0001$), final abalone weight ($F_{4,10} = 164.67$, $P < 0.0001$), SGR ($F_{4,10} = 88.84$, $P < .0001$), LGR ($F_{4,10} = 45.23$, $P < 0.0001$) and CF ($F_{4,10} = 9.27$, $P = 0.002$) as determined by one-way ANOVA (Table 11).

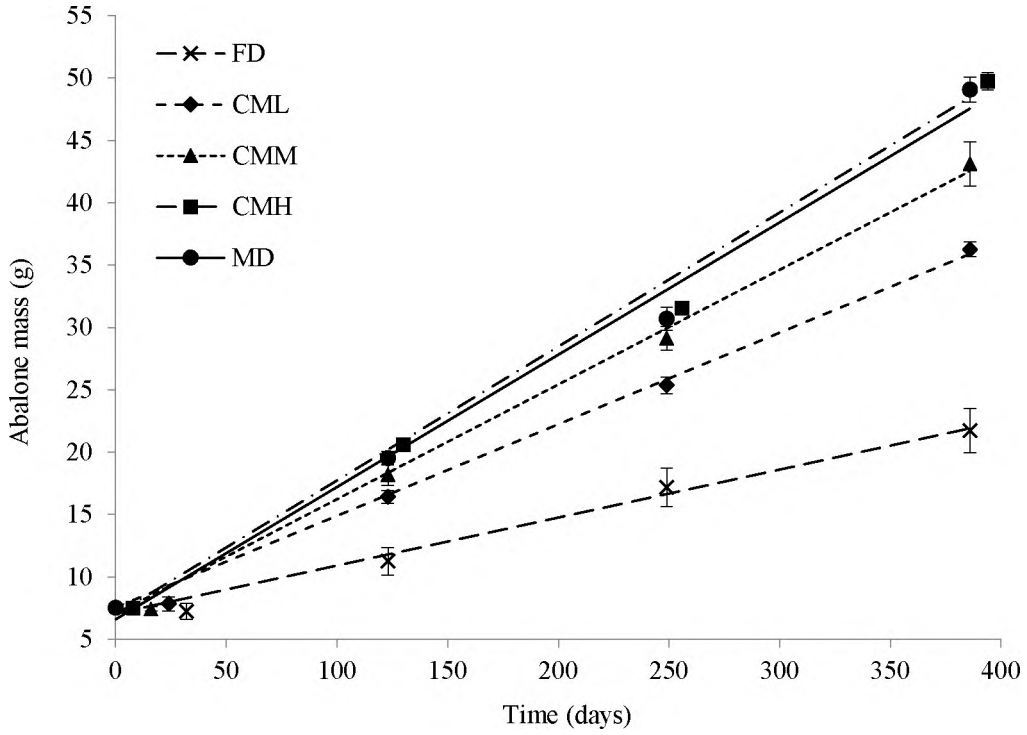


Figure 25. Linear regressions of mean weight data per replicate of juvenile *H. midae* reared for 386 days on a formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CMH, CMM & CML). Means of weight data for each treatment at each measurement interval are presented. Error bars indicate standard deviation.

Table 11. Growth, condition and survival parameters for juvenile *H. midae* reared for 386 days on a formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CML, CMM & CMH).

	Diet				
	FD	CML	CMM	CMH	MD
Initial weight (g)	7.43 ± 0.67 [a]	8.06 ± 0.57 [a]	7.60 ± 0.20 [a]	7.73 ± 0.34 [a]	7.69 ± 0.13 [a]
Initial length (mm)	37.20 ± 1.96 [a]	37.45 ± 0.33 [a]	37.24 ± 0.66 [a]	37.91 ± 0.81 [a]	38.09 ± 0.46 [a]
Final weight (g)	22.6 ± 1.75 [a]	37.23 ± 0.64 [b]	44.37 ± 1.66 [c]	51.64 ± 0.76 [d]	49.23 ± 2.37 [d]
Final length (mm)	55.62 ± 0.95 [a]	63.92 ± 0.81 [b]	65.85 ± 0.78 [bc]	68.55 ± 0.53 [d]	66.94 ± 1.03 [cd]
SGR (% bw day^{-1})	0.29 ± 0.02 [a]	0.40 ± 0.01 [b]	0.45 ± 0.00 [c]	0.48 ± 0.01 [c]	0.47 ± 0.01 [c]
LGR (μm day^{-1})	47.7 ± 3.9 [a]	68.6 ± 1.3 [b]	74.1 ± 3.7 [bc]	79.4 ± 3.5 [c]	74.7 ± 3.0 [bc]
Final CF	0.76 ± 0.09 [a]	0.83 ± 0.03 [ab]	0.90 ± 0.01 [bc]	0.93 ± 0.03 [bc]	0.95 ± 0.02 [c]
Mortality (%)	1.3 ± 1.3[ab]	0.4 ± 0.7 [b]	0.4 ± 0.7 [b]	0.4 ± 0.7 [b]	7.9 ± 5.6 [a]

Values reported are treatment means of three replicates and standard deviation. Values with the same superscript character in each row are not significantly different ($P < 0.05$)

A general trend of a significant positive relationship was evident between all growth parameters measured and macroalgal representation in the diet, with a plateau at medium to high macroalgal inclusion treatments (Figure 26 - Figure 28). The Tukey's HSD test revealed that final abalone weight increased significantly with increasing macroalgal representation in the diet until diet CMH, which was not significantly different from MD. Final abalone length showed a similar trend, with high macroalgal diets MD and CMH significantly different from the low macroalgal diets FD and CML, which were significantly different from each other. Diet CMM bridged these two groups as it was not significantly different from either diet CML or MD. SGR increased significantly with macroalgal consumption up to CMM, after which there was no significant difference between treatments CMM, CMH and MD. LGR was significantly higher for diet CMH compared to diets FD and CML, which were significantly different from each other. Diets CMM and MD were not significantly different from either CML or CMH. Final CF was significantly higher for diet MD compared to diet FD, while combination diets were not significantly different from each other. Mortality rates were generally low for combination diets (< 1%) although significantly higher (7.9 %, $F_{4,10} = 4.63$, $P = 0.023$) for diet MD.

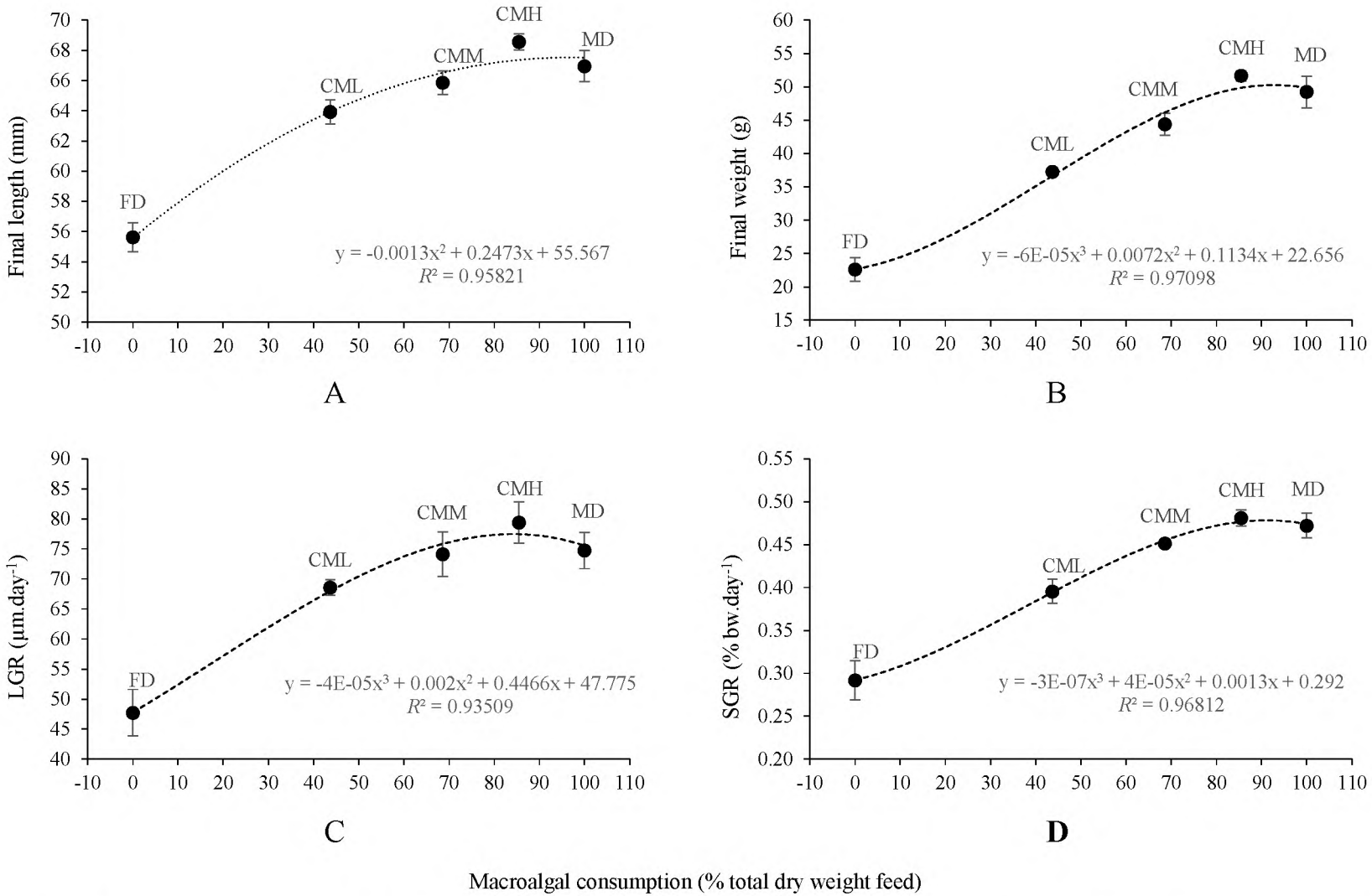


Figure 26. Polynomial regression of final abalone length (A; mm), final abalone weight (B, g), linear growth rate (C; µm day^{-1}) and specific growth rate (D; % bw day^{-1}) against macroalgae consumption (% total dry weight feed ingested) for abalone fed a formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CMH, CMM & CML). Values plotted are means of replicate means and SD.

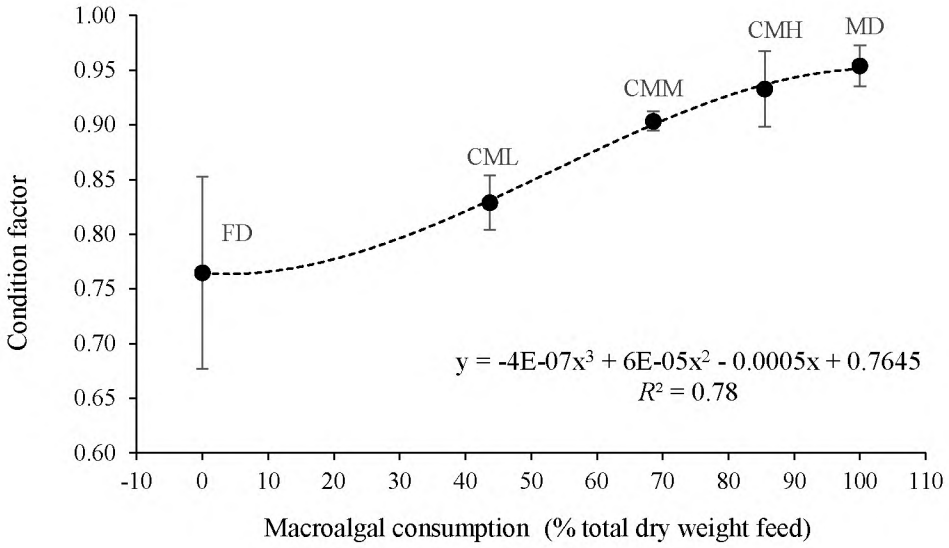


Figure 27. Polynomial regression (y = -4E-07x3 + 6E-05x2 - 0.0005x + 0.7645, $R^2 = 0.78$; $P < 0.001$) of abalone condition factor (CF) and macroalgae consumption (% total dry weight feed ingested) for abalone fed a formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CMH, CMM & CML). Values plotted are means of replicate means and SD.

Nutritional indices also exhibited significant differences between dietary treatment (Table 9). FCR was significantly lower for combination diets CML and CMM compared to diet MD, although there were no significant differences between diets FD and all three combination diets ($H_{4,15} = 12.17$, $P = 0.016$). There was a significant positive relationship between the proportional intake of macroalgae in the diet and both PER (Figure 28) and PER^f (Figure 29). PER values ranged from 2.42 ± 0.28 for diet FD to 4.04 ± 0.22 for diet MD, with diet FD significantly less than the combination (CML, CMM and CMH) and macroalgal (MD) diets ($F_{4,10} = 48.74$, $P < 0.001$). PER^f exhibited a similar trend, with significantly improved protein efficiency in the combination diets (CML, CMM and CMH) compared to diet FD ($F_{3,8} = 19.91$, $P < 0.001$).

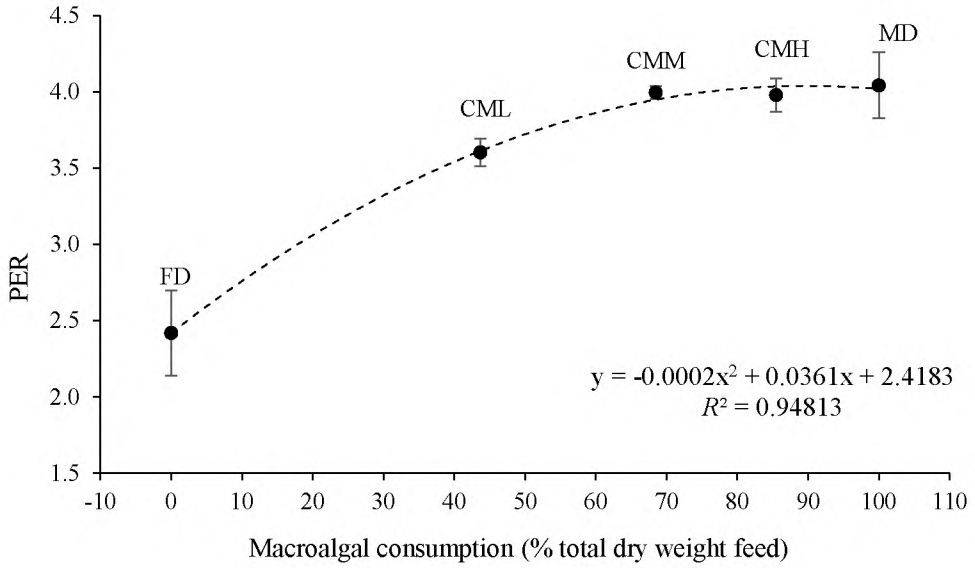


Figure 28. Polynomial regression (y = -0.0002x2 + 0.0361x + 2.4183, $R^2 = 0.94813$; $P < 0.001$) of abalone protein efficiency ratio (PER) and macroalgae consumption (% total dry weight feed ingested) for abalone fed a formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CMH, CMM & CML). Values plotted are means of replicate means and SD.

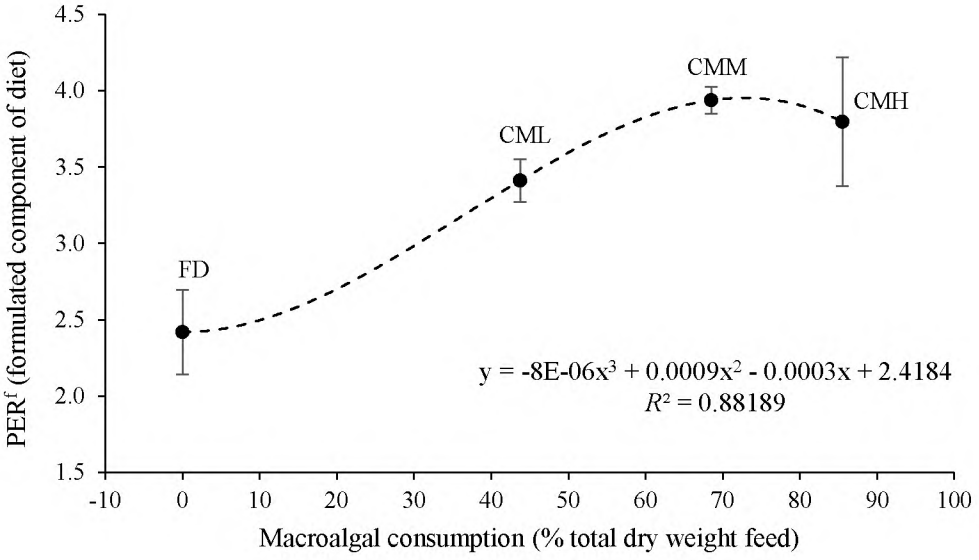


Figure 29. Polynomial regression (y = -8E-06x3 + 0.0009x2 - 0.0003x + 2.4184
$R^2 = 0.88$; $P < 0.001$) of abalone protein efficiency ratio of the formulated component of the diet (PER^f) and macroalgae consumption (% total dry weight feed ingested) for abalone fed a formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CMH, CMM & CML). Values plotted are means of replicate means and SD.

3.3.4 Stable isotopes

Macroalgal $\delta^{13}C$ values were generally stable over the first two periods of the growth trial (day 0 – 123 and day 124 – 249), but with a statistically significant enrichment in the third period (day 250 – 386) (*Ulva* - $F_{2,50} = 12.36$, $P < 0.001$; *E. maxima* - $F_{2,48} = 4.90$, $P = 0.012$; *Gracilaria* – $F_{2,49} = 13.96$, $P < 0.001$). Macroalgal $\delta^{15}N$ showed no significant shift over the period of the trial (Table 12). Over the total growth period (day 0 – 386), $\delta^{13}C$ and $\delta^{15}N$ values for all macroalgal types were significantly different from each other ($\delta^{13}C$ – $F_{2,153} = 72.23$, $P < 0.001$; $\delta^{15}N$ – $F_{2,153} = 253.1$, $P < 0.001$) with mean values of: (1) *Ulva* -9.9 ± 2.8 $\delta^{13}C$ and -0.7 ± 2.1 $\delta^{15}N$, (2) *E. maxima* -14.1 ± 1.5 $\delta^{13}C$ and 7.6 ± 1.1 $\delta^{15}N$ and (3) *Gracilaria* -18 ± 3.2 $\delta^{13}C$ and -3.1 ± 3.3 $\delta^{15}N$. Abalone abductor muscle $\delta^{13}C$ values showed no significant shift within treatments over time apart from a slight significant decrease in treatment FD at 249 days. Abalone $\delta^{15}N$ values however showed significant enrichment over time in treatments CML ($F_{2,8} = 8.4$, $P = 0.025$) and FD ($F_{2,9} = 49.49$, $P < 0.001$). Significant differences were observed between treatments for both $\delta^{13}C$ and $\delta^{15}N$ at day 123 ($\delta^{13}C$ - $F_{4,10} = 40.63$, $P < 0.001$; $\delta^{15}N$ - $F_{4,10} = 82.68$, $P < 0.001$), day 249 ($\delta^{13}C$ - $F_{4,9} = 28.39$, $P < 0.001$; $\delta^{15}N$ - ($F_{4,9} = 101.3$, $P < 0.001$) and day 386 ($\delta^{13}C$ - $F_{4,10} = 131.0$, $P < 0.001$; $\delta^{15}N$ - $F_{4,10} = 225.60$, $P < 0.001$). By day 386, Tukey's *post hoc* test showed significant separation for both $\delta^{13}C$ and $\delta^{15}N$ values between treatments FD, CML, CMM and a group comprising of MD and CMH, that were not significantly different from each other (Figure 30).

SIAR-estimated contributions ($SIAR_{EC}$, 90% credibility) of dietary components, when compared to proportional contribution to diet derived from feed consumption data (PCFC), revealed noticeable discrepancies between proportional consumption and proportional abductor muscle deposition for some dietary components (Figure 31). $SIAR_{EC}$ above PCFC for a specific dietary component would suggest preferential utilization of that component for foot muscle deposition. In treatment MD, *Ulva* and *Gracilaria* $SIAR_{EC}$ (90% credibility) exceeded the boundaries of PCFC, while *E. maxima* PCFC fell within the boundaries of $SIAR_{EC}$. In the combination diets CML, CMM and CMH, *Gracilaria* $SIAR_{EC}$ followed a similar pattern to MD, remaining above PCFC while *E. maxima* and *Ulva,* $SIAR_{EC}$ fell within the boundaries of PCFC. The $SIAR_{EC}$ for formulated feed in all combination diets was PCFC. Within the formulated feed dietary treatment FD, $SIAR_{EC}$ of Soya and Starch-2 were within the

boundaries of PCFC, while fishmeal and Starch-1 were markedly below and above PCFC respectively.

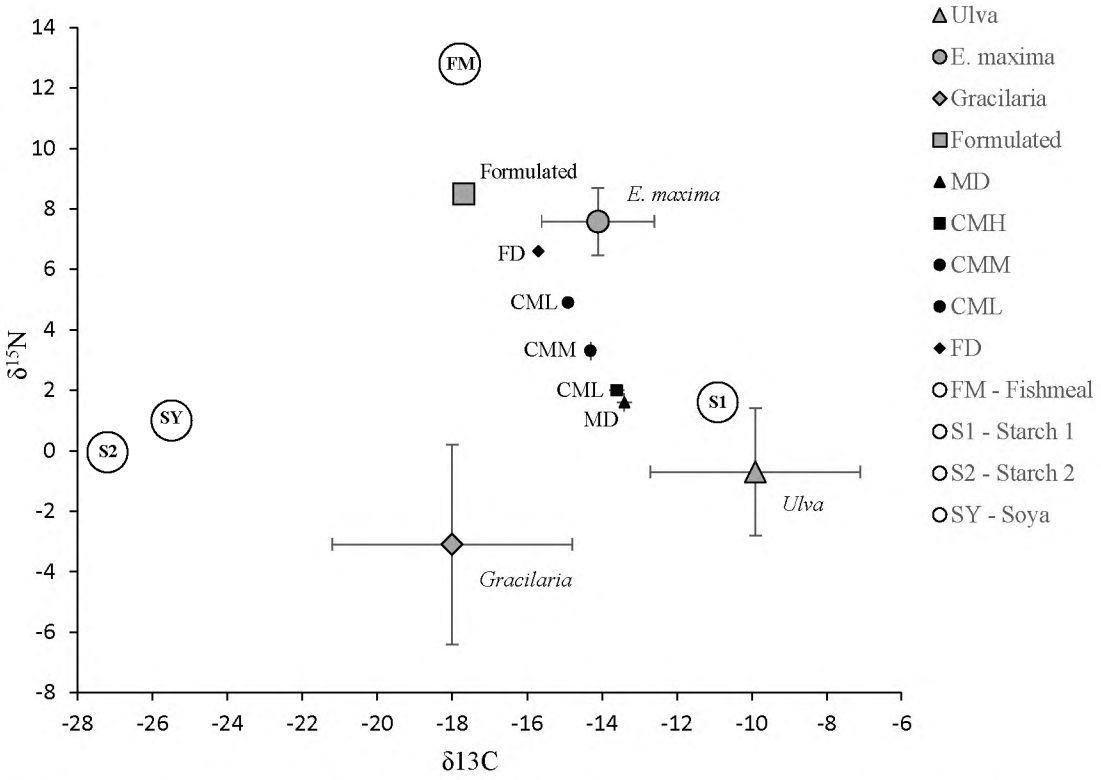


Figure 30. Mean (± SD) carbon (δ13C) and nitrogen (δ15N) isotope values of macroalgae (*Ulva*, *Gracilaria* and *E. maxima*; 53 weekly samples), a formulated pellet and its major constituent ingredients and abalone abductor muscle tissue (collected at day 386) from 5 dietary treatments fed mixed macroalgae (MD), formulated feed (FD) and three combination diets of formulated feed and macroalgae fed together with low (CML; 43.7%), medium (CMM; 68.5%) and high (CMH; 85.5%) macroalgal supplementation levels.

Table 12. Carbon (δ13C) and nitrogen (δ15N) stable isotope values for macroalgal samples (weekly), formulated feed (whole pellet and component ingredients) and abalone abductor muscle tissue (sampled at day 124, 250 and 386).

		Period (days)			
		0-123	124-249	250-386	0-386 (x)
Samples (n)		17	17	19	53
Dietary components					
Macroalgae					
$\delta^{13}C$	*Ulva*	-7.9 ± 2.3 ^{a}	-9.6 ± 2.6 ^{a}	-11.8 ± 2.2^{b}	$_{x}$ -9.9 ± 2.8
	E. maxima	-13.3 ± 1.3 ^{a}	-14.1 ± 1.7 ^{ab}	- 14.8 ± 1.1^{b}	$_{y}$ -14.1 ± 1.5
	Gracilaria	-15.9 ± 2.7 ^{a}	-17.6 ± 3.0 ^{a}	-20.4 ± 2.1^{b}	$_{z}$ -18.0 ± 3.2
$\delta^{15}N$	*Ulva*	-1.0 ± 1.2 ^{a}	-0.9 ± 1.7 ^{a}	-0.2 ± 2.9^{a}	$_{x}$ -0.7 ± 2.1
	E. maxima	7.7 ± 0.8 ^{a}	7.6 ± 1.3 ^{a}	7.7 ± 1.3^{a}	$_{y}$ 7.6 ± 1.1
	Gracilaria	-3.2 ± 0.9 ^{a}	-4.6 ± 1.1^{a}	-1.6 ± 1.2^{a}	$_{z}$ -3.1 ± 3.3
Formulated feed					
$\delta^{13}C$	Whole pellet				-17.7
	Fishmeal				-17.8
	Soya				-25.5
	Starch 1				-10.9
	Starch 2				-27.2
$\delta^{15}N$	Whole pellet				8.5
	Fishmeal				12.8
	Soya				1.0
	Starch 1				1.6
	Starch 2				-0.3
Abalone abductor muscle					
n		15	15	30	
$\delta^{13}C$	MD	$_{m}$ -13.3 ± 0.2 ^{a}	$_{m}$ -13.7 ± 0.4 ^{a}	$_{m}$ -13.4 ± 0.2 ^{a}	
	CMH	$_{m}$ -13.6 ± 0.2 ^{a}	$_{m}$ -13.7 ± 0.3 ^{a}	$_{m}$ -13.6 ± 0.2 ^{a}	
	CMM	$_{n}$ -14.6 ± 0.4 ^{a}	$_{m}$ -14.6 ± 0.3 ^{a}	$_{n}$ -14.3 ± 0.1 ^{a}	
	CML	$_{n}$ -14.9 ± 0.1 ^{a}	$_{n}$ -15.4 ± 0.1 ^{b}	$_{o}$ -14.9 ± 0.1 ^{a}	
	FD	$_{o}$ -15.6 ± 0.3 ^{a}	$_{o}$ -16.3 ± 0.5 ^{a}	$_{p}$ -15.7 ± 0.1 ^{a}	
$\delta^{15}N$	MD	$_{m}$ 1.3 ± 0.2 ^{a}	$_{m}$ 1.1 ± 0.2 ^{a}	$_{m}$ 1.6 ± 0.3 ^{a}	
	CMH	$_{m}$ 1.8 ± 0.0 ^{a}	$_{m}$ 1.9 ± 0.2 ^{a}	$_{m}$ 2.0 ± 0.1 ^{a}	
	CMM	$_{n}$ 2.7 ± 0.6 ^{a}	$_{n}$ 3.0 ± 0.3 ^{a}	$_{n}$ 3.3 ± 0.3 ^{a}	
	CML	$_{o}$ 3.8 ± 0.3 ^{a}	$_{o}$ 4.2 ± 0.4 ^{ab}	$_{o}$ 4.9 ± 0.2 ^{b}	
	FD	$_{p}$ 6.0 ± 0.3 ^{a}	$_{p}$ 5.2 ± 0.1 ^{b}	$_{p}$ 6.6 ± 0.1 ^{c}	

Values reported for macroalgae for each growth period are mean and SD of weekly samples. Values reported for abalone abductor muscle are the means of treatments mean values and SD. Different superscript letters after values indicate significant different values within the row. Different subscript letter before values indicate significantly different values within the column grouping.

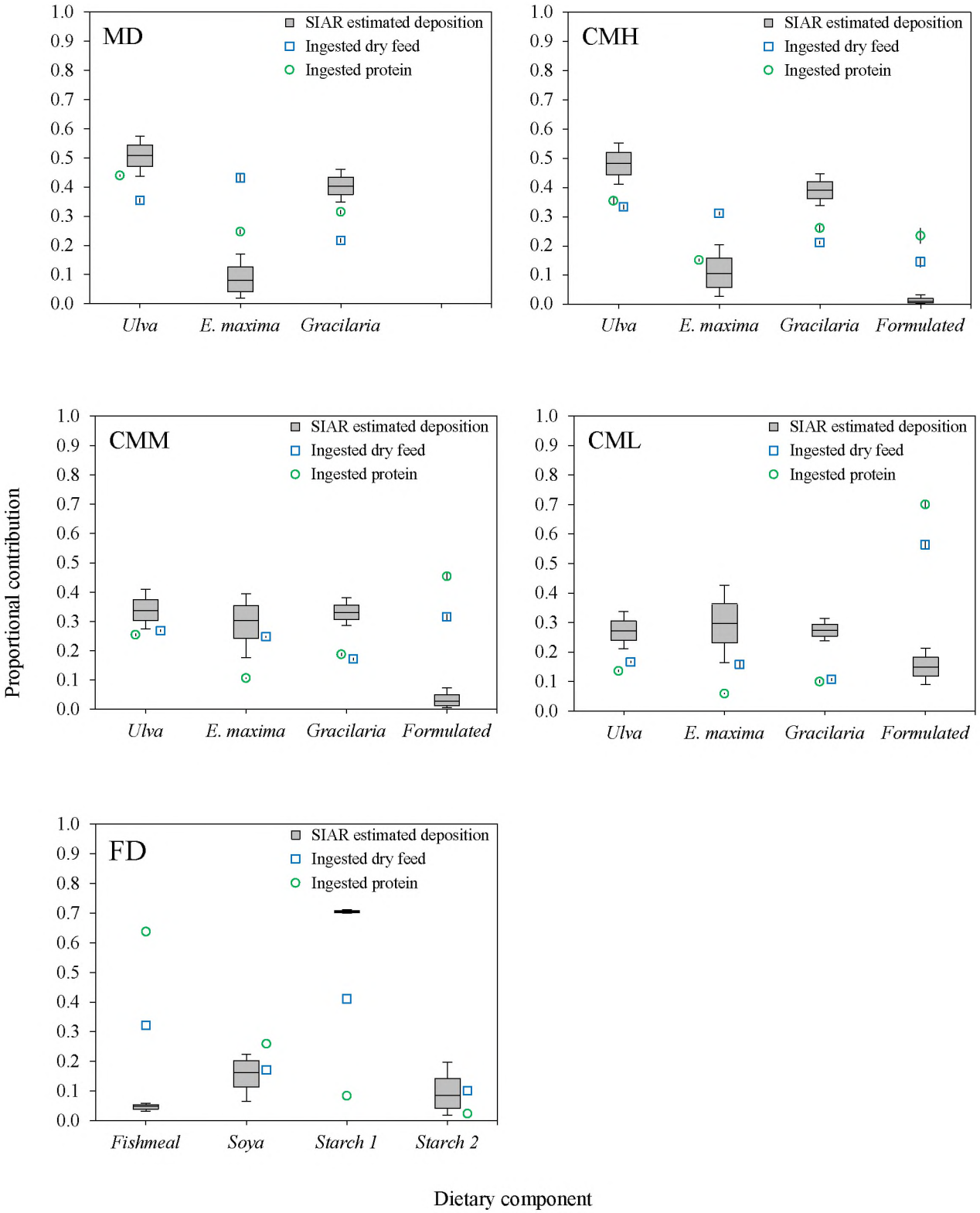


Figure 31. SIAR model estimated contribution ($SIAR_{EC}$) of dietary components to abalone abductor muscle tissue deposition (box plots; box limits on 25th and 75th percentile, bars represent range of 90% credibility) and the proportional contribution of dietary components to abalone diet as total dry mass (□) and dry protein (○) as determined using feed consumption and proximal composition data.

3.3.5 Canning

Significant differences in the proportional contribution of abalone component structures to whole-abalone wet weight were observed for shell and viscera but not for meat or mucous (Figure 32). Proportional contribution of shell was significantly higher for diet FD compared to all other treatments, which were not significantly different from each other ($F_{4,10} = 11.56$, $P < 0.005$). Viscera contribution was significantly higher for diets CMH and MD compared to diets FD and CML ($F_{4,10} = 18.68$, $P < 0.005$), while diets CML and CMM were not significantly different from each other.

Processing loss was significantly lower for diet FD compared to other treatments ($F_{4,10} = 5.47$, $P = 0.013$) except diet CMH, although no significant differences in the can-in yield between treatments was observed (Figure 33). Canning loss, although markedly lower for diet FD was not significantly different, but final commercial yield was significantly higher for diet FD compared to all other treatments ($H_{4,15} = 10.1$, $P = 0.039$). Water loss was significantly lower in treatment FD compared to treatment CML, and both these treatments were significantly lower that MD. Combination treatments CML, CMM and CMH were not significantly different from each other ($F_{4,10} = 25.9$, $P < 0.0005$). Dry yield was significantly higher for diet FD compared to diet CML and both these diets were significantly higher that diets CMM, CMH and MD. Strong positive relationships were observed between the formulated feed proportion of the diet and; (1) commercial yield (2) dry yield and (3) muscle glycogen content were observed (Figure 34). Furthermore, muscle glycogen content was positively correlated to dry yield (Figure 34).

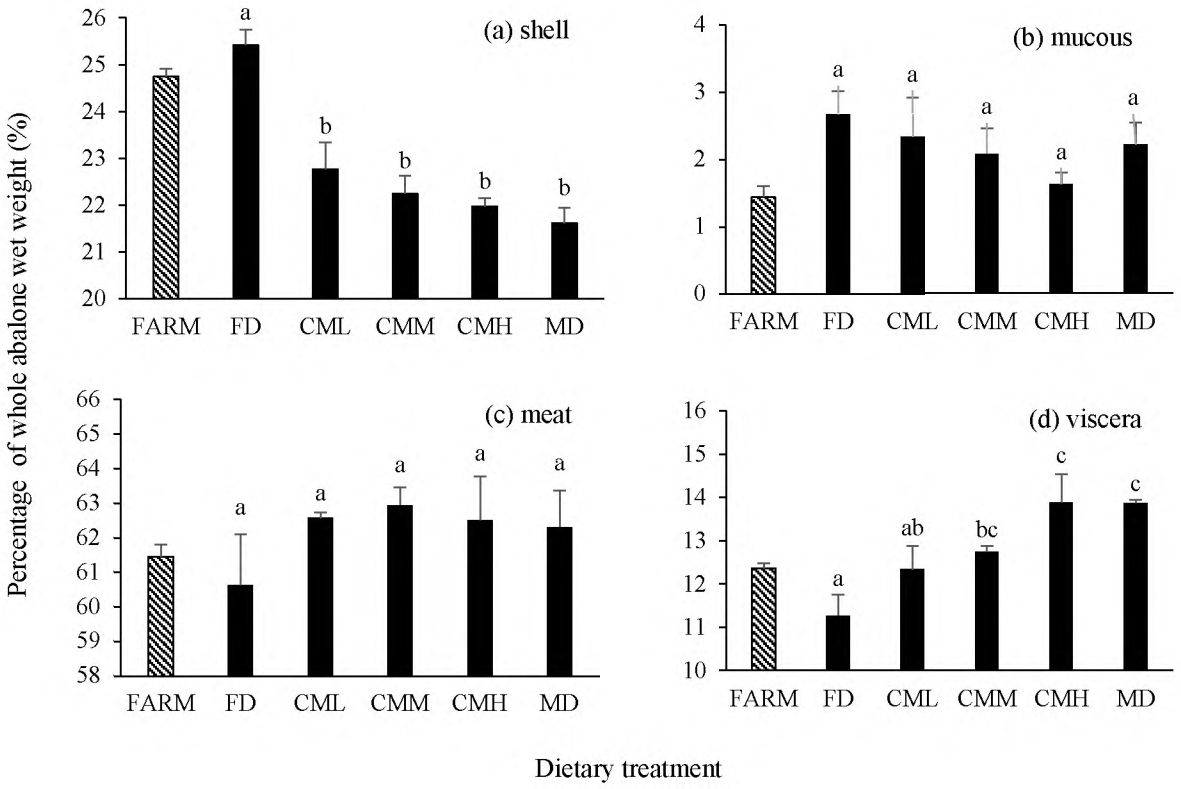


Figure 32. Body component breakdown from a simulated canning trial of juvenile abalone *H. midae* reared for 386 days on formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CML, CMM & CMH). A reference group from comparable sized farmed abalone reared on formulated feed (Abfeed™ S34) in outdoor raceways is included (FARM). Solid bars represent treatment means derived from replicate means (n = 3). Error bars indicate standard deviation.

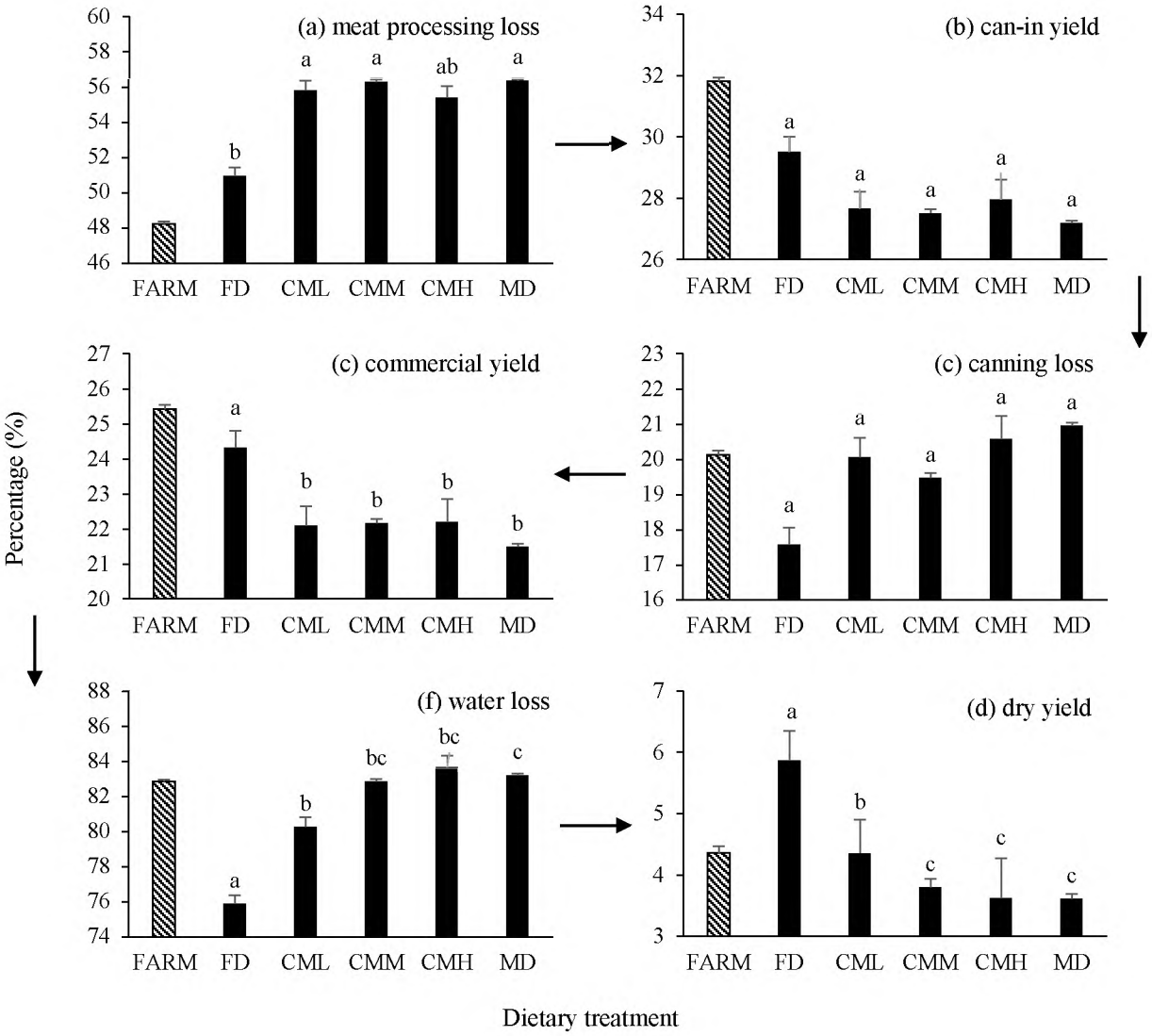


Figure 33. Processing losses (% weight loss between two processing stages) and yields from a simulated canning trial of juvenile abalone *H. midae* reared for 386 days on formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CML, CMM & CMH). A reference group from comparable sized farmed abalone reared on formulated feed (Abfeed™ S34) in outdoor raceways is included (FARM). Solid bars represent treatment means derived from replicate means (n = 3). Error bars indicate SD.

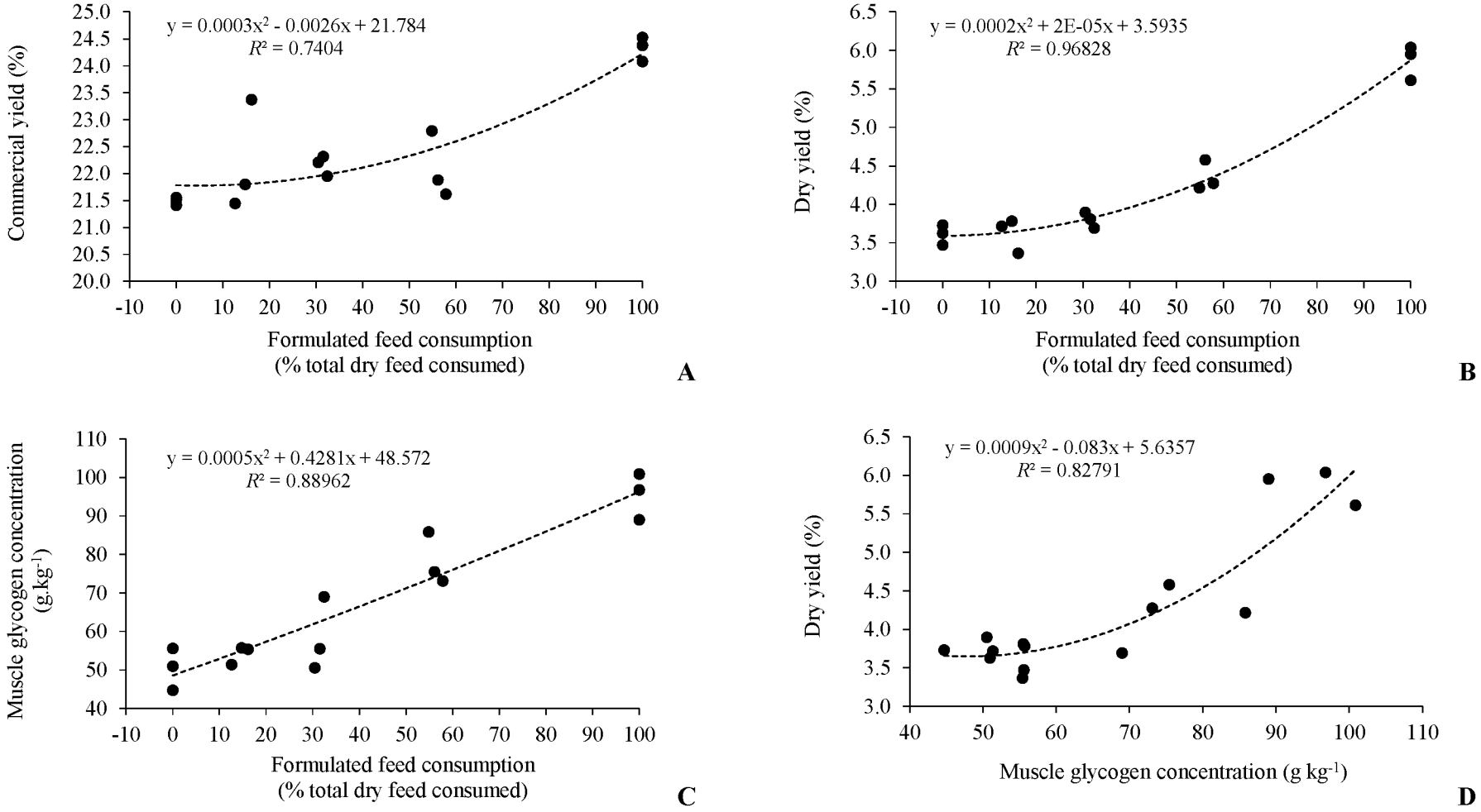


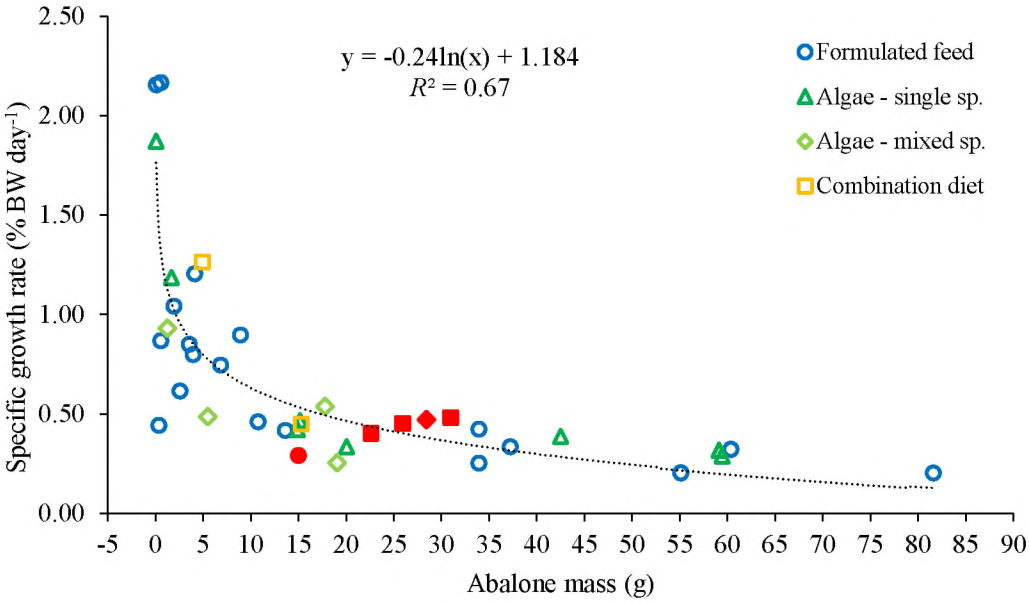
Figure 34. Polynomial regression of commercial yield (A; % whole abalone wet weight); dry yield (B; % whole abalone wet weight) and muscle glycogen concentration (C; g kg^{-1}) against formulated feed consumption (% total dry feed intake) and dry yield following canning (D; % whole abalone wet weight) against muscle glycogen concentration (g kg^{-1}) for juvenile abalone *H. midae* reared for 386 days on formulated pellet (FD), fresh macroalgae (MD) and three combination diet regimes (CML, CMM & CMH).

3.4 Discussion

Abalone growth

The composition of the diet significantly affected the growth rate of abalone in the current study, with a positive relationship between growth rate and the level of macroalgae in the diet up to a proportional macroalgal contribution of 68.5%, above which growth rates generally plateaued. However, before examining the biological relevance of between-treatment differences in growth in dietary trials, it is useful to locate the absolute growth rates achieved within the context of the previously demonstrated capacity for growth of the given species. Reduced absolute growth rates in the presence of suitable feeds can potentially indicate the influence of confounding or masking factors on growth, such as water quality parameters (Naylor et al., 2011, 2013, 2014; Reddy-Lopata et al., 2006). Furthermore, given the log-linear relationship between specific growth rate and animal mass in abalone (Day & Fleming, 1992), animal size should be taken into consideration when drawing comparisons. It is evident that growth rates reported for *H. midae* in the literature vary widely (Table 13). However, if one assumes that water quality conditions were not limiting for abalone growth in these studies, and growth rates are adjusted for temperature (to 18 °C as per Britz et al. (1997b), then it is possible to gain some insight into what constitutes "good" growth in *H. midae* at any particular body size by modelling the growth rates of the best performing treatments against animal size from the studies in Table 13. Only the best performing treatments within the four dietary categories (formulated feed, macroalgae; single species, macroalgae; mixed species and combination; formulated feed and macroalgae) were selected to provide a best-case-scenario for *H. midae* growth. There was no significant trend between linear growth rate and abalone size, which confirms that abalone maintain a constant rate of shell-length increment with increasing size (Figure 35). The mean linear growth rate reported for these studies is 59.7 ± 16.1 µm day^{-1} (1.82 mm $month^{-1}$) at 18 °C. Furthermore, the absence of a marked pattern in the distribution of specific dietary treatments in relation to the mean indicates the lack of consensus in the literature regarding which diet treatment results consistently in good growth rates. In terms of weight increase, the significant log-linear relationship between abalone weight and specific growth rate is clearly demonstrated (Figure 35). Comparison of the absolute linear (shell) and specific mass growth rates achieved in the current study with the linear models derived from previous studies indicates that they

were on par with or exceeded the reference models (Figure 35), with the notable exception of the FD treatment. The relatively slow growth of abalone in treatment FD requires further scrutiny, particularly given the fact that the formulated feed used in the current study is used extensively in the commercial culture of abalone in South Africa and can support linear growth rates of 2.00 mm $month^{-1}$ under favourable environmental conditions.



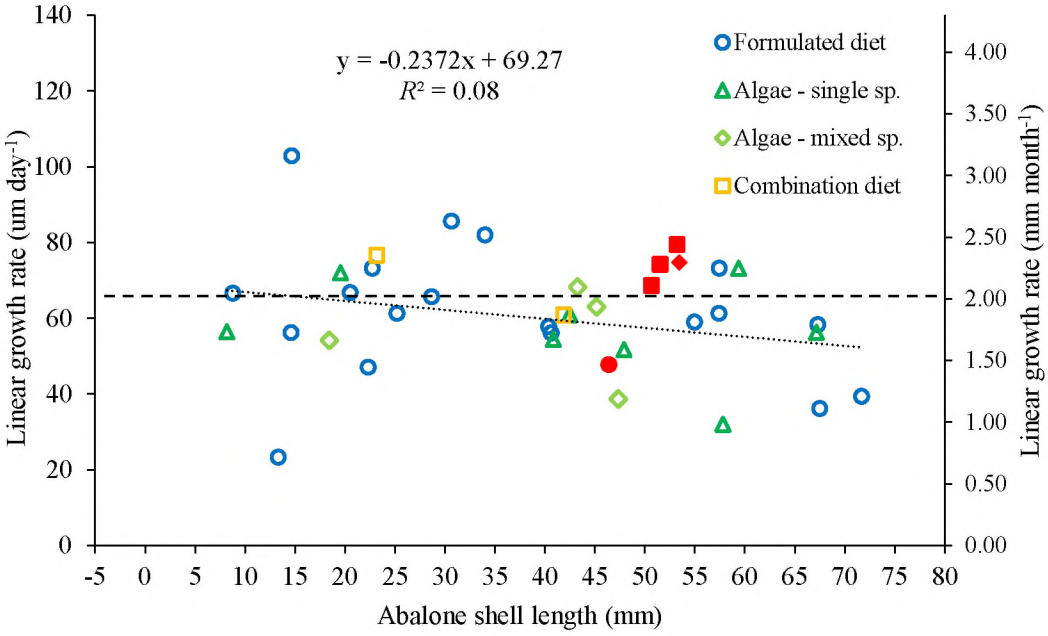


Figure 35. Regressions of best achieved specific (% BW day^{-1}; y = -0.24ln(x) + 1.184; R^2 = 0.67; P < 0.001) and linear (μm day^{-1}; y = -0.2372x + 69.27; R^2 = 0.08; P = 0.11) growth rates, relative to abalone mass and length respectively, of the abalone *H. midae* fed formulated feed, single species macroalgae, mixed species macroalgae and combination diets from 18 studies (Table 13). Reported abalone length and mass values are calculated median values during growth studies. Only the best performing dietary treatment per category for each study is reported. Growth rates were standardised to 18°C using median reported water temperatures. When not reported, length and weight values were calculated using the length-weight relationship as follows: Weight = 0.1531 × Length$^{3.0899}$. Solid symbols (red) represent data from the current study.

The cause of variable or poor growth rates for the abalone provided with a formulated feed may be the result from factors related to the timing of dietary cues on the modulation of the organism's digestive enzyme system. Anecdotal observations suggest that abalone weaned from microalgae onto macroalgae, and then later switched to formulated feeds, exhibit reduced growth rates, which suggests that the timing of weaning from natural to formulated feeds may play a role in the ability of abalone to utilise formulated feed during grow-out. If one applies a monogastric, endogenous-enzyme-driven digestive model to haliotids, then the digestive efficiency of abalone will be strongly correlated to their ability to secrete suitable enzymes in suitable quantities for the hydrolysis of ingested feed (Bansemer et al., 2016a). Abalone in the natural environment undergo a series of dietary shifts in their early ontogeny. Initial post-settlement feeding on biofilms and mucous is followed by radula development and a transition to diatoms, turf and crustose algae following radula development (Johnston et al., 2005). The final dietary shift is to macroalgae, which has been reported to occur at sizes ranging from 5 – 20mm depending on species (Hahn, 1989; Jarayabhand & Paphavasit, 1996; Kawamura et al., 2001). The ability to successfully adapt to shifts in diet would require both a change in the morphology of key feed-acquisition structures such as the radula (Johnston et al., 2005; Kawamura et al., 2001; Roberts et al., 1999), as well as a concomitant ability to alter enzyme secretion that matches the nutritional composition of new diets. A shift in culture diet from natural macroalgae to formulated feeds is generally associated with an increased availability of dietary protein. It can be hypothesised that the ability of abalone to effectively digest and utilise high levels of protein in formulated feeds during later grow-out is determined, to a degree, by the calibration of the proteolytic enzyme system in early ontogeny when multiple dietary shifts occur and plasticity is likely to be greatest. While abalone have been shown to exhibit an adaptable enzymology in response to variations in diet (García-Carreño et al., 2003; Garcia-Esquivel & Felbeck, 2006; Johnston et al., 2005; Knauer et al., 1996), there is some evidence to indicate that this capacity may vary according to the ontogenetic stage of the animal.

Table 13. Best achieved specific and linear growth rates per dietary treatment category (formulated diet, algae (single sp.), algae (mixed sp.). and combination diets (formulated feed and algae) standardised to 18 °C from 19 feeding studies conducted on the abalone *H. midae*.

Study	Period (days)[1]	System[2]	Best Diet[3]	Temperatture[4] (°C)	Conversion factor[5]	Initial Mass (g)	Initial length (mm)	Final Mass (g)	Final Length (mm)	SGR (% BW day^{-1})	LGR (um/day)	SGR – 18°C (% BW day^{-1})	LGR – 18°C (um day^{-1})	LGR18 mm/month
FORMULATED DIET														
Britz et al. (1997)	*91.5*	LR	FD	18	1.00	1.10	17.50	2.85[6]	23.60[7]	1.04[8]	66.70	1.04	66.7	2.03
Naidoo et al. (2006)	270	FF	ABF (35% Pr)	15.5	1.14	7.45	34.04	19.85	47.27	0.36[8]	49.00[9]	0.42	56.1	1.71
Shipton and Britz (2001a)	316	LR	FD (22% Pr)	20.1	0.88	19.60	44.40	48.30	65.60	0.29[8]	67.09[9]	0.25	58.9	1.80
	316	LR	FD (21% Pr)	20.1	0.88	0.52	13.90	4.72	30.80	0.70[8]	53.48[9]	0.61	47.0	1.43
Dlaza et al. (2008)	346.5	FF	ABF (35% Pr)	14.5	1.20	0.30	12.72	8.01	32.78	1.00	60.79	1.20	73.1	2.23
Macey and Coyne (2005)	252	LF	FD with probiotic	16.5	1.087	2.00	20.74	15.92[10]	40.58[7]	0.82[8]	78.73[9]	0.89	85.6	2.61
	252	LF	FD with probiotic	16.5	1.087	63.00	67.15	100.3[10]	76.27[7]	0.18[8]	36.19[9]	0.20	39.3	1.20
Sales et al. (2003b)	95	FF	FD (29% Pr)	15.7	1.133	4.79	30.58[11]	8.94	37.5[11]	0.66[8]	72.30[9]	0.74	81.9	2.50
Green et al., (2011a)	84	LR	FD (24% Pr)	18	1	2.70	25.93	5.27[10]	31.44[7]	0.80[8]	65.57[9]	0.80	65.6	2.00
	84	LR	FD (34% Pr)	18	1	50.41	66.02	59.96[10]	69.05[7]	0.21[8]	36.07[9]	0.21	36.1	1.10
Simon et al. (2004)	390	FF	ABF	14.6	1.199	18.88[11]	47.50	55.62[11]	67.39[7]	0.28[8]	51.00	0.33	61.2	1.87
Britz and Hecht (1997)	72	LR	FD (34% Pr)	18	1	0.20	11.00	0.95	18.40	2.16[8]	102.8[9]	2.16	102.8	3.13
	142	LR	FD (44% Pr)	18	1	7.40	36.30	14.20	44.50	0.46[8]	57.75[9]	0.46	57.7	1.76
Britz et al. (1997a)	117	LR	FD (30% Pr)	20	0.884	0.29	11.80	0.52	14.9[11]	0.50[8]	26.27[9]	0.44	23.2	0.71
Britz (1996b)	124	LR	FD (29% Pr)	19	0.942	1.76	21.20	5.37	29.26[7]	0.90	65.00	0.85	61.2	1.87
Green et al. (2011b)	70	LR	FD (26% Pr)	18	1	28.99	54.90	38.93[10]	60.02[7]	0.42[8]	73.11[9]	0.42	73.1	2.23
Francis et al. (2008)	183	FF	FD (26% Pr)	13.8	1.243	45.65	63.13	75.20	71.52	0.26	46.84	0.32	58.2	1.78
Knauer et al. (1996)	30	FF	FD (36% Pr)	15.8	1.127	0.10	7.94	0.17	9.69	1.91	59.00	2.15	66.5	2.03
Shipton et al. (2002)	80	LR	FD (25% Pr)	20	0.884	0.37	12.10	0.81[10]	17.2[11]	0.98	63.47[9]	0.87	56.1	1.71
This study	386	LR	ABF (34% Pr)	18	1	7.43	37.20	22.60	55.62	0.29	47.70	0.29	47.7	1.45

Table 13 con.

ALGAE (SINGLE SP.)													
Naidoo et al. (2006)	270	FF	K	15.5	1.14	7.61	34.45	22.81	47.27	0.41[8]	47.48[9]	0.47	54.3	1.66
Francis et al. (2008)	189	FF	K	13.8	1.24	46.47	64.31	72.45[10]	51.33[7]	0.24	25.73	0.29	32.0	0.98
Simon et al. (2004)	390	FF	K	14.6	1.199	18.88[11]	47.50	66.18[11]	71.29[7]	0.32[8]	61.00	0.39	73.2	2.23
Simpson and Cook (1998)	183		P	18.5	0.971	13.95[11]	43.07	26.17[11]	52.80	0.34[8]	53.17[9]	0.33	51.6	1.57
	183		P	18.5	0.971	0.33[11]	12.78	3.05[11]	26.33	1.22[8]	74.04[9]	1.19	71.9	2.19
Robertson-Andersson et al. (2011)	244	FF	K	15.5	1.145	8.69	36.04	21.19	48.96	0.37	53.15	0.42	60.8	1.86
Francis et al. (2008)	183		K	13.8	1.243	45.65	63.13	72.67	71.22	0.26	45.22	0.32	56.2	1.71
Knauer et al. (1996)	30	FF	D	15.8	1.127	0.07	7.43	0.11	8.94	1.66	50.00	1.87	56.4	1.72
ALGAE (MIXED SP.)													
Naidoo et al. (2006)	270	FF	K + U^c + G^c	15.5	1.14	7.83	37.74	27.78	52.60	0.47[8]	55.04[9]	0.54	63.0	1.92
Simpson and Cook (1998)	183		K + P / A / U (Rotation)	18.5	0.971	14.60[11]	43.71	23.13[11]	50.99	0.26[8]	39.78[9]	0.25	38.6	1.18
	183		K + P (Rotation)	18.5	0.971	0.37[11]	13.36	2.16[11]	23.55	0.96[8]	55.68[9]	0.93	54.1	1.65
Robertson-Andersson et al. (2011)	244	FF	K + U^c	15.5	1.145	8.69	36.04	2.31	50.52	0.42	59.59	0.48	68.2	2.08
This study	386		K + U^c + G^c	18	1	7.69	38.09	49.23	66.94	0.47	74.70	0.47	74.7	2.28
COMBINATION													
Naidoo et al. (2006)	270	FF	K + ABF (35% Pr)	15.5	1.14	7.92	34.75	22.75	49.09	0.39[8]	53.11[9]	0.45	61	1.85
Dlaza et al. (2008)	346.5		ABF + U + K	14.5	1.20	0.30	12.72	9.60	33.71	1.05	63.61	1.26	76	2.33
This study	386		CML	18	1	8.06	37.45	37.23	63.92	0.40	68.60	0.40	68.6	2.09
	386		CMM	18	1	7.60	37.24	44.37	65.85	0.45	74.10	0.45	74.1	2.26
	386		CMH	18	1	7.73	37.91	51.64	68.55	0.48	79.40	0.48	79.4	2.42

[1] As reported in days, or month data converted to days using 1 month = 30.5 days. [2] Culture system codes as follows: FF = Farm + flow-through, FR – Farm + recirculating, LF = Laboratory + flow-through, LR = Laboratory + recirculating. [3] K = Harvested Kelp *Ecklonia maxima*, U = *Ulva* sp., U^c = Cultured *Ulva* sp., G^c = Cultured *Gracilaria* sp., P = *Porphyra*, A = *Aeodes*, D = diatoms, FD + Formulated diet, ABF= Abfeed, Pr = Protein. [4] Temperature used is mean reported or calculated as the mean from the range reported. [5] Temperature conversion factor from Britz et al. (1997): 5.78% increase in growth per 1°C increase in temperature from 12 – 20 °C. [6] Read from graph. [7] Calculated from reported linear growth rate. [8] Calculated as follows: SGR = [ln(Final mass) - ln(Initial mass)] ÷ days × 100. [9] Calculated as follows: = [Final length (mm) – Initial length (mm)) ÷ days] × 1000. [10] Calculated from reported mass growth rate. [11] Calculated from corresponding mass/length data using the length: weight relationship: Mass (g) = 0.1531 × length (cm) $^{3.0899}$.

A number of studies conducted on small individuals (3 – 17 mm) show an upregulation of proteolytic enzymes in response to formulated feeds with elevated protein levels when compared to algal feed. For example, trypsin activity was significantly higher in *H. rubra* (11.3 ± 0.13 mm SL) given formulated feed compared to diatoms (Johnston et al., 2005), while Knauer et al. (1996) reported significantly higher protease activity for *H. midae* (3.22 – 11.29 mm SL) fed a formulated diet (35.5% protein) compared to diatoms (5.0% protein). A similar pattern of the upregulation of proteolytic enzymes in response to protein level has also been reported in macroalgae-only diets. García-Carreño et al. (2003), for example, reported that in *H. fulgens* (17.3 ± 2.2 mm) fed three species of seaweed, activities for acid proteinases, serine proteinases and six of nine amino-peptidases were highest for the macroalgal diet with the highest protein level. In contrast, older animals that have already progressed to digesting a macroalgal diet appear to show proteolytic enzyme stasis or even downregulation in response to increased dietary protein. Edwards and Condon (2001) reported no significant difference in protease activity for wild-caught *H. rubra* (30 – 100 mm) fed a formulated pellet (42.6% protein) compared to a natural diet of rhodophytes (10 - 20% protein). Similarly, both one-year (23.3 mm) and two-year (56.5 mm) old *H. laevigata*, showed no significant upregulation of trypsin activity in response to dietary protein increase in a formulated feed over a range of 9% (Bansemer et al., 2016c). Furthermore, Garcia-Esquivel & Felbeck (2006) showed consistently lower protease activity for *H. rufescens* (4.2 ± 0.01 cm SL) fed balanced diets (25% and 38% protein) when compared to the kelp *Macrocystis pyrifera* (9% protein). Although animals in the current study were weaned on-farm from diatoms onto a combination of macroalgae and formulated pellets, macroalgae was always present in the tanks and as such the intake of the formulated component post-weaning is unknown. Observations in the current trial suggested that formulated feed uptake was low when macroalgae was present in the tank. Therefore, the slow growth of abalone fed formulated feed in the current study could in part be attributed to a deficiency of the proteolytic enzymes required to utilise high dietary protein efficiently. The significantly lower protein efficiency ratio exhibited by animals fed a formulated feed diet (2.42) would support this, assuming the poor utilisation can be attributed to low digestibility and subsequent excretion, rather than post-absorption diversion for maintenance energy over somatic growth requirements. In addition, the culture system utilised in the current study may have played a role in the reduced growth rates attained on formulated feed when compared to those reported under farm conditions. The lighting provided did not support the growth

of a surface film of unicellular algae on the upper basket walls and feeder plate as is commonly observed in the culture baskets on farm situated in direct sunlight or partially shaded conditions. Under farm conditions, abalone actively graze on this biofilm and, although being fed exclusively formulated feed, are essentially existing under an unintentional combination diet (CD) regime.

Combination diets

The incorporation of macroalgae into the diet of abalone to form CDs in the current study resulted in a significant increase in growth and protein utilization efficiency over abalone fed exclusively on a formulated feed. These results generally concur with those obtained for *H. rufescens* in the previous study (see section 2.3.1). However, *H. midae* showed a significant increase in growth rate at a lower proportional contribution level of macroalgae (43.7%) compared to *H. rufescens* (76.1%). This would indicate that, although the study on *H. rufescens* lacked the resolution for experimental validation, the inclusion level of macroalgae required to stimulate significantly higher growth in *H. rufescens* may well be markedly lower than 76%. To the authors knowledge, no studies to date have investigated graded incorporation levels of dietary components in CDs for abalone. However, in instances where a single combination diet can be compared to its formulated component fed exclusively, combination diets resulted in similar (Lee et al., 1997) or significantly higher (Dang et al., 2011; Dlaza et al., 2008; Durazo-Beltrán et al., 2003b; Hernández & Uriarte, 2009; Ju et al., 2016; Mulvaney et al., 2013a; Naidoo et al., 2006) growth rates. The potential role that macroalgae plays in enhancing growth as part of CD regimes, as initially discussed in the previous chapter (see section 2.4), can be broadly categorised as either nutritional or functional in nature. Nutritionally, macroalgae may be providing essential nutrients either directly or as products from the activity of the gut microbiome. These could include amino acids, fatty acids, vitamins or minerals not available from formulated feeds that compensate for nutritional deficiencies in the formulation of artificial feeds (Iehata et al., 2009, 2014). This has been previously suggested as a possible explanation for the superior growth of multi-species macroalgal diets over monospecific diets (Day & Fleming, 1992; Qi et al., 2010; Stuart & Brown, 1994)

Several possibilities exist from a functional perspective that may explain the enhanced growth observed under CD regimes. Macroalgae contain bio-active compounds that have shown anti-bacterial, anti-viral and anti-oxidant potential in mammals and fish

(Chojnacka et al., 2012; O'Sullivan et al., 2010; Trichet, 2010). Evidence of macroalgae consumption resulting in enhanced antiviral activity in abalone has also been reported (Dang et al., 2011). It is likely that macroalgae may support abalone health and improve resilience to on-farm stressors, thereby reducing energetic allocation to pathogen control and improving scope for growth (Bansemer et al., 2014). Macroalgae also act as a feeding stimulant, from both a chemosensory and tactile perspective (Allen et al., 2006; Bansemer et al., 2014; Jan et al., 1981a). Total feed intake increased significantly with an increase in the proportion of macroalgae present in the diet of *H. midae* (Table 9) and a similar pattern was evident for *H. rufescens* in the previous study (Table 5). However, while it likely that macroalgae may have been more palatable than formulated feed, linking growth rates to total feed intake can be problematic as feed requirements would increase as abalone increase in size, whether that increase was the result of increased feed intake or superior food quality. Perhaps more important than total feed intake, are indicators and mechanisms associated with nutrient utilization efficiency. The protein efficiency ratio (PER) improved significantly with the incorporation of macroalgae to form combination diets, even as this addition ultimately resulted in a decrease in total available dietary protein. If the assumption holds that PER of macroalgae remains similar whether fed individually or in combination with formulated feed, as per the calculation in PER^{f}, then the driver of increased PER in combination diets would be the increased utilisation of protein from the formulated component. This outcome could be expected if macroalgae were modulating either protein availability/uptake during digestion or, alternatively, intermediary protein metabolism and allocation post-absorption. The potential for macroalgal polysaccharides to function as prebiotics, thereby modulating intestinal microflora and associated enzyme activity, has been discussed previously (Nel et al., 2017b, Chapter 2).

A potential mechanism that may explain the enhanced protein utilisation of formulated feed when fed in combination with macroalgae relates to how the structural nature of ingesta derived from macroalgae may influence gut function in abalone. The action of the rhipidoglossan radula of abalone macroalgae during macroalgae feeding results in a mixture of coarse and fine food fragments of feed entering the digestive tract (Campbell, 1965). By the time ingested feed reaches the stomach, it still includes large fragments of macroalgae that are likely passed directly from the crop to the stomach, bypassing the caecum (Campbell, 1965). The regular presence of large food fragments is supported by the existence of valve-like structures at the digestive gland (DG)

opening(s), whose function is purported to prevent the movement of large algal particles into the digestive gland (Crofts, 1929). The stomach contents are formed into the protostyle, a mucous bound faecal rod, by the muscular contraction of the style sac and rotation by cilia (Morton, 1953). Owing to the lack of cilia in the DG tubules, these muscular contractions are indicated as the primary mechanism that drives the flux of material and fluid between the crop/stomach region and the digestive gland, an important site of nutrient uptake and a major source of digestive enzymes (Campbell, 1965; Garcia-Esquivel & Felbeck, 2006; McLean, 1970; Owen, 1966). The preparation of formulated feeds involves the milling of ingredients to achieve a fine particle size, which allows good mixing and binding of ingredients, and easy passage through the die during extrusion. Ingesta present in the stomach following feeding on formulated feed would likely be much finer than that of abalone feeding on macroalgae, particularly if initial digestion of the starch binding component of the diet occurs in the post-oesophagus.

Differences in the bulkiness of the stomach contents would require differential levels of activity of the style-sac musculature and cilia to prepare the protostyle from the stomach contents for movement into the intestine. The increased style-sac activity associated with processing bulky macroalgal ingesta would potentially lead to an increased flux of digested material and enzymes between the digestive gland and post-oesophagus/stomach regions as well as increased movement of the protostyle against the gastric shield and stomach sorting grooves, thereby improving the efficiency of digestion. The extended gastric evacuation time, and by association degree of style-sac processing, recorded for abalone fed macroalgae (24 - 48+ hours depending on algal toughness) (Day & Cook, 1995), compared to formulated feeds (18 – 24 hours) (Britz et al., 1996) would support this hypothesis. The increased time of gastric residence for tough phaeophytes, such as kelp, over softer chlorophytes and rhodophytes (Day & Cook, 1995) could provide an inherent adaptive compensatory mechanism that maximises the digestive capacity of abalone when faced with tough nutritionally-poor feed. By feeding combination diets, it is possible that gastric processing time is extended, leading to more efficient digestion and subsequently improved growth. Confirmation of this mechanism as a factor in the improved growth of abalone fed formulated diets would require suitably designed digestibility studies. However, overcoming the practical considerations of developing experimental protocols that allow

feeding of isonitrogenous and isoenergetic treatments composed of nutritionally disparate feed groups, such as formulated feeds and macroalgae, remain a challenge.

Shell condition

The proportion of abalone with shell damage increased with an increase in both growth rates (LGR and SGR), as well as the proportion of macroalgae in the diet, with the majority of damage on the growing edge of the shell. Abalone in this study, as has been reported for other molluscs, exhibited an ability to repair previously damaged shell and develop the brown, non-mineralized layer around damaged regions typical of the first, pre-mineralization stages of repair (Fleury et al., 2008). Shell repair would be expected to be costly in terms of the energy balance of the organism, potentially contributing to the significantly reduced individual growth rates observed for abalone with shell damage compared to those without (Table 10). Abalone shell is formed by the biologically-controlled mineralization of calcium carbonate resulting from the secretions of the thin, polarized mantle epithelium (Fleury et al., 2008; Marin, 2012). Most of the shell (95 – 99%) is composed of calcium carbonate, representing both the calcite and aragonite polymorphs, combined with an organic matrix (1 – 5%) composed of proteins and polysaccharides (Zhang et al., 2004). The organic matrix strongly influences shell toughness, improving fracture resistance 3000 times over simple crystalline aragonite. The organic matrix controls several aspects of the mineralization process, including selecting the $CaCO_3$ polymorph and regulating crystallite shape and orientation (Fleury et al., 2008; Zhang et al., 2004). Depending on species, abalone shell can be calcitic, aragonitic or a combination of both (Dauphin et al., 1989). The influence of specific nutritional components on abalone shell formation has been demonstrated in abalone, with the level of dietary inclusion of Vitamin A and D (Zhang et al., 2004), zinc (Mai et al., 2003) and guaiacol (Zhang et al., 2008) resulting in significant effects on the minerology and pattern of soluble matrix proteins in new shell. These studies provided evidence of a positive correlation between the ratio of acidic/basic (A/B) amino acids in the shell and the calcite/aragonite ratio. This relationship was used by Tung and Alfaro (2012) to make inferences regarding the strength of abalone shell based on shell amino acid profiles, with diets resulting in low A/B amino acid ratios presumed to have higher aragonite content and be associated with greater strength. Significant differences in the A/B amino acid profile of the shell for different dietary protein sources were observed, indicating the potential role of diet in shell minerology. Of interest were the low A/B ratios reported for fishmeal-based diets. The positive

relationship between shell damage and macroalgal dietary contribution in the present study could be a result of the effect of a decreasing contribution of particular amino acids, potentially derived from fishmeal, associated with the formulated feed, and the subsequent effect on organic matrix composition and resulting shell minerology. Alternatively, if the diet did not affect shell composition directly, the parallel increase in growth rate associated with the increasing proportion of macroalgae in the diet would require more rapid shell generation. The shell growth rates of the fastest growing treatments were in the region of 2.3 – 2.4 mm month^{-1}, which can be considered rapid for *H. midae* (Table 13). It is plausible that shell thickness was reduced to support the generation of sufficient shell surface-area coverage for the rapidly increasing soft body. Interestingly, farmers anecdotally report thinner shell growing edges associated with rapid growth. The negative trend in proportional contribution of shell weight to whole abalone wet weight, compared to macroalgae contribution to diet, would support this (Figure 32). Shell damage resulting from reduced shell integrity in rapidly growing abalone would be compounded by the concomitant increase is density associated with rapid growth, reducing available surface area and increasing the likelihood of abalone:abalone interactions that could damage the growing edge of the shell.

Stable isotopes

To the authors knowledge, this is the first study to use stable isotope analysis and Bayesian mixing models in an attempt to quantify the contribution of dietary ingredients in a formulated feed, and dietary components under combination feeding regimes, to the deposition of pedal muscle tissue in a closed nutritional system. The stable isotope signatures of abalone foot muscle already showed significant separation between dietary treatments by as early as 123 days and, by 386 days, the pattern of separation for both $\delta^{13}C$ and $\delta^{15}N$ was similar, with treatments FD, CML, CMM and a group comprising CMH and MD all significantly different from each other. Given the significant separation in isotopic signatures both between both treatment groups and amongst available food sources, isotopic mixing models provide a useful methodology for estimating the proportional contribution of these sources (food types) to a mixture (consumer) (Parnell et al., 2010). However, application of the model requires consideration of the assumptions associated with it.

The first assumption is fairly ubiquitous among all mixing models, and requires that consumers lie within the isotopic mixing polygon created by the discrimination-factor adjusted isotopic values of the sources (Parnell et al., 2010; Phillips & Koch, 2002). The isotopic signatures of abalone in all dietary treatments fell within the 95% mixing region determined from their contributing dietary components (Figure 36), indicating that a solution that satisfies the geometry of the mixing model can be found. Bayesian mixing models differ fundamentally from simple linear models in that the source data are incorporated as distributions rather than fixed points, thereby presenting difficulties in defining discrete mixing polygons and subsequent assessment of point-in-polygon for consumers (Smith et al., 2013). These models will attempt to fit a solution even if sources are outside the mixing polygon (Parnell et al., 2010), leading to potentially erroneous interpretations of source contributions if the model is not evaluated for point-in-polygon adherence. Smith et al. (2013) provide a quantitative methodology based on Monte Carlo simulation, whereby many possible mixing polygons are generated and tested for point-in-polygon (i.e. the ability to establish mass balance). The proportion of iterated polygons that exhibit point-in-polygon are interpreted as the frequentist probability that a consumer's isotopic signature can be explained by the mixing model, given the provided variation in the isotopic signatures of the consumer and its proposed sources (Smith et al., 2013). The 95% mixing region represents that area within which the mixing model can establish mass balance for consumers with 95% confidence and is proposed as the cut-off point for model validation (Smith et al., 2013). In essence, the Monte Carlo simulation evaluates whether the model has a logical solution (Smith et al., 2013) and the Bayesian mixing model determines that solution (Parnell et al., 2010). Whether abalone isotopic signatures adhered to the mixing region also provides information regarding the validity of the discrimination factors employed in the model.

That the isotopic signatures of abalone fell within their respective 95% mixing regions is an expected result given that the dietary treatments were closed nutritional systems with known inputs. However, this result also validates the diet-derived methodology employed for estimating discrimination factors (Caut et al., 2009), as the discrimination factors calculated using this method did not cause consumers to fall outside the mixing polygon. This a particularly so for the high $\Delta\delta^{15}N$ for the seaweeds *Gracilaria* ($\Delta\delta^{15}N$ – 5.03) and *Ulva* ($\Delta\delta^{15}N$ – 4.28), which defined the lower border of the polygon. These values were higher than those determined from the single laboratory study that has

attempted to provide an estimate of discrimination factors in abalone ($\Delta\delta^{15}N$ – 2.2, $\Delta\delta^{13}$ – 1.6), although this study relates to the consumption of a formulated feed (Won et al., 2008). Given the significant linear relationship between a diet's isotopic signature and its subsequent discrimination factor when assimilated (Caut et al., 2008, 2009), and the disparity between the isotopic signature of formulated diet fed by Won et al., 2008) and food types in the current study, it would not have been useful to apply the discrimination factors defined by Won et al. (2008) in this study. Low $\delta^{15}N$ values, at times below those of the primary producers understood to comprise the diet of the abalone, have been reported in ecological studies and may be indicative of low $\Delta\delta^{15}N$ values when consuming algae (Pinnegar & Polunin, 2000; Vega-García et al., 2015). However, this apparent disparity was attributed to the unquantified contribution of other food sources with a low $\delta^{15}N$ signature, such as of N_2-fixing cyanobacteria or detritus from a food web with alternative primary producers. While the sensitivity of Bayesian mixing models to variation in discrimination factors has been demonstrated (Bond & Diamond, 2011; Moore & Semmens, 2008), in the context of the current study diet-derived discrimination factors (Caut et al., 2009) will likely provide the best method available currently to estimate discrimination factors in the absence of specific laboratory-derived values.

Isotopic routing was less of a concern in the current study as the dietary components were known and the contribution of these dietary components to a specific tissue (i.e. pedal muscle) was of interest. When employing the SIAR modelling approach, as with all other mixing models, there is the fundamental assumption that nutrients are broken down into their elemental components, these essentially entering a shared pool and then being reassembled into various tissues as required (Martínez Del Rio et al., 2009; Parnell et al., 2010). However, observations that different tissues can have contrasting isotopic signatures suggest that this is not always the case. This apparent differential allocation of isotopically divergent dietary components to certain tissues is referred to as isotopic routing (Martínez Del Rio et al., 2009; Schwarcz, 1991). This phenomenon can have major implications for ecological diet-reconstruction studies in that different tissue would illicit variable and even completely incorrect results when incorporated into isotope mixing models (Martínez Del Rio et al., 2009). The application of mixing models to stable isotope signatures of foot muscle does not attempt to define the importance of feed components to the whole animal, but rather their importance to foot

muscle deposition, essentially aware that isotopic routing may be occurring to produce the isotopic signatures observed.

Estimates using the SIAR model estimates of the proportional contributions ($SIAR_{EC}$) of *Ulva* and *Gracilaria* to foot muscle deposition (FMD) in treatment MD were above their proportional consumption while the kelp *E. maxima,* which accounted for over 40% of ingested macroalgae, exhibited a $SIAR_{EC}$ below its proportional consumption (Figure 31). These macroalgae fed showed distinct differences, however, in their nutritional composition, particularly in terms of protein content. *Gracilaria* (18.9 ± 4.8%) and *Ulva* (18.3 ± 3.9%) both exhibited significantly higher ($F_{2,147} = 65.6$, $P < 0.001$) protein levels (% dry weight) over the growth period compared to *E. maxima* (8.4 ± 1.8%) (Figure 18). When integrated with consumption data, the proportional contribution of *Ulva*, *Gracilaria* and *E. maxima* to total protein in the MD treatment was 43.9 ± 0.4, 31.4 ± 0.7, and 24.6 ± 0.8% respectively. So, although total dry consumption values for macroalgae did not match well with the $SIAR_{EC}$ to FMD, their proportional contribution to total dietary protein showed much greater alignment. The dietary protein level of macroalgae as a determinant of abalone growth, and by association tissue deposition, has been indicated in a number of studies, with perhaps the strongest supporting evidence coming from those that controlled for macroalgal species but manipulated protein level (Robertson-Andersson et al., 2011; Shpigel et al., 1999; Viera et al., 2011). Furthermore, the concept of protein and amino acid availability as a major limiting factor in the growth of abalone is further reinforced by the results from studies conducted using formulated feeds (Bansemer et al., 2014; Britz, 1996a; Britz & Hecht, 1997; Coote et al., 2000; Mai et al., 1995; Sales et al., 2003b).

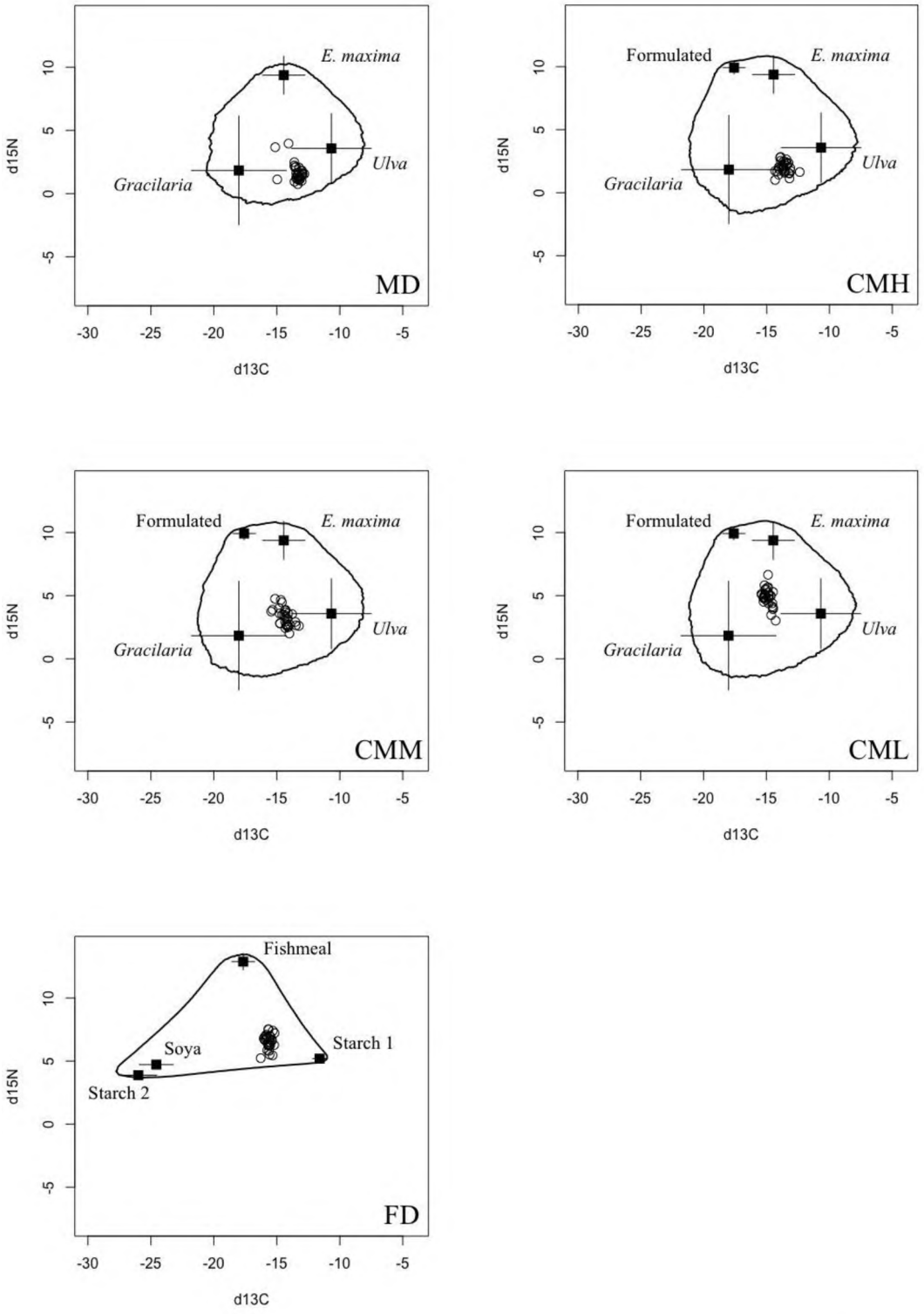


Figure 36. A biplot of stable isotope signatures (± SD) for consumers (dietary treatments MD, CMH, CMM, CML and FD) and sources (*Ulva*, *Gracilaria*, *E. maxima*, formulated pellet and the formulated pellet ingredients of starch 1, starch 2, fishmeal and soya). Polygons represent the boundary of the 95% mixing region (i.e. the region in which the mixing model can establish mass balance for all consumers in the system with 95% confidence) as determined using the Monte Carlo simulation method detailed by Smith et al. (2013).

While protein contribution to the diet was a better indicator of FMD than total dry mass contribution for macroalgae species, it did not fully align with the $SIAR_{EC}$. The $SIAR_{EC}$ of *Gracilaria* and *Ulva* was slightly higher than their proportional protein contribution, while *E. maxima* was lower. This is likely related to differences in aspects of nutrient availability between the macroalgal types. Fragments of the phaeophyte *E. maxima* ingested by *H. midae* have been demonstrated to break down significantly slower (> 48 h) than rhodophytes and chlorophytes such as *Plocamium*, *Porphyra* and *Ulva* that were unrecognisable within 48 h (Day & Cook, 1995). Similarly, soft rhodophyte species where digested more rapidly than phaeophytes by *H. rubra* (Foale & Day, 1992). The reduced digestibility of phaeophytes has been attributed to their structural toughness and the presence of anti-nutritional polyphenolic compound (Day & Cook, 1995; McShane et al., 1994; Stepto & Cook, 1996; Winter & Estes, 1992). Macroalgal fragments from *Ulva* and *Gracilaria* may have been more digestible than those from *E. maxima* resulting in a disproportionate contribution to the pool of absorbable nutrients and consequently $SIAR_{EC}$ values exceeding their proportional contribution to the diet. An alternative and potentially complementary explanation would be that aspects of the nutritional composition of *Ulva* and *Gracilaria*, such as amino acid profile or storage carbohydrate chemistry, better supported FMD in the form of both new protein formation and glycogen deposition compared to *E. maxima*. Interestingly, while the pattern of $SIAR_{EC}$ to the proportional contribution of dry feed and protein remained similar for *Ulva* and *Gracilaria* when fed as part of a combination diet, *E. maxima* exhibited a distinct shift (Figure 31). The $SIAR_{EC}$ for *E. maxima* reached parity with its proportional contribution of protein in diet CMH before exceeding it in diets CMM and CML as the proportion of formulated feed in the diet increased within the combination diets. This result would indicate that the nutritional profile of *E. maxima* is in fact suitable to promote FMD, although when more readily available food sources such as *Ulva* and *Gracilaria* are available, as in treatments MD, it's contribution is reduced. The high prevalence of *E. maxima* in the diet in wild abalone in some locations (Barkai & Griffiths, 1986; Day & Cook, 1995; Zeeman et al., 2012) and the successful culture of *H. midae* when using it as the sole feed for commercial operations would support this (Troell et al., 2006). Furthermore, the inclusion of *E. maxima* into a formulated feed has been shown to improve the growth rate of *H. midae* compared to a formulated feed without supplementation (Nel et al., 2017b). This finding also suggests caution regarding the assumption for the calculation of PER^f that the PER of macroalgae

remains the same whether fed alone or in combination with a formulated feed, at least in cases when mixed macroalgal diets are used to benchmark the calculation of PER^f.

The utilization of formulated feed for FMD relative to it proportional contribution to the diet was poor in all combination diets, even when contributing over 50% of total dry feed and 70% of dry protein, as in diet CML. Data from the FD treatment would suggest that this could be attributed in part to the poor utilisation of fishmeal as a protein source in the formulated feed (Figure 31). This is an anomalous outcome as fishmeal as a protein source has generally been demonstrated to yield good growth in abalone (Britz, 1996b; Fleming et al., 1996; Sales & Janssens, 2004; Tung & Alfaro, 2012). In instances where fishmeal has resulted in poor growth (Uki et al., 1985a, 1985b), it has been attributed to excessive heat treatment during processing (Uki & Watanabe, 1986). Differences in fishmeal source, and by association processing method, may explain the wide range of apparent protein digestibility coefficients (APDs) reported for this feed ingredient in abalone. Vandepeer and Van Barneveld (2003) reported values of 46% – 56% for Tasmanian fishmeal in *H. laevigata* and *H. rubra*, while (Fleming et al., 1998) reported a value of 43% for fishmeal of unknown origin in *H. laevigata*. In *H. midae*, APD values ranging from 68 – 88% have been reported for fishmeal of various sources, with low-temperature Danish fishmeal producing consistently high APD values (> 80%) (Sales & Britz, 2001a, 2003; Shipton & Britz, 2002). Locally sourced steam-dried, formalin-free fishmeal was used in the current study and it is possible that the poor utilization of this ingredient for growth may be related to irregularities in its quality relating to supplier processing inconsistencies. However, the secondary protein source soya, showed a contribution to FMD within the bounds of the $SIAR_{EC}$, potentially indicating that differences in the nutritional composition, such amino acid profile, could explain the differential utilisation observed. It is important to note that although the fishmeal in diet FD was apparently not being directed towards FMD in this study, it does directly imply that digestibility, and by association the availability of nutrients, is low. Nutrients from fishmeal could alternatively be playing a role in viscera and shell growth, or as an energy substrate for maintenance metabolism.

The very high contribution of Starch 1 to FMD indicated by $SIAR_{EC}$ (~70%), despite a low contribution to total ingested protein (8.3%), would suggest it is contributing

largely to the carbohydrate component of pedal muscle tissue. The energy metabolism of many gastropods (including abalone) is carbohydrate based, with excess energy stored in the form of glycogen that may constitute up to 40% of the dry weight of abalone foot muscle tissue (Braid et al., 2005; Fluckiger et al., 2011; Goddard & Martin, 1966; Livingstone & De Zwaan, 1983; Plisetskaya & Joosse, 1985). Abalone in the current study exhibited a significant positive relationship between the proportional contribution of formulated feed to the diet and muscle glycogen content, with maximum tissue concentrations of 96 ± 6 g kg^{-1} wet weight for the FD treatment (Figure 34). This would equate to 370 - 440 g kg^{-1} glycogen as dry weight based on muscle tissue moisture content of 74 - 78% (Hatae et al., 1996; Hughes, 2014; Olaechea et al., 1993). Similarly, *H. midae* (70 mm shell length) on formulated feed exhibited significantly elevated muscle glycogen values compared to those fed the kelp *E. maxima* and kelp/formulated-feed rotational diets (Laas & Vosloo, 2010). Although values reported are not directly comparable to the current study due to differences in the methods used to determine glycogen (chemical *vs.* enzymatic), muscle glycogen concentrations as high as 140 g kg^{-1} wet weight were reported (Laas & Vosloo, 2010). It is evident that the consumption of formulated feed by *H. midae* and, as shown by stable isotope analysis, particularly the dominant starch element, results in the elevated deposition of muscle glycogen compared to abalone fed macroalgae.

Canning

Canning is a popular method of processing farmed *H. midae* in the South African abalone culture industry. Differences in the chemical composition of abalone foot meat in response to diet have the potential to affect changes in muscle tissue during the chemical and thermal processing associated with canning, thereby influencing subsequent yield outcomes (Brown et al., 2008). The proportional contribution of shell and viscera were significantly higher and lower respectively for abalone fed formulated feed (FD) when compared to those fed macroalgae (MD). However, the proportion of body weight as foot muscle showed no significant difference between treatments, so any differences observed in yield could be attributed to differences in mass loss during processing. Treatment FD had a significantly higher commercial canned meat yield compared to treatment MD. This was the result of the combined effect of a significantly lower proportional mass loss due to non-thermal meat processing (scrubbing, salting and refrigeration) prior to canning, and reduced (although not significant; $P = 0.08$)

proportional mass loss during canning. Similar results were reported for *H. rufescens* in the previous chapter, although there was no differentiation between thermal and non-thermal processing loss. This study provides evidence supporting the hypothesised elevated glycogen muscle concentrations associated with feeding formulated feeds suggested in the previous study on *H. rufescens*. While the water-binding capacity of glycogen (Greenleaf et al., 1969; Przybylski et al., 2006) may play a role in initially retaining water during initial non-thermal processing, it is unlikely to be responsible for reduced processing loss during canning, and may even have the opposite effect, as this water is thought to be freed during the cooking process (Fernandez et al., 1991; Monin et al., 1987). Muscle tissue is composed predominantly of water, which contributes approximately 75% of total weight (Traore et al., 2012). Cooking results in substantial weight loss (20 - 40%) which is ascribed to the denaturation of myofibril and connective tissue proteins resulting in structural shrinkage. This causes the expulsion of water from the meat, with the degree of loss affected by both temperature profile and tissue structure (Bertram et al., 2004). While initial denaturation of abalone muscle protein (myofibrils and collagen) can occur at temperatures as low as 47-58 °C, extensive solubilisation of collagen only occurs above 90 °C (Gao et al., 2001; Øiseth et al., 2013; Zhu et al., 2011). A 9-fold increase in soluble collagen (compared to uncooked meat) has been reported for *H. discus* foot muscle boiled for 60 min at 90 °C (Zhu et al., 2011), while Hatae et al. (1996) reported a significant decrease in the collagen content of *H. discus* meat following boiling (98 °C for 60 min). These studies would indicate the conversion of collagen to gelatine following extended high-temperature, submerged heat treatment similar to those encountered during the canning process (114 °C for 45 min). Øiseth et al., (2013) describes differences in the muscle structure between abalone farmed using formulated feed and wild abalone presumably consuming predominantly macroalgae, particularly with the presence of large collagen-rich areas in wild abalone. It is possible that similar differences occurred in the abalone in this study, with the increased canning loss for abalone feeding on macroalgae (MD) potentially being the result of the loss of gelatinised collagen to cooking water. This hypothesis would be supported by the significantly higher water content of muscle tissue post-cooking observed for treatment MD, with gelatinised collagen possibly being replaced by water in the remaining protein structure during the cooking.

4 POSTPRANDIAL NUTRITIONAL PHYSIOLOGY OF THE PERLEMOEN *H. MIDAE*.

4.1 Introduction

The traditional outcomes of nutritional studies, such as growth or specific nutrient utilization efficiency, are the result of a complex suite of interactions and processes collectively forming an organism's nutritional physiology. Studies focussing on nutritional physiology provide a means to elucidate some of underlying mechanisms driving the outcomes observed in growth studies. By applying this mechanistic understanding to the development of feeds and feeding protocols for abalone, it is potentially possible to generate a more targeted approach to realising specific production outcomes.

The growth of an organism in an aquaculture context in generally reported as an increase in either length or mass (or both) over a given period, as these are often the most relevant and simplest metrics for a producer. However, growth can also be viewed in terms of the energy content of an organism, essentially equivalent to the energy retained in the chemical bonds of the organic molecules accumulated over time (Wootton, 2011). Viewing growth from the perspective of energy implies consideration of the laws of thermodynamics. First, the prediction of increasing entropy requires that an ordered entity such as an abalone, requires a constant input of energy to remain ordered, irrespective of growth (Nelson, 2011). Secondly, energy conservation requires that for growth to occur, energy input must exceed energy output (Wootton, 2011). Energy input comes in the form of ingested food, which provides chemical-bond energy and high-energy electrons, as well as minerals and macromolecules not synthesised endogenously (Nelson, 2011). This energy supports a suite of energy consuming, manipulative and storage chemical reactions, collectively termed the organisms

metabolism (ref here). A standard bioenergetics equation provides a useful framework to understand the energy budget of an organism, particularly with regards to the allocation of energy post-ingestion (Figure 37).

$$C = (F + U) + (R_S + R_A + R_{SDA}) + (P_S + P_{ST} + P_T)$$

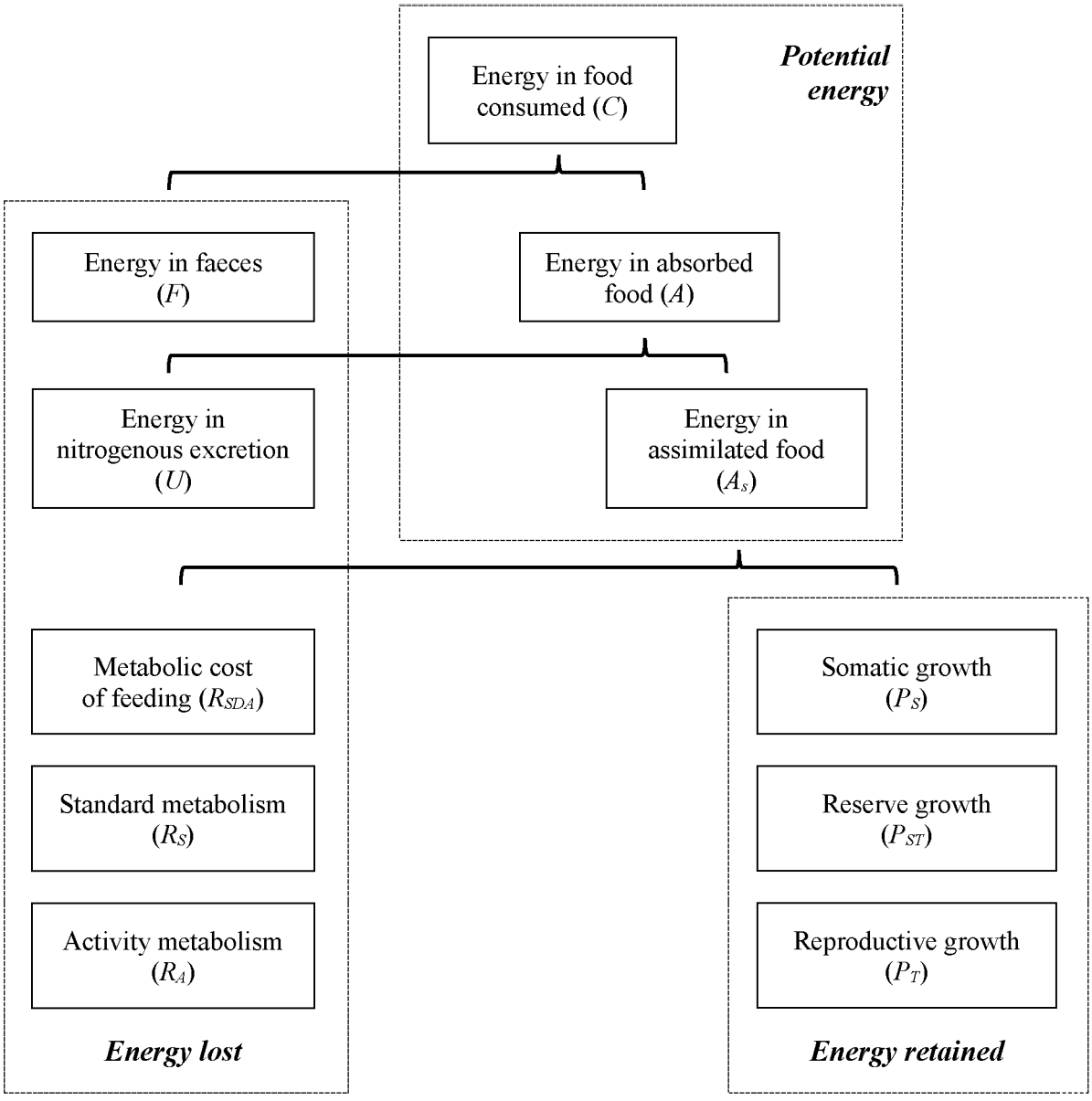


Figure 37. A standard energy budget and flow chart of energy allocation in an organism. Diagram adapted from Nelson & Chabot (2011) amd Wootton (2011).

The efficiency of energy use in growth studies is often quantified as the food conversion efficiency (FCR: dry feed intake ÷ wet weight gain). FCR's approaching 1 are not

uncommon in abalone but, although this is a useful metric for producers interested in whole organism yield relative to feed input, it does not consider that much of an abalone is composed of water and $CaCO_3$. Energy use in abalone, as with other organisms, is thermodynamically inefficient, with a large proportion of the potential energy in feed lost as heat (Nelson, 2016), a by-product of the myriad of reactions comprising its metabolism. Measuring the generation of this heat, termed direct calorimetry, is currently the best estimate of metabolic activity, although the high heat capacity of water and low metabolic rate of aquatic ectotherms results in technical constraints (Nelson & Chabot, 2011). Indirect calorimetry, which measures the uptake or production of molecules associated with the catabolic conversion of food to ATP energy, O_2 and CO_2, provides a more practical estimate of metabolism. Given the difficulties of measuring CO_2 in water, especially given the complex carbonate chemistry in seawater, determination of O_2 consumption (MO_2) as a surrogate for metabolic rate is generally employed as the more practical method (Nelson, 2016). Energy is preferentially allocated to mandatory components of an organisms' energy budget. These include maintenance energy requirements related to the homeostatic activities of cells and whole organisms, termed the standard metabolic rate (SMR), comprised broadly of the biosynthesis of macromolecules, the chemical work of moving ions or compounds against concentration gradients and internal mechanical work (i.e. heart contractions) (Chabot et al., 2016b; Wootton, 2011). Furthermore, energy is required to fund the cascade of cellular activities associated with the ingestion, digestion, absorption, transport and assimilation of a meal, commonly termed specific dynamic action (SDA) (Secor, 2011, 2009). Finally, routine (i.e. foraging) or environmentally induced (i.e. predator evasion) activity place further demands on available energy. The mandatory nature of SMR, SDA and routine activity mean that diet-related differences in these parameters can affect the proportion of energy available for the growth components of the energy budget.

Given the marked differences in the structural and chemical composition between formulated feeds and macroalgae, it is plausible that these differences could affect the energy required for the SDA response. Accurately estimating both the SDA and SMR of an organism are fundamentally linked, as SDA requires an accurate estimate of SMR as a baseline, and SMR requires that organisms be post-absorptive. Furthermore, SMR requires that organisms be inactive. To the author's knowledge, only a single previous

study has provided an estimate of SDA in abalone. Montaño-vargas et al. (2005), in calculating an energy budget for *H. corrugata*, briefly measured oxygen consumption (4 measurements at midday) for abalone fed the previous night. This measurement was reported as SDA, although technically it represents metabolic rate at *x* hours post-feeding. By definition, SDA represents the accumulated energy expended from the ingestion, digestion, absorption and assimilation of a meal (Jobling, 1994; Secor, 2009). The accurate measurement of SDA requires preprandial determination of SMR with suitable purging and respirometer-acclimation periods, a standardised meal, continuous estimation of metabolic rate postprandially at a suitable resolution to calculate the various commonly reported parameters of the SDA response and a suitable methodology to account for the effect of activity on SMR and SDA. Numerous studies have reported metabolic rate values for abalone in relation to extrinsic environmental factors such as temperature (Gaty & Wilson, 1986; Lyon, 1995; Paul & Paul, 1998; Vosloo & Vosloo, 2010), dissolved oxygen level (Gaty & Wilson, 1986; Harris, 1999; Paul & Paul, 1998; Taylor & Ragg, 2005), ammonia concentration (Harris et al., 1998c), salinity (Lim et al., 2014), light and circadian rhythm (Ahmed et al., 2008; Barkai & Griffiths, 1987; Chacon et al., 2003), water flow (Taylor & Ragg, 2005), activity (Baldwin et al., 2007), starvation (Carefoot et al., 1993; Gaty & Wilson, 1986; Segawa, 1991) and diet (often as input data for energy budget calculations) (Gómez-Montes et al., 2003; Montaño-vargas et al., 2005; Peck et al., 1987; Viana et al., 2007a). Intrinsic factors such as body size have also been explored (Barkai & Griffiths, 1987; Farías et al., 2003; Gaty & Wilson, 1986; Peck et al., 1987). Common to these studies, bar a few isolated cases, are the generally low resolution of the data collected, usually from single incubations (or a small sample of repeated incubations) using closed-cell respirometry techniques. Furthermore, any comparisons between studies, and often between treatments within studies, are limited by the general failure to apply a mass-scaling coefficient to compensate for allometric differences. In addition, both purging and respirometer acclimation time (if reported) are highly variable, ranging from 10 minutes to 72 hours. Combined with a lack of an empirical method to account for spontaneous activity, the potential for confounding effects of both activity, stress and SDA on presented values remains both unaccounted for and generally unexplored.

The aerobic catabolism of amino acids is responsible for a component of the oxygen consumption recorded during SMR and SDA measurements. Following the hydrolysis

of proteins by proteolytic enzymes in the gut, amino acids are absorbed and enter circulation. They either form the precursors for protein anabolism and specialized biomolecules (i.e. hormones) or, as they cannot be stored, undergo oxidative deamination whereby the amino group is cleaved and excreted as ammonia and the remaining carbon skeleton oxidised to forms alpha-keto acids. These form major metabolic intermediaries that enter the Kreb's cycle, ultimately resulting in the liberation of usable energy in the form of ATP. Determining the ammonia excretion (NH_4^E) dynamics of abalone not only provides information that can feed directly into energy budget calculations and aquaculture system design but, by integrating MO_2 and NH_4^E data, the nature of the substrates undergoing catabolism can be inferred (Hatcher, 1991). The O:N atomic ratio provides a useful index for estimating the relative utilization of protein for energy metabolism (Widdows, 1985). Low O:N ratios indicate the high utilization of protein relative to lipids and carbohydrates. Although defining a theoretical minimum value of O:N for protein-dominated catabolism is complex given the interactions between the specific amino acids being catabolised, the ammonia formation mechanisms and intermediary metabolism, values of 3 – 16 are suggested for protein-dominated catabolism (Mayzaud & Conover, 1988). Values of 50 – 60 are indicative of equal utilization of protein and lipid, while O:N ratios for carbohydrate-dominated catabolism theoretically approach ∞ (Hatcher, 1991; Ikeda, 1977; Mayzaud & Conover, 1988).

Knowledge of the substrate(s) being used for energy metabolism is particularly interesting from an aquacultural nutrition perspective, especially when designing and evaluating formulated feeds. As protein is generally an expensive component to supply in the diet, nutritionists prefer to harness alternative energy sources such as lipid and carbohydrate to fund metabolic energy requirements, thereby sparing amino acids from general oxidative purposes for use in tissue synthesis. Determining O:N ratios in relation to feeding offers a useful qualitative indicator of the degree of protein deamination occurring postprandially, and the resulting use of α-keto acids in energy-producing pathways (Mayzaud & Conover, 1988). Studies investigating ammonia excretion in abalone are limited, with only a handful of these integrating MO_2 data to calculate O:N ratios (Ahmed et al., 2008; Barkai & Griffiths, 1987; Farías et al., 2003; Montaño-vargas et al., 2005; Peck et al., 1987; Segawa, 1991). These estimates reflect a snapshot view of the substrate being utilized for energy metabolism, as the oxygen

consumption and ammonia excretion data were collected over short incubation periods, generally following a purging period. When one considers the expected postprandial fluctuations in oxygen consumption resulting from the SDA response and circadian rhythm, fluctuation in ammonia excretion related to the absorption of dietary amino acids and the inability of abalone to store amino acids effectively, it is likely that shifts in the O:N ratio, and consequentially the substrates fuelling energy metabolism are likely. The integration of extended time-series data for NH_4^E and MO_2 following feeding can potentially provide valuable insights into the fate of absorbed amino acids and, by association, the efficiency of protein utilization for tissue synthesis.

Although some studies have presented O:N ratios indicative of the utilisation of a proportion of protein as an energy substrate, especially during starvation (Segawa, 1991) or when fed a high-protein formulated feed (Farías et al., 2003; Montaño-vargas et al., 2005), it is generally understood that abalone share the carbohydrate-based energy metabolism described for many gastropod molluscs (Livingstone & De Zwaan, 1983). D-Glucose appears to be the dominant circulating monosaccharide, with energy storage in the form of polysaccharide glycogen (Livingstone & De Zwaan, 1983). The enzymes required for glycolysis and the pentose-phosphate pathway have been reported in abalone, as well as the incorporation of radiolabelled glucose confirming glycogen synthesis (Bennett & Nakada, 1968). There is little indication of the role of galactogen in the development of gametes, as has been reported for terrestrial or freshwater gastropods such as *Helix*, *Vivipparus*, *Austrolobus* and *Otal*a (Healy & Schulte, 2012). A carbohydrate-centred energy metabolism and storage physiology is not unexpected for the *Haliotidae*, given the generally high carbohydrate and low lipid and protein content of their natural macroalgal feed, combined with the low lipid content of abalone tissue. However, macroalgal carbohydrates are predominantly ingested in the form of cell-bound polysaccharides, which differ markedly from the gelatinized terrestrial starches present in formulated feeds. Elevated haemolymph glucose levels in response to a high carbohydrate diet have been reported in other gastropod species. For example, feeding *Megalobulimus oblongus* with a high carbohydrate diet increased haemolymph glucose by 46% compared to a lettuce diet (Rossi & Silva, 1993), while feeding *Lymnaea stagnalis* a high carbohydrate diet (Bemax) elevated haemolymph glucose 3 – 9-fold over a lettuce control diet (Veldhuijzen, 1974; Veldhuijzen & van Beek, 1975). The potential for an increase in abalone in the peak and duration of the postprandial

haemolymph glucose elevation following ingestion of refined carbohydrates in abalone therefore exists. The ability of abalone to regulate haemolymph glucose levels, whether through endocrine control or other mechanisms, is not well understood. Evidence from other mollusc species, particularly bivalves, indicates the role of insulin or insulin like substances (ILS) in decreasing elevated haemolymph glucose and increased glycogen-synthetase activity (Livingstone & De Zwaan, 1983; Plisetskaya & Joosse, 1985), and similar peptides have been reported in abalone (van der Merwe et al., 2011; York et al., 2012). The degree to which these control mechanisms exist in abalone, and their plasticity in adapting to potentially greatly elevated glucose loads resulting from novel terrestrial-carbohydrate-based diets remains unexplored.

The objectives of the suite of metabolic studies undertaken in the present study were therefore threefold. The first was to accurately determine the SMR and SDA response of abalone fed a standard meal of either a commercially formulated feed or macroalgae, using high-resolution intermittent-flow respirometry techniques. Secondly, determine the ammonia-excretion dynamics over a matching feeding cycle, to allow the integration of $\dot{M}O_2$ and NH_4^E data in estimation of the postprandial O:N atomic ratio profile. Finally, assess the dynamics of postprandial haemolymph glucose concentration using a serial sampling approach.

4.2 Materials and Methods

4.2.1 Culture system & experimental animals.

Following the conclusion of the primary 55-week growth trial described in Chapter 3, abalone were grown for a second culture period of 45 weeks, continuing their previous dietary treatments. Due to logistical constraints, during the second culture period kelp was removed from the diet of all treatments receiving macroalgae. The macroalgal component was therefore composed of equal proportions by wet weight of *Ulva* sp. and *Gracilaria* sp.. Furthermore, the day:night cycle in the controlled environment (CE) room housing the recirculating aquaculture system (RAS) was shifted from a 6am:6pm 12L:12D photoperiod to a 2am:2pm photoperiod over an eight-week acclimation period from weeks 59 – week 66. The cycle was adjusted by 30 minutes each week over this period. Shifting the onset of darkness in the CE room to earlier, relative to natural daylight hours, facilitated operation of physiology experiments that required ongoing human monitoring during the initial period of the dark phase, when abalone are most active.

Three trials investigating the effect of diet on aspects of abalone nutritional physiology were conducted during this second culture period. Three of the original dietary treatments were selected for further investigation, namely the formulated feed diet (FD), the macroalgal diet (MD) and the low inclusion combination diet (CML). Respirometry experiments assessing the metabolic cost of digestion were conducted over a 10-week period from weeks 68 – week 77. A total ammonia nitrogen (TAN) excretion trial was conducted in week 89, followed by trials assessing the effect of feeding on haemolymph glucose concentration (HGC) in weeks 98 and 99. Trial-specific methodologies are presented in section 4.2.3, 4.2.4 and 4.2.6.

4.2.2 Feed ration

Each of the nutritional physiology experiments required that abalone were fed during the trial to allow measurements during both the pre- (fasted) and postprandial states. A standard ration, matching the animal's dietary treatment, was fed. For all trials, the target ration was 0.5% of body weight as dry-weight feed. The ration mass for each

abalone was calculated using an expected dry-weight conversion factor for each dietary component used, and the mass of the abalone as measured at the start of the trial. A true dry-weight conversion factor for each dietary component was then determined for each trial (oven drying duplicate samples to a constant mass at 100 °C), and then used to calculate the true ration fed.

Macroalgal rations used for the respirometry and TAN excretion trials were composed of equal proportions (on a dry weight basis) of freshly harvested *Ulva* sp. and *Gracilaria* sp., and rehydrated *E. maxima* frond (saltwater submersion for 6 hr; previously oven dried at 38 °C for 24 hours and stored at -20 °C). Due to spoilage of stored kelp, only *Ulva* sp. and *Gracilaria* sp. were used for the HGC trial. Weighed rations of macroalgae were formed into parcels and secured using a cable tie. Combination-diet rations were formed as for macroalgae-only diets, with the formulated feed pellet component sandwiched within the macroalgae (Figure 38). The formulated-feed-only rations consisted of whole pellet(s) held by a cable tie. The tag end of the cable tie was clipped off following ration preparation for the respirometry trial, so as not to hinder abalone movement in the respirometers. Tag ends were retained for the TAN and HGC trials, to facilitate placing rations through the lid openings into the incubation chambers and removing the cable tie post-feeding with minimal disturbance to the abalone. Unfed control animals received a dummy cable tie with no feed.

Figure 38. Food parcel for a combination diet treatment, secured with a cable tie.

Experimental timing was denoted in hours, centred on T0 as the time of feeding and commencement of the dark phase. Prior to feeding, abalone were fasted for 96 hours in all trials, with feeding just prior to T0 minimising the pre-consumption soak time before abalone began actively foraging at the onset of darkness. Uneaten feed was collected by siphon from the chambers at T6 (TAN and HGC trials), sorted into dietary components, oven dried at 100 °C for 24 h and then weighed. Individuals that consumed less than 50% of the fed ration were excluded from further analyses.

4.2.3 Determination of oxygen consumption.

4.2.3.1 Experimental system

The oxygen consumption rate of abalone was determined using intermittent-flow respirometry. This technique was employed as it effectively combines the best aspects of closed respirometry and flow-through respirometry, while circumventing, or at the least minimising, their intrinsic problems (Svendsen et al., 2016). Closed respirometry involves sealing an organism in a metabolic chamber and calculating its oxygen consumption rate from the decrease in oxygen concentration in the chamber over time. Although simple to construct and operate, results from closed respirometry systems should be considered with caution owing to the confounding effects of other metabolites. Progressive hypoxia and hypercapnia, combined with the accumulation of nitrogenous waste products (particularly ammonia and nitrite), prevent steady-state conditions in the metabolic chamber during the measurement period (Svendsen et al., 2016). The duration of the measurement phase of an experiment is therefore limited by the accumulation of these metabolites and the rate of oxygen depletion. Respirometry studies often attempt to quantify the standard metabolic rate (SMR), essentially the minimum aerobic metabolic rate of a resting, post-absorptive, non-reproducing poikilothermic organism (Chabot et al., 2016b). As oxygen consumption in the acclimation period is not measured when employing closed respirometry, the multiple measurements over a time series require use of a new organism at each time interval, making development of experimental protocols that accurately target suitable windows for determining SMR challenging. Flow-through respirometry, by contrast, does not

employ a sealed chamber, but rather relies on quantifying the difference in oxygen concentration in the water entering and leaving a flow-through chamber. This information, when combined with the flow rate through the chamber per unit time, allows determination of the oxygen consumption rate of the organism in the chamber. The introduction of flow to the metabolic chamber, if flow rates are high enough, effectively removes issues of hypoxia, hypercapnia and nitrogenous waste accumulation. Unfortunately, flow-through systems are susceptible to mixing and equilibration issues, particularly relating to oxygen sensor error or drift, as well as changes in flow rate (Steffensen, 1989).

Intermittent-flow respirometry combines short measurement phases in a closed, recirculating chamber with flush periods of clean water at an exchange rate that is sufficient to remove accumulated carbon dioxide and nitrogenous wastes while replenishing dissolved oxygen to pre-measurement levels (Steffensen, 1989; Svendsen et al., 2016). Figure 39 describes the three-part operation cycle of an intermittent respirometry system (flush, wait and measurement periods), including the timing of pump operation that facilitates this cycle and the expected nature of dissolved oxygen concentration fluctuations that are observed. As the cycle of operation is looped and generally automated, this method facilitates long-term observations of the oxygen consumption of an organism with good temporal resolution.

During experiments, animals were housed in two parallel metabolic chambers. Each chamber and its associated plumbing was submerged in independent respirometry tanks (10 l volume; bucket shaped) which were themselves submerged in a single large water bath (500 mm × 600 mm × 300 mm; fiberglass) (Figure 40). Water from the water bath was pumped via a submersible pump (Resun; SP 980), though a UV steriliser (Ultrazap; 8 Watt) and into a biological filter (25 l; air-fluidised plastic media). Outflow from the biological filter passed through a felted screen (~ 100 micron) before flowing by gravity through flexible, clear PVC hose (internal ∅ 12 mm) to the respirometry tanks. The hose-end was located at the base of the tank to ensure mixing and prevent the introduction of bubbles that could enter the circulation pumps and ultimately the respirometry system. A surface level outflow returned water to the water bath. A bleeder valve from the submersible pump and airstones ensured mixing in the water bath. The system was housed in a controlled environment room to regulate water temperature (18 °C) and light:dark cycle (12L:12D) as required.

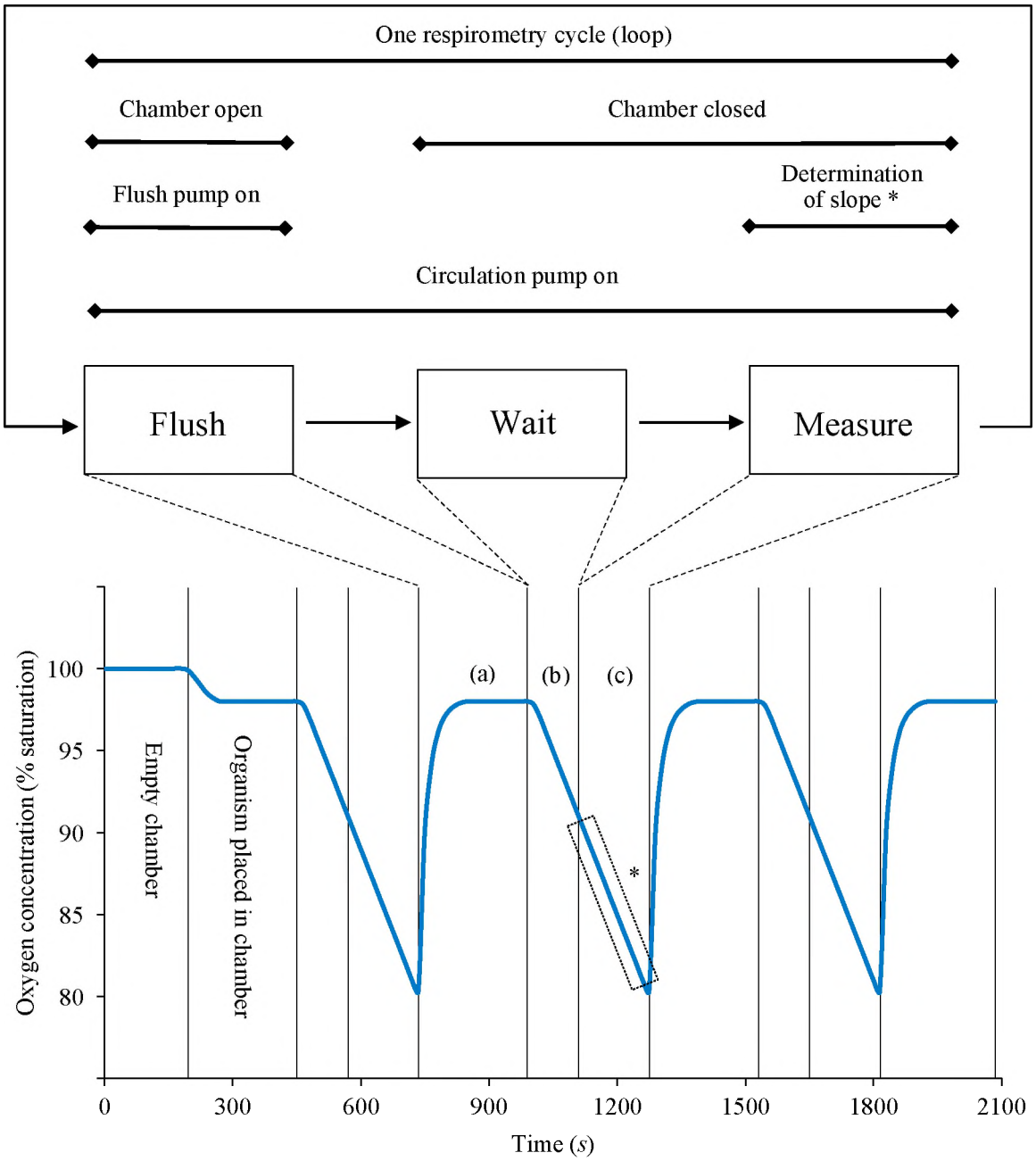


Figure 39. Intermittent-flow respirometry experiments consist of a series of repeated loops each composed of three distinct periods. During the flush period (a) the metabolic chamber is open and water exchange occurs driven by a flush pump. At the termination of this phase, the chamber is sealed, the flush pump shuts off and the circulation pump moves water either directly within the chamber or through a sub-sampling loop if the probe is not located in the chamber. The wait period (b) follows during which the flush water and chamber water are given time to mix and dissolved oxygen (DO) levels to equilibrate. The decline of DO in the chamber can be non-linear in this phase owing to mixing and the response time of the oxygen probe. The final phase is the measurement period (c) where the decline in DO concentration is generally linear and organismal oxygen consumption can be calculated from the slope of the linear decrease in oxygen content over time. The DO concentration at the end of the measurement period should not drop below 80% saturation to prevent hypoxia from affecting respiration rates. The cycle then repeats with the next flush period to flush metabolic waste products and replenish DO, which increases in an exponential manor (Svendsen et al., 2016). Figure adapted from Svendsen et al., (2016)

The metabolic chambers (Figure 41) were disc-shaped and constructed from a section of clear plexiglass tubing (50 mm tall, internal ∅ 100 mm, wall thickness 5mm). The section of tubing was aligned horizontally and solvent-welded centrally to a square plate (120 mm x 120 mm x 6 mm), which formed a base [Figure 41(*q*)]. A second plate had a circular aperture (∅ 110 mm) reamed into its centre and was solvent-welded in place, level with the upper surface of the tubing to form a collar [Figure 41(*o*)]. A third square plate had a circular disc of plexiglass (∅ 98mm, 4mm thick) welded to its centre [Figure 41(*u*)]. This served to hold a rubber O-ring [Figure 41(*v*)] in place and centre the square plate when placed as a lid. Four corner-mounted stainless-steel wing nuts [Figure 41(*n*)] pulled the lid plate and collar together, providing compression on the O-ring to seal the chamber. A perforated baffled plate (∅ 80 mm, 4 mm thick, 4 mm gap from lid) was attached centrally to the inside of the lid to diffuse water entering the chamber and to prevent abalone from blocking the inlet/outlet ports [Figure 41(*t*)]. Four ports in the lid (ID ∅ 5 mm) allowed for water movement in and out of the chamber [Figure 41(*r*)].

Opposite pairs of ports served as the inlet/outlet ports for the flush and recirculation water flow systems respectively (Figure 41). The flush system consisted of a submersible pump (Resun SP 500) [Figure 41(d)] pumping water along clear PVC tubing (internal ∅ 7 mm), through a non-return valve [Figure 41(*f*)] and into the chamber through an inlet port. Water then exited through the opposite outlet port, passing through a second non-return valve before exiting the tubing back into the respirometry tank. The recirculating system consisted of water being drawn from the metabolic chamber through the remaining outlet port using a second submersible pump [Figure 41(k)], then pumped within PVC tubing into a flow-through cylindrical probe chamber [Figure 41(l)] and returned to the metabolic chamber through further PVC tube [Figure 41(e)] and via the opposite inlet port. The probe chamber (plexiglass cylinder; internal ∅ 30 mm; height 30 mm; 11,75 ml volume with probe tip inserted) had a basal inflow port to direct incoming water directly onto the membrane of the galvanic oxygen probe, which was inserted 15mm into the chamber through an O-ring-sealed port at the top of the chamber. Water left the probe chamber through a wall-mounted outflow port.

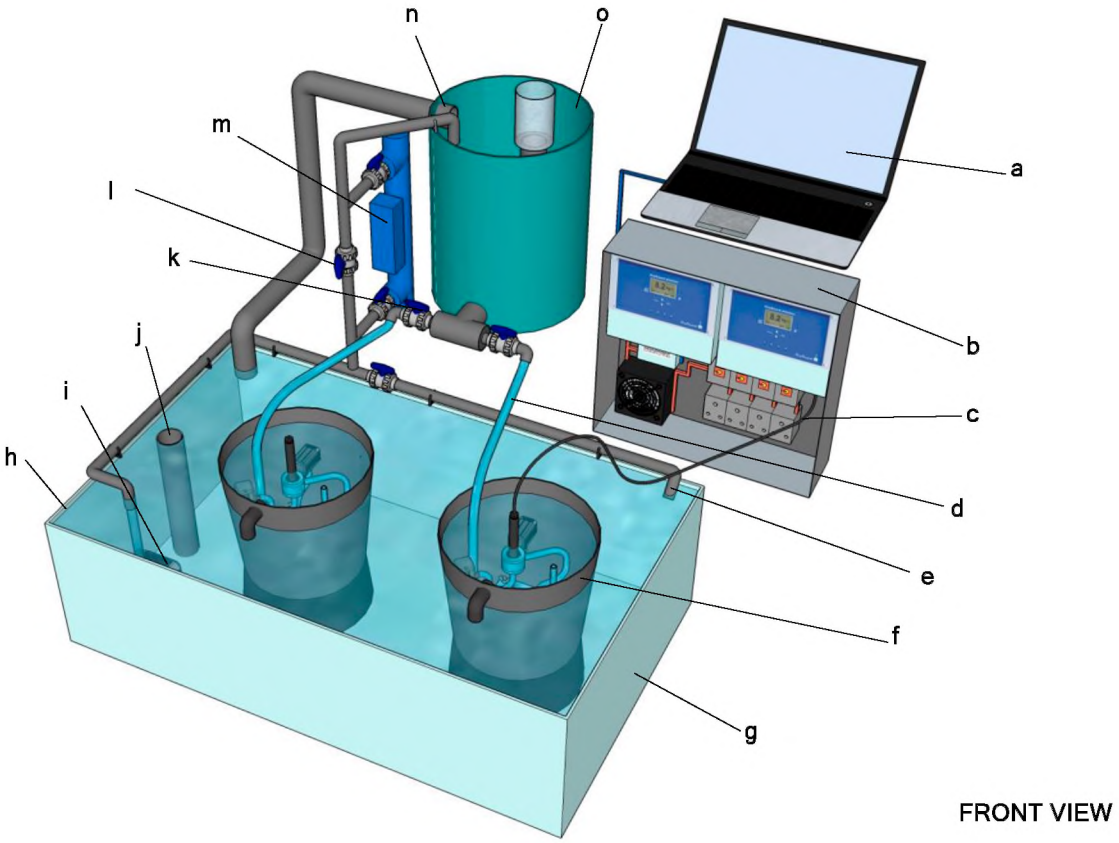
The respirometry system was operated and monitored through a multi-component control panel interfaced through a laptop computer. The dissolved oxygen (DO)

concentration in the water from the metabolic chamber passing through the probe chamber was measured using a Oxyguard® Mini galvanic, self-polarising, temperature-compensated probe [Figure 41(j)]. This probe was connected to an Oxyguard® Atlantic single-channel DO/temperature meter [Figure 40(p)]. This meter received signals from the external oxygen probe (mV) and integrated them with barometric pressure (internal barometer) and salinity (manual input) values to provide temperature-, pressure- and salinity-compensated dissolved oxygen readings (mg l^{-1} or % saturation) that could be displayed on the built-in display as well as output as an analogue signal (4-20 mA). The analogue output was transmitted to a Measurement Computing™ multifunction USB Data Acquisition (DAQ) device [Figure 40(u)], which converted the analogue signal to a digital signal that provided output to the laptop computer via USB interface. The DAQ unit also served to relay instructions from the laptop to four solenoid switches (Figure 40(r)], which controlled current supply to the four plug points [Figure 40(s)] from which the submersible pumps were powered. The control panel was powered using a multi-voltage Power Supply Unit (PSU) [Figure 40(v)], as used in desktop computer towers. Data logging of timestamps, cycle number, DO, temperature, period (as well as peripheral device control pumps) was managed through Visual Basic (VB) scripts run in Microsoft Xcel [Figure 40(a)]. VB code was adapted from the open source software package AquaResp (www.aquaresp.com).

4.2.3.2 Assumptions and calibration

Background respiration

The oxygen consumption rates of aquatic organisms obtained using respirometry techniques can be affected by background respiration from suspended and attached bacteria occurring within the experimental system. To reduce the contribution of bacterial respiration and provide a standard starting point for each trial, the respirometry system (RS) was cleaned prior to each trial according to the following protocol: (1) respirometry tanks and their associated metabolic chambers, PVC tubing and pumps were removed from the water bath and drained; (2) the tanks were filled with a 1% solution in warm water of Liquinox Critical-Cleaning Laboratory Detergent (Alconox Inc.; http://www.alconox.com) and allowed to circulate with both pumps running for 30 minutes; (3) tanks were drained of the solution and all components rinsed in running tap water; (4) tanks were then refilled with a 1:30 bleach (5.25% sodium hypochlorite) solution and allowed to circulate for a further 20 minutes and (5) all components were again rinsed with tap water before a series of three rinses with seawater. A pilot study was conducted to establish the background respiration in empty chambers following the above cleaning treatment and submersion for 72 hours in circulating seawater to approximate the mid-point in a 6-day respirometry trial. A flush/wait/measure (200 sec/80 sec/200 sec) protocol was employed for 250 cycles (33 h) in parallel chambers. The mean R^2 values over time for the slope of oxygen concentration in blank chambers during the measurement periods was 0.21 ± 0.18 and 0.30 ± 0.23 (n = 250) for chambers 1 and 2 respectively. Furthermore, the mean range of DO values over the measurement period in the chambers was 0.04 ± 0.02 and 0.05 ± 0.01 mg l^{-1} for chambers 1 and 2 respectively. This was well within the specified repeatability range of 0.08 ± 0.001 mg l^{-1} calculated for the DO probe used (OxyGuard® Technical Specifications indicate repeatability of ± 0.5% of measured value; mean chamber DO during trial of 7.51 ± 0.10 mg l^{-1}). These two points would indicate that within the time frame of measurement period used, fluctuations in DO in empty metabolic chambers were non-directional and within the expected inherent repeatability range for the DO probe used. Such background respiration was considered negligible and disregarded in later analyses.



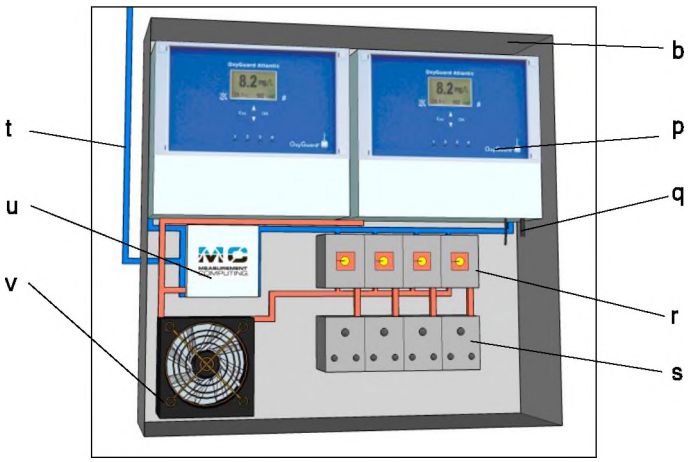


Figure 40. Three-dimensional plan of experimental system (front view) used to conduct respirometry trials on abalone. The system components are keyed as follows: (a) laptop computer, (b) control box, (c) oxygen probe cable, (d) respirometry tank water supply line, (e) bleeder valve return outlet, (f) respirometry tank, (g) water bath, (h) water level in water bath, (i) recirculating pump, (j) water bath upstand overflow, (k) respirometry tank supply control valve, (l) biological filter supply control valve, (m) UV steriliser, (n) biological filter overflow, (o) biological filter tank, (p) Oxyguard® Atlantic single channel dissolved oxygen(DO)/temperature meter, (q) DO probe input, (r) relay switch controlled power outputs to submersible pumps, (s) plug outlets to submersible pumps, (t) USB cable, (u) Measurement Computing™ multifunction USB Data Acquisition (DAQ) device and (v) multi voltage Power Supply Unit (PSU).

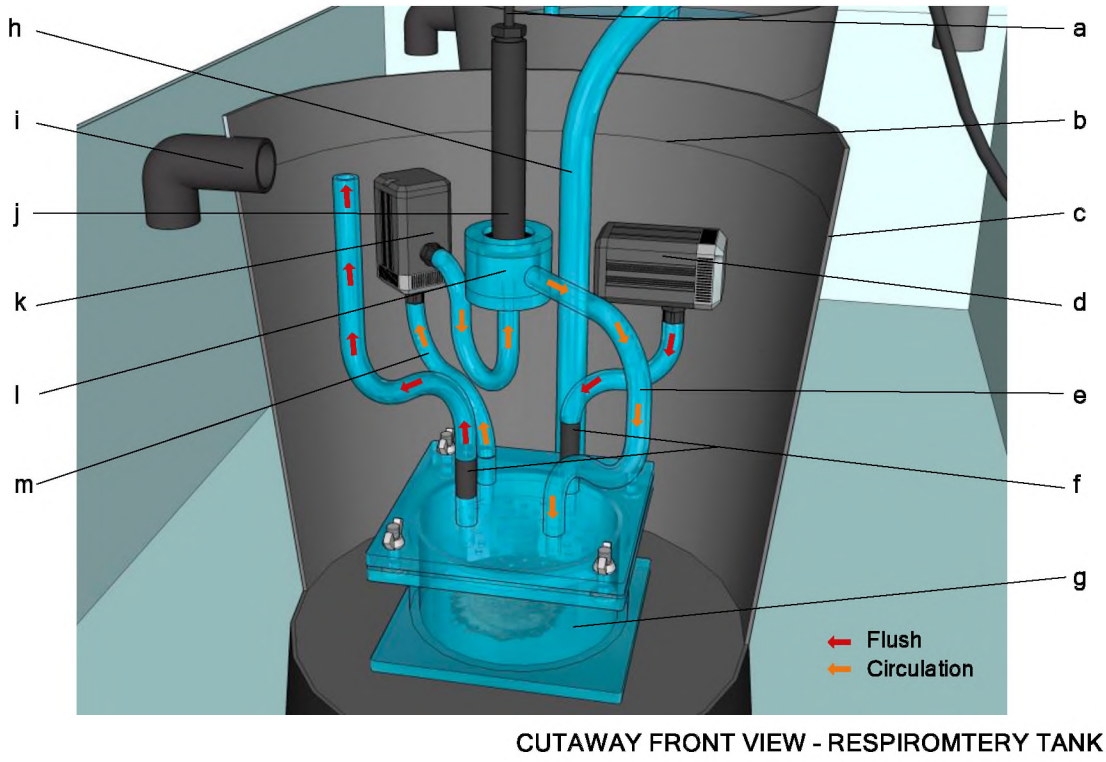


Figure 41. Three-dimensional plan of respirometry tank and chamber (front view) used to conduct respirometry trials on abalone. The system components are keyed as follows: (a) oxygen probe cable, (b) water level, (c) respirometry tank, (d) flush submersible pump, (e) circulating return piping line, (f) non-return valve, (g) plexiglass respirometer chamber, (h) respirometry tank water supply piping, (i) respirometry tank overflow, (j) Oxyguard® Mini dissolved oxygen probe, (k) circulating submersible pump, (l) flow-through DO probe chamber, (m) circulating outflow piping line, (n) stainless steel bolt and wingnut, (o) chamber collar plate, (p) abalone, (q) chamber base plate, (r) inflow/outflow nipples, (s) chamber lid, (t) baffle plate, (u) alignment disc and (v) O-ring.

Mixing and washout

The flush-pump flow rate of approximately 100 l h^{-1} resulted in a flush volume ratio of 20 over a 240-sec flush cycle. This is double the flush volume ratio of 10 recommended by Svendsen et al. (2016) to ensure a fractional wash-in of 99.9%. Therefore, it can be assumed that near-complete washout of nitrogenous waste was achieved. A dye plume test showed homogenous mixing of the recirculating water body within a 100 sec post-flush recirculation period.

Respirometry system volume determination

As dissolved oxygen in water is generally measured as a concentration (i.e. O_2 mass relative to volume; mg l^{-1}), the determination of the volume of water being measured is crucial for calculating oxygen uptake rates. The volume of each of the parallel respirometry systems (RS) (i.e. the metabolic chamber and associated recirculation plumbing and pump) was determined gravimetrically as follows:

1. The flush system inflow and outflow lines were sealed at the non-return valves and a dummy probe plug was inserted into the probe chamber.
2. The RS was submerged with the lid open in a freshwater bath and with the recirculation pump running.
3. All bubbles were dislodged for the chamber surface, and the pump and tubing walls.
4. The chamber lid was sealed underwater
5. The RS was removed from the water, the pump unplugged from the control panel, and the whole unit wiped dry with tissue paper
6. The RS was then placed on a rack in a convection drying oven at 40 C for 60 min.
7. The mass of the RS was determined using a digital scale.
8. The RS was dismantled and all water drained from the system, wiped dry using a paper towel and returned to the drying oven for a further 60 min.
9. The mass of the RS was again determined using a digital scale.
10. The volume of water in the system was calculated as follows:

$$V_{RS} = (M_{full} - M_{empty}) \div D_{fw}$$

where V_{RS} is the system volume in l, M_{full} is the mass (kg) of the RS when full of water, M_{empty} is the mass (kg) of the RS when empty and D_{fw} is the density of freshwater at 20°C (0.9982 g cm^{-3}).

The above methodology was repeated in triplicate for each chamber with volumes (mean ± SD) of 386.56 ± 0.1 and 379.71 ± 0.1 ml calculated for chambers 1 and 2 respectively. However, to accurately calculate the volume of water in the RS during experimental trials, it was necessary to determine the volume of abalone in the chamber, since this volume of water would be displaced. Donovan & Carefoot (1997) propose the following equation for determining the volume of individual abalone (V_A):

$$V_A = (M_{air} - M_{water}) \div D_{sw}$$

where M_{air} is the mass of the abalone in air, M_{water} is the mass of abalone in water and D_{sw} the density of seawater (1.025 g cm^{-3}). Unfortunately attempts to employ this method in this study were unsuccessful due to limited accuracy of available equipment. Donovan & Carefoot (1997) did not provide a mass volume ratio for *H. Kamtschatkana*, although recent work by Lee et al. (2015) describe a mass:volume relationship [weight (g) = 1.224 × volume (cm^3)] for *H. discus hannai* which was adopted in the current study as a proxy for converting *H. midae* mass to volume.

Probe calibration

The mV output from the OxyGuard® Mini galvanic probes were calibrated using the Oxyguard® Atlantic meter according to the manufacturer's instructions. Internal software regulation in the Atlantic meter prevented successful calibration if temperature or oxygen partial-pressure readings were unstable. A full calibration was undertaken before each trial was started.

4.2.3.3 Experimental protocol

A total of 9 experimental trials (n = 18 abalone) were conducted to determine the oxygen consumption rate of individual abalone over a 10-week period from weeks 68 – week 77. Animals from the three dietary treatments were selected as follows: (1) 10 individuals from the formulated diet (FD), with animals per replicate basket of 4:4:2; (2) 6 animals from the macroalgal diet (MD), with animals per replicate basket of 2:2:2 and (3) 2 animals from a single basket of the low-macroalgae combination diet (CML). The data from a further two CML animals were discarded due to anomalies arising from malfunction of the oxygen meter unit. No further trials were attempted after this point. Experimental timing is denoted in hours, centred on the time of feeding as T0.

Each experimental trial was initiated (T-96) with the gentle removal of two healthy abalone (i.e. no visible shell or foot damage) by hand from the general population in their treatment basket. Abalone were placed on a foam pad, their shells dried of excess water using a paper towel and weighed. Each abalone was then placed in an individual holding basket that was housed in a holding tank that formed part of the culture RAS. Abalone were not fed while in the holding baskets. At T-48, each abalone was removed from its holding basket and placed into a metabolic chamber. The oxygen consumption rate of the abalone was then measured continuously using intermittent respirometry for a period of 120 hours (T-48 – T72). A flush/wait/measure protocol of 240 sec/120 sec/200 sec was employed for abalone < 60 g and 240 sec/120 sec/140 sec for abalone >60 g. At T0, 48 hours after entering the metabolic chambers, abalone were fed a standard ration corresponding to their treatment.

During the last flush period prior to the onset of the dark phase in the CE room (T0), the lid of the metabolic chamber was removed and the feed introduced into the chamber with the abalone. Bubbles trapped in the feed parcels were removed prior to placing them into the metabolic chamber. Chambers were observed using a red-light source at 6 hours post-feeding (T6) to confirm that feed had been consumed. No visible feed was observed during these observations. At T72 the abalone were removed from their respective chambers, weighed again and measured to the nearest 0.01 mm using digital vernier callipers.

Data preparation

A linear regression was fitted to dissolved oxygen (Y) against time (X) data for each measurement phase. Where the R^2 value was < 0.9, indicating poor linearity in oxygen decline, the data for that measurement phase were excluded from any further analyses (Figure 43).

Determination of oxygen uptake rates

Absolute oxygen uptake rates were calculated for each measurement phase as follows:

$$M\mathrm{O}_2 = -S \times V$$

Where $M\mathrm{O}_2$ is the oxygen uptake rate (mg O_2 h^{-1}), S is the slope of the linear regression of the dissolved oxygen (Y; mg O_2 l^{-1}) and time (X; hour) data during the measurement phase and V is the volume of the metabolic chamber (l) corrected for abalone volume.

Due to variable growth rates between dietary treatments, there was marked variation in the size of abalone selected for respirometry trials (Table 19). The metabolic rate of an organism generally changes with body mass is a non-proportional manner (Nelson & Chabot, 2011), often described by the simple power function:

$$R = aM^b$$

Where R is the rate of metabolism, a is the scaling coefficient (antilog of the intercept in a log-log plot), M is body mass and b is the scaling exponent (slope of log-log plot) (Glazier 2014). To compare different sized organisms of the same species, a suitable scaling exponent needs to be applied to weight data when calculating metabolic rates. Extensive empirical research has pointed towards a general interspecific b value of 0.75 in both ectotherms and endotherms, often referred to as the ¾-power law, although a commonly accepted explanative mechanistic model remains elusive (Glazier, 2014; Nelson & Chabot, 2011; Savage et al., 2004; Schmidt-Nielsen, 1984). In the absence of data for the species being studied, using a b value of 0.75 would be acceptable, but if data are available, best practice would be to define a species-specific scaling factor (Nelson & Chabot, 2011). This was undertaken for abalone in the current study using $M\mathrm{O}_2$ data representing standard metabolic rate (SMR) as per the quantile method (p = 0.2) (Chabot et al., 2016b) over a size range of 39.89 – 95.98 g (Figure 42).

The calculated mass exponent (b) was 0.7539, which was applied for the determination of standardised mass-specific oxygen uptake rates in the current study as follows:

$$\dot{V}O_2 = ((-S \times V) \div M) \times (M \div M_S)^{(1-b)}$$

Where $\dot{V}O_2$ is the mass-specific oxygen uptake rate (mg O_2 kg^{-1} h^{-1}), S is the slope of the linear regression of the dissolved oxygen (Y; mg O_2 l^{-1}) and time (X; hour) data during the measurement phase, V is the volume of the metabolic chamber (l) corrected for abalone volume, M is the mean wet mass (kg) of the abalone as measured at the start and end of the experimental trial and Ms is the standardised mass calculated as the mean mass of all abalone measured to the nearest gram (64 g) (Steffensen et al., 1994; Stoffels, 2015).

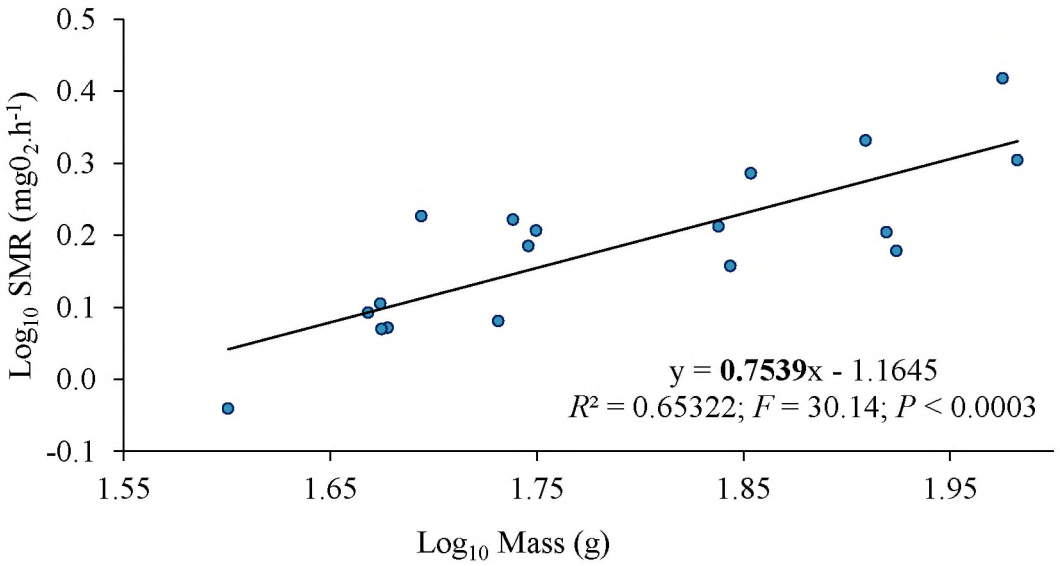


Figure 42. Log_{10} standard metabolic rate (SMR; $mg0_2$ h^{-1}) as a function of Log_{10} Mass (g) for the perlemoen *H. midae* (n = 18) in the weight range of 39.89 – 95.98 g. The mass scaling exponent for oxygen consumption was calculated using linear regression to be 0.7539 (R^2 = 0.65322; F = 30.14; P < 0.0003).

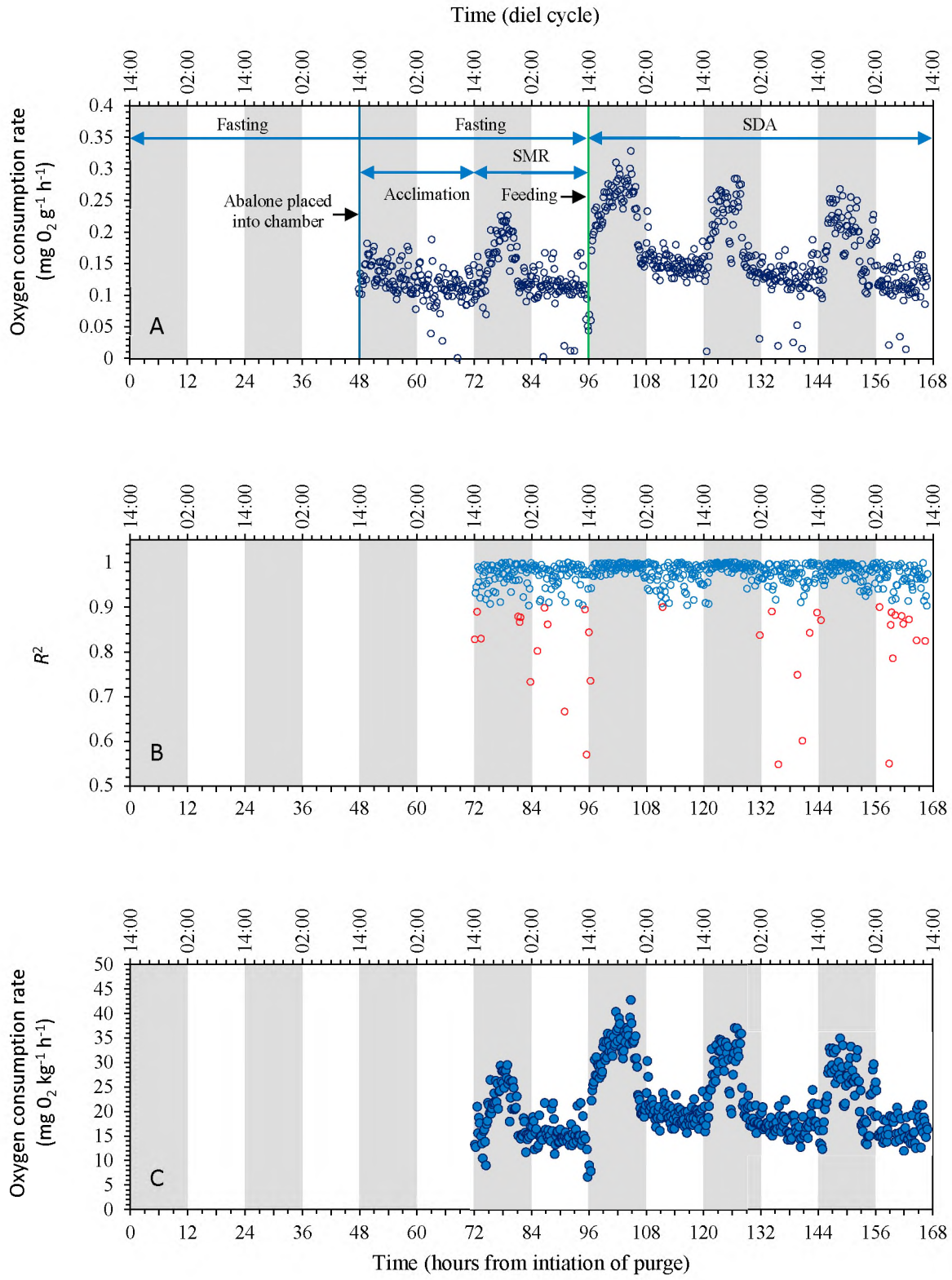


Figure 43. Timeline depicting key experimental phases and raw mass specific MO_2 data (A), R^2 values for the slope of the linear regression of the dissolved oxygen (Y; mg O_2 l^{-1}) and time (X; hour) data during the measurement phase of each intermittent respirometry cycle (B; red colour depicts those R^2 values below 0.9) and adjusted mass specific MO_2 data after the removal of data points where $R^2 < 0.9$ (C).

Standard Metabolic Rate (SMR)

Determining the standard metabolic rate of an organism (i.e. the minimum aerobic metabolic rate of a resting, post-absorptive and non-reproducing poikilotherm) of an organism is a prerequisite as a baseline for assessing any subsequent increases in metabolism associated with the consumption, digestion and absorption of a meal (Chabot et al., 2016b; Svendsen et al., 2016). Several points regarding experimental design and statistical analysis need to be considered when attempting to obtain accurate estimates of SMR using respirometry. Key practical issues related to the determination of SMR in this study, and the experimental approach undertaken to address these, are presented in Table 14. Furthermore, the statistical approach used to calculate SMR from a pool of MO_2 values requires careful consideration, especially when the animal exhibits spontaneous activity that is not recorded and corrected for. Selecting the lowest n number of values from a dataset of MO_2 values as an estimate of SMR (Crear & Forteath, 2000; Roche et al., 2013) tends to underestimate SMR, as it fails to take into account any temporal variation in SMR and/or measurement error (Chabot et al., 2016b). If it is assumed that when an animal is functioning at SMR, with occasional activity, then the dataset of MO_2 values will be normally distributed around the true SMR, mixed in with varied higher values above SMR due to spontaneous activity, for which a quantile approach would be suitable. The dataset can be split into the p smallest and 1 - p largest values, and a suitable p chosen to represent SMR (Chabot et al., 2016b). However, in a large dataset the distribution of MO_2 values will often be bi- or even multimodal, with values related to SMR forming one distribution, and those related to spontaneous activity a second one with a higher mean (Chabot et al., 2016b; Steffensen et al., 1994). Under these circumstances, the mean of the lowest normal distribution (MLND) provides a robust method for estimating SMR, with the variability of that distribution accounting for biological error and measurement error (Chabot et al., 2016b).

The 24-h period (T-48 – T-24) following the introduction of abalone into the metabolic chamber was considered an acclimation period and the data generated during this period were excluded from any further analyses. MO_2 measurements collected over the next 24-h period (T-24 – T0; 72 h post purge) were selected for the determination of SMR using the methodology proposed by Chabot et al. (2016b). These data were first

examined for any diurnal cycle. Over a full 24-h period, abalone exhibited a mean MO_2 that was 38 ± 19% (n = 18) higher during the dark phase compared to the light phase. Owing to this, data from the dark phase were excluded from further analyses related to SMR as it likely reflected high levels of spontaneous activity. The VO_2 from the light phase (T-12 – T0; 84 h post-purge), when abalone were generally quiescent, was used to determine SMR. Both the MLND method and the quantile method ($p = 0.2$) were calculated using the R-script *calcSMR.R* provided as Appendix I to Chabot et al. (2016b). In this script, the MLND is calculated using the function Mclust in the R-package mclust (Fraley et al., 2012), limited to a maximum of 4 distributions. According to Chabot et al. (2016b), the MLND is preferred as it is the only available method that does not assume a fixed number or proportion of values that are at or below SMR, and can therefore adjust the data without bias. It is considered a reliable estimator of SMR, but only when the coefficient of variation for the MLND ($C.V._{MLND}$) is low (< 5.4). In cases where $C.V._{MLND} > 5.4$, the quantile method ($p = 0.2$) is indicated.

Table 14. Key practical considerations for the accurate estimation of standard metabolic rate (SMR) (Chabot et al., 2016) and measures taken in the current study to mitigate potential confounding factors.

Practical considerations for the accurate determination of SMR	Experimental approach of current study.
Respirometer design	
Respirometry system should be capable of providing multiple MO_2 readings to elucidate fluctuations related to acclimation, diel cycle and activity.	Intermittent flow respirometry employed (minimum of 6 measurements h^{-1})
Metabolic chamber should be suitably sized to avoid stress, minimise excessive activity and provided optimum water quality.	Abalone could undergo a full range of movement in the chamber while minimising available travel distance. Shading of the chamber from direct light prevented refuge-seeking behaviour. A biological filtration system combined with UV sterilisation-maintained water quality.
Experiment duration	
A suitable acclimation period to allow for recovery from handling stress associated with transfer to the respirometer.	A 24-h acclimation period was instituted. Abalone were removed from holding baskets by initiating locomotion using a light source followed by rapid hand removal. Care was taken to avoid abalone clamping to the basket surface and the use of lifting tools. Weighing occurred rapidly in a darkened room.
Measurements should be conducted over a 24 h period to account for a diurnal cycle in MO_2.	Measurements were conducted over a 24 h period.
The fasting period should be sufficient to ensure the organism is in a post-absorptive state.	Abalone were fasted for 76 h prior to the initiation of SMR measurements. Owing to the diurnal cycle observed, only data from the light period were analysed, essentially giving a true fasting period of 88 h.
Water temperature should be controlled and animals thermally acclimated to experimental temperature.	The respirometry system was regulated at 18 °C in a controlled environment room. Abalone where cultured for 67 weeks at this temperature ensuring thermal acclimation.

Table 14 con.

Body Mass / life stage	
As metabolic rate scales non-proportionally with mass, an appropriate scaling coefficient needs to be applied if comparing animals of different sizes.	A metabolic scaling coefficient (0.7539) was applied to all data (refer section 4.2.3.2)
The ontogenetic stage of an organism can affect SMR.	All abalone tested were from the same spawning event.
Reproductive stage, and the associated metabolic costs of gonad development, can affect SMR.	The reproductive status of abalone could not be accurately determined as they were not sacrificed post-experiment.
Locomotor activity	
Spontaneous activity can cause errors in the estimation of SMR as locomotion can cause a 300 – 500% increase in $\dot{M}O_2$.	Abalone are generally sedentary during the light phase. $\dot{M}O_2$ measurements used to determine SMR were restricted to this quiescent period to reduce the effect of spontaneous activity.

Metabolic cost of feeding

The increased energy expenditure associated with meal ingestion and digestion has been studied extensively for well over 200 years (Secor, 2009). Originally coined 'specific dynamic action' or 'SDA' from Rubner's (1902) erroneously translated "spezifisch-dynamische wirkung", the postprandial metabolic response has since acquired multiple aliases. This occurred as researchers became aware of the multiple mechanical and biochemical processes associated with feeding (beyond Rubner's original limits of the ingestion and post-absorptive fate of protein, carbohydrates and fats), and took umbrage with the term "action" to describe what was the cumulative effect of these multiple processes (Secor, 2009). Alternative terms include, but are not limited to, heat increment of feeding (HIF), heat increment (HI), diet-induced thermogenesis (DIT), thermic effect of feeding (TEF) and postprandial thermogenesis (PPT). For the current study, the term "apparent SDA" is defined as the accumulated energy expended from the ingestion, digestion, absorption and assimilation of a meal (Jobling, 1994; Secor, 2009). The term 'apparent' is included to imply the total postprandial response, with no distinction between the mechanical and biochemical costs (Beamish, 1974; Secor, 2009).

Apparent SDA can be quantified according to a number of variables that define the profile of the response. These include baseline metabolism (generally SMR), peak, time to peak, duration, SDA, SDA scope and SDA coefficient (Figure 44, Table 15). Determining these variables is straightforward when the SDA response is short and the animal can be kept quiescent. Difficulties in estimating SDA become apparent when activity, either spontaneous or related to a diurnal rhythm, increase $\dot{M}O_2$ values over and above the SDA response after feeding. Failure to adjust for this activity can lead to inaccurate estimations of virtually all SDA variables (Figure 44B).

Table 15. Variables commonly used to define the profile of the SDA/apparent SDA response (Chabot et al., 2016a; McCue, 2006; Secor, 2009)

Variable	Definition
Baseline metabolism	Generally, the standard metabolic rate (SMR; i.e. minimum aerobic metabolic rate of a resting, post-absorptive and non-reproducing poikilotherm) is used. Routine metabolic rate (RMR; SMR plus normal spontaneous activity) is occasionally used when attempting to correct for activity during the measurement of SDA.
Peak	Maximum postprandial metabolic rate.
Time to peak	Time post-feeding/ingestion taken to reach maximum postprandial metabolic rate.
Duration	Time from feeding/ingestion to when metabolic rate returns to baseline metabolism.
SDA	Accumulated metabolic cost above the baseline metabolism for the duration of the SDA response (i.e. the area under the $\dot{M}O_2$ curve bounded by the baseline metabolism)
SDA coefficient	SDA energy (KJ) divided by meal energy.
SDA scope	SDA peak divided by baseline metabolism.

Variables illustrated in Figure 44.

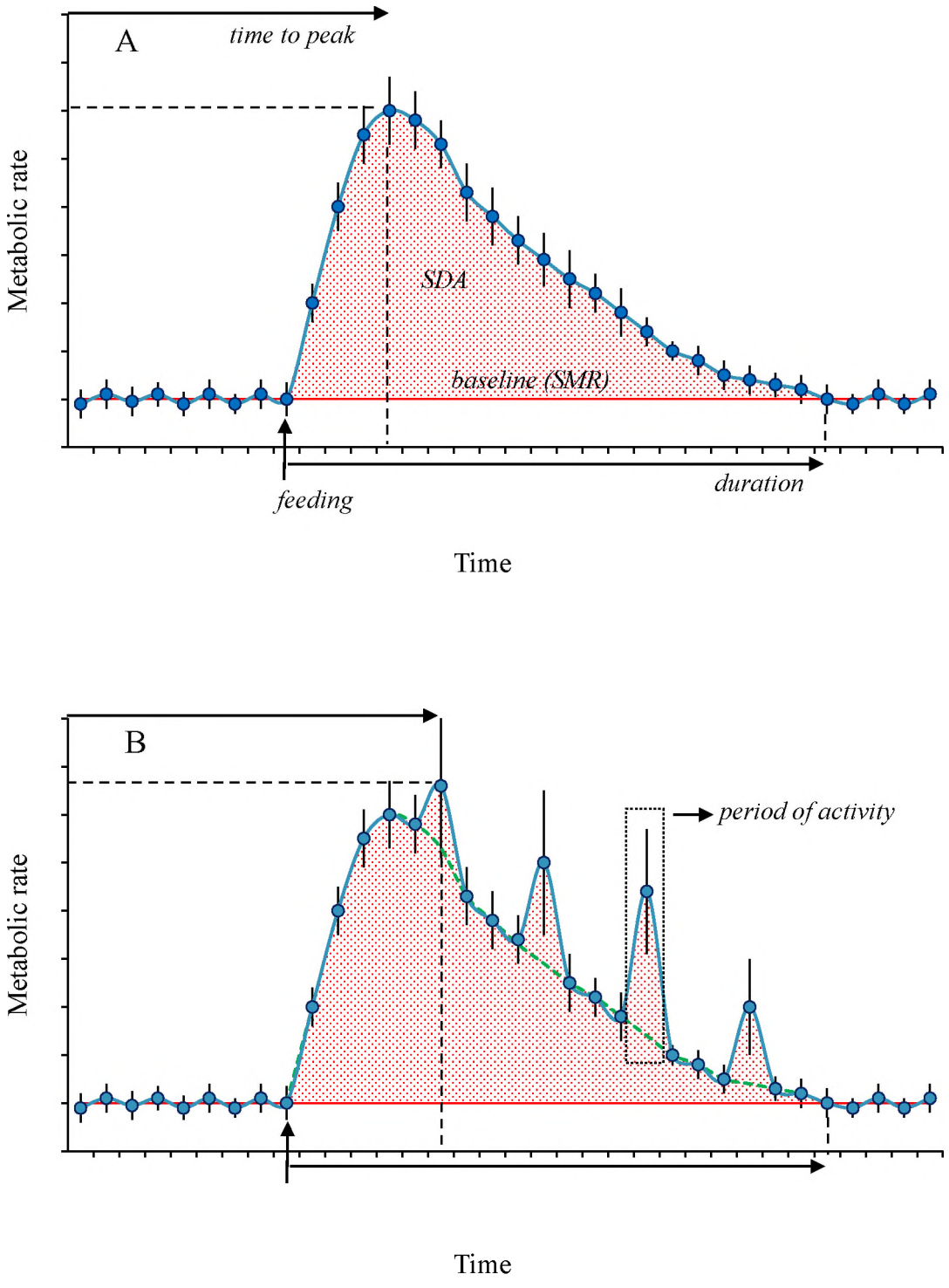


Figure 44. Hypothetical apparent SDA responses in the same organism, one showing a quiescent animal (A) and the same animal, with periods of spontaneous/diurnal activity (B). Data points are mean ± S.D. metabolic rate values calculated from repeated measurements over equal periods of time.

Abalone in the current study showed a marked preprandial diurnal cycle in $\dot{M}O_2$, which when combined with the long period of the SDA response (> 48 hours), indicated the need to assess the SDA response using a method that accounts for this activity. Previous studies have sought to tackle this problem by various means including: (1) using the RMR in place of SMR; (2) correcting SDA data for activity based on parallel unfed control animals; (3) correcting SDA data on an individual basis based on preprandial activity profiles and (4) implementing an enforced exercise regime (induced swimming) to standardise activity levels (Chabot et al., 2016a). These methods rely, however, on the assumption that activity before feeding (or in parallel unfed animals) is a valid proxy for postprandial activity or that enforced activity does not alter the SDA response. Chabot et al. (2016a) provide an alternative robust methodology, centred on quantile regression, which effectively corrects for the effect of periods of activity on the estimation of the SDA response. Briefly, the reasoning behind their approach is follows. After feeding, any $\dot{M}O_2$ reading will be the combination of oxygen demand (O) as follows:

$$\dot{M}O_2 = O_{SMR} + O_{SDA} + O_A + V$$

where O_A is activity and V is variability. In any given postprandial plot of $\dot{M}O_2$ against time, the lowest values are assumed to represent periods of very minimal or no activity:

$$\dot{M}O_{2\,LOW} = O_{SMR} + O_{SDA} + V$$

Chabot et al. (2016b) contend that the $\dot{M}O_2$ of a quiescent animal varies around a mean (SMR) punctuated with some periods of elevated $\dot{M}O_2$ related to activity. A quantile approach was shown to be a valid method to estimate SMR given these assumptions. Quantile regression extends the idea of sample quantiles to the estimation of conditional quantile functions depending on covariates in the same way that least-squares regression extends sample means to conditional mean functions. A non-parametric approach is employed as it does not infer the shape of the SDA curve, with the penalty method of Koenker et al. (1994) considered best suited. Chabot et al. (2016a) implement their

aproach through the function rqss in the R-package quantreg and provide scripts (R language) for estimating and plotting SDA, as Appendix S1 to their study.

The analysis is dependent on two inputs when defining the conditional quantile functions namely: (1) λ which is the penalty parameter and; (2) τ which is the quantile of interest. Chabot et al. (2016a) provide the following reccomendations for choosing appropriate values for τ and λ. The choice of τ is dependent on the method used to detrmine SMR, with $\tau = p$ if the quantile method was used, or alterntaively 0.2 or 0.25 are suitable if the MLND was employed. λ should be higher than duration of activity cycles (i.e. 12 if there is a distinct diurnal pattern), although this value is not always impervious to activity cycles when τ is 0.2. A range of 24 – 30 is suggested, with 30 being especially resistant to activity cycles. As the quantile method ($p = 0.2$) was used to determine SMR in the current study, and a distinct daily cycle in MO_2 were evident, a τ of 0.2 and λ of 30 were used to determine the profile of the SDA response.

4.2.4 Determination of total ammonia nitrogen (TAN) excretion.

4.2.4.1 Experimental system

The total ammonia nitrogen (TAN = NH_3-N + NH_4^+-N) excretion rate of abalone was determined using timed-interval water sampling of closed incubation chambers. The experimental system consisted of 36 bucket-shaped PVC incubation chambers (1080 ml volume) [Figure 45(j)] suspended in two linked water baths [Figure 46(d)]. Each chamber was fitted with a clip-on lid to prevent abalone escaping the chamber. The lid had two holes (∅ 6 mm) to allow PVC tubing supplying water and air to pass through as well as two openings (∅ 20 mm) for water overflow (Figure 46). An air stone in each chamber provided mild aeration to ensure oxygen saturation during incubation and provide adequate mixing [Figure 46 (m)]. Water to each chamber was supplied by gravity via PVC hose (outer ∅ 6mm) from a central header tank [Figure 45 (l)]. The header tank was supplied with water from the culture RAS (post-treatment by biological filter and UV sterilization unit) [Figure 45(b)] that was passed through a second biological filter (25 L; air fluidised plastic media; outflow felted-screen of ~ 100

micron) [Figure 45(d,p)]. An independent base outlet for each hose from a shared header tank, and equal length tubing to each chamber, ensured equal water flow rates to each chamber. During the flush phase the water in the chamber was exchanged every 15 mins. Water overflow from the chambers entered the water baths and drained to the culture RAS. Water flow from the culture RAS through the water baths (surface inflow / base outflow) ensured mixing of the water column in the water baths [Figure 45(h,n)]. The system was housed in a controlled environment room to regulate water temperature (18 °C) and photoperiod (12L:12D).

4.2.5 Experimental protocol

A trial was conducted to test the effect of feeding on the TAN excretion rate of abalone fed three diets namely mixed macroalgae (MD), a formulated pellet (FD) and a combination diet (CML). Using timed-interval water sampling from a closed incubation chamber, the TAN excretion rate of an abalone could be determined as follows:

$$\text{TAN excretion (mg g}^{-1}\text{ h}^{-1}) = (TAN_{\text{final}} - TAN_{\text{initial}}) \times V) \div (W \times T)$$

Where TAN_{final} is the TAN concentration in the sample (mg l^{-1}) at the end of the incubation period; TAN_{initial} is the TAN concentration in the sample (mg l^{-1}) at the start of the incubation period; V is the volume of water in the experimental chamber (l) adjusted for abalone volume using a mass:volume ratio of 1.224 (Lee et al., 2015); W is the mass of the abalone (g; mean of values determined at the start and end of the trial); and T is the duration of the measuring period (h).

Of the 36 experimental chambers, twelve were allocated to each dietary treatment. One served as a system control and remained empty throughout the trial. A further nine chambers were stocked with abalone from each of the three dietary treatment replicate baskets (three abalone per basket) and were fed during the trial. The remaining two chambers were each stocked with a single abalone from two of the dietary treatment baskets and served as a duplicate non-feeding control. Sampling times are denoted in hours, centred on the time of feeding as T0.

The trial was initiated (T-96) with the gentle removal of healthy abalone (i.e. no visible shell or foot damage) by hand from the general population in their treatment basket.

Abalone were placed on a foam pad, their shells dried of excess water using a paper towel and weighed. Animals, separated by treatment, were then placed in holding baskets housed in a holding tank that formed part of the culture RAS. Abalone were not fed while in the holding baskets. At T48 abalone were removed from their holding basket and placed into the incubation chambers to acclimate. Preprandial baseline TAN excretion rates were determined for all abalone in both the light and dark phase. Water flow to the chamber was shutoff at T-18 (dark phase), a 10ml water sample taken, an incubation period of 4 hours allowed to pass and a then a second water sample taken at T-14. This was repeated at T-6 – T-2 (light phase).

At T0, 48 hours after being placed into chambers, abalone were fed the diet corresponding to their treatment. The protein content of each sample was estimated using mass-spectrophotometry derived N values converted to protein content using the N-Prot factors derived by Lourenço & Barbarino (2002) for the taxonomic groupings of red (n = 9, N-Prot 4.59), green (n = 6, N-Prot 5.13) and brown (n = 4, N-Prot 5.38) macroalgae. A N-Prot factor of 6.25 was used for the formulated feed sample. The TAN excretion rate was then measured postprandially over the following incubation periods: T6 - T9, T10 - T12, T16 - T19, T28 - T31, T40 - T43 and T64 - T67. Water samples taken at each sampling event were either processed immediately or frozen at -16 °C for up to 7 days, within the recommended maximum storage period of 2 weeks (Parson et al., 1984). The TAN concentration in water samples was determined using the indophenol method (refer section Appendices: Appendix 3). Following the last measurement period, abalone were removed from the chambers and weighed as at the start of the trial, and shell length measured using digital callipers.

To compare TAN excretion between abalone of variable mass in the absence of a suitable data-derived scaling exponent, a general scaling exponent (b) of 0.75 was applied to standardise TAN excretion rates (mg g^{-1} h^{-1}) to a common mass as follows:

$$\text{Standardised TAN excretion rate} = (TAN_E) \times (M \div M_S)^{(1-b)}$$

Where TAN_E is the mass-specific TAN excretion rate (mg g^{-1} h^{-1}), M is the mean wet mass (kg) of the abalone as measured at the start and end of the experimental trial and Ms is the standardised mass calculated as the mean mass of all abalone measured to the nearest gram (87 g).

The profile of postprandial TAN excretion was characterised as per the variables defined in Table 16.

Table 16. Variables employed to describe the postprandial total ammonia nitrogen (TAN) excretion dynamics of the abalone *H. midae*.

Variable	Definition
Baseline TAN excretion rate	Mean of two postprandial TAN excretion rates (light & dark phase).
Cumulative TAN excretion rate	Area under the TAN excretion curve bound by the baseline TAN excretion rate and a set time-period (first to last postprandial TAN excretion rate measurement; T6 – T67)
Peak	Maximum postprandial TAN excretion rate measured.
Scope	TAN excretion peak divided by baseline TAN excretion rate.
Nitrogen (N) coefficient	Total nitrogen excreted divided by meal nitrogen content.

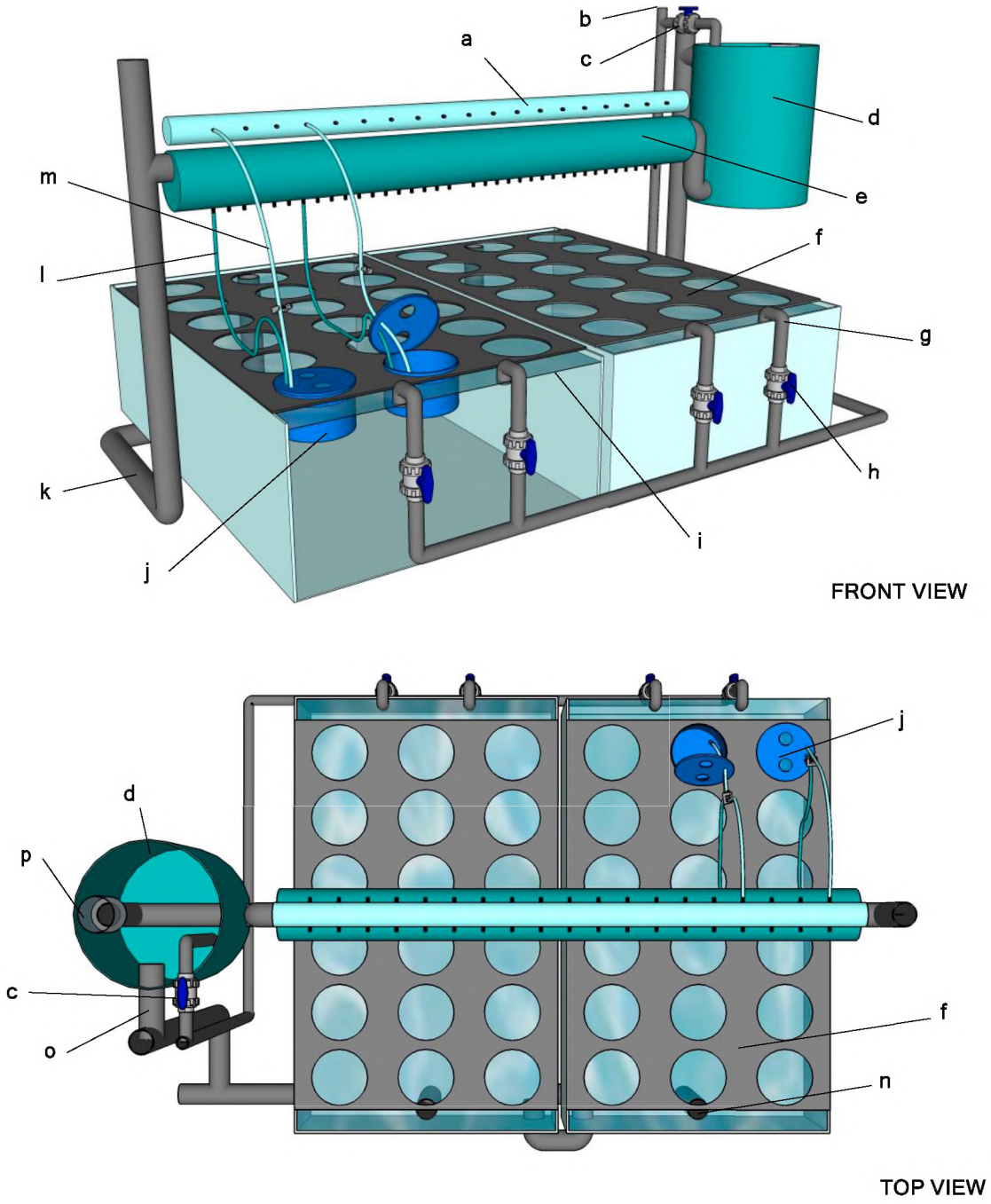


Figure 45. Three-dimensional plan (front and top view) of the of the experimental system used to house abalone for ammonia excretion and haemolymph glucose trials. The system components are keyed as follows: (*a*) air supply manifold, (*b*) water supply inlet from main recirculating aquaculture system, (RAS) (*c*) biological filter inflow control valve, (*d*) biological filter (*e*) water supply manifold, (*f*) experimental chamber support manifold, (*g*) water bath inflow, (*h*) water bath flow control valve, (*i*) water level, (*j*) experimental chamber, (*k*) overflow drainage to main RAS, (*l*) tubing water supply line, (*m*) tubing air supply line, (*n*) water bath overflow, (*o*) biological filter emergency overflow and (*p*) mesh screen over biological filter overflow draining to supply manifold.

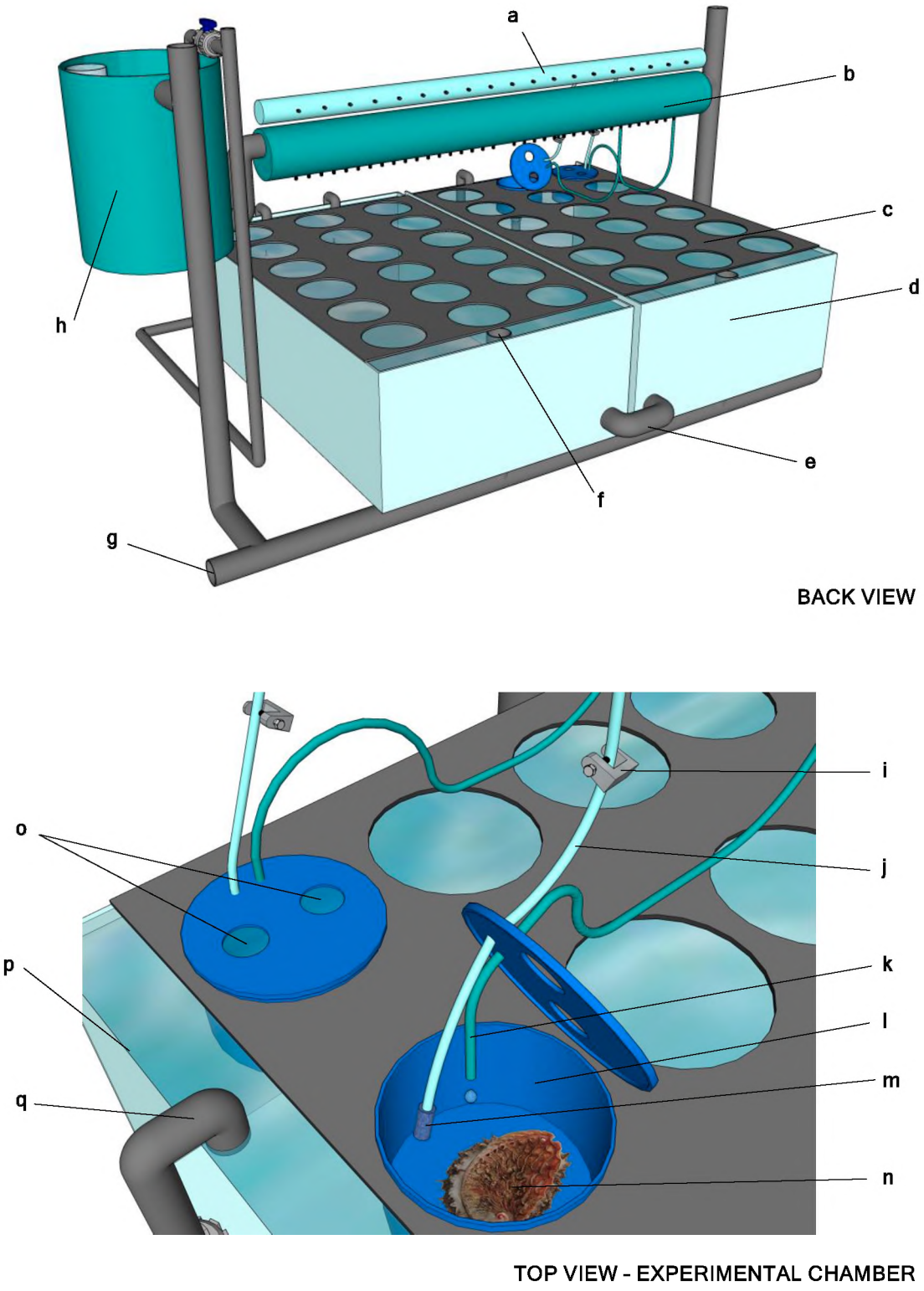


Figure 46. Three-dimensional plan (back and top view) of the of the experimental system used to house abalone for ammonia excretion and haemolymph glucose trials. The system components are keyed as follows: (a) air supply manifold, (b) water supply manifold, (c) experimental chamber support manifold, (d) water bath, (e) linking plumbing, (f) overflow drainage, (g) drainage outlet, (h) biological filter, (i) air flow regulation clamp, (j) tubing air supply line, (k) tubing water supply line, (l) experimental chamber, (m) airstone, (n) abalone on floor of chamber, (o) openings in lid to allow water overflow and introduction of feed, (p) water level and (q) water bath inflow.

4.2.6 Determination of haemolymph glucose concentration (HGC).

4.2.6.1 Experimental system

Abalone were housed in the same 36-chamber experimental system (ES) and under the same conditions as described for the ammonia excretion trial (refer section 4.2.4.1). Each chamber (1080 ml volume) received continuous water flow during the experiment at a rate of 4 exchanges h^{-1}.

4.2.7 Experimental protocol

A trial was conducted to test the effect of feeding on the haemolymph glucose concentration of previously fasted abalone fed three diets namely mixed macroalgae (MD), a formulated pellet (FD) and a combination diet (CML). Each trial used a total of 52 abalone sampled randomly and at equal proportions from the replicate baskets for each treatment. At each sampling time, haemolymph samples were obtained from abalone and analysed for HGC per the method detailed in Appendix 4. In brief, this involved extracting haemolymph samples from the pedal sinus using a hypodermic needle and analysis for HGC using an enzymatic assay. Sampling times are denoted in hours, and centred on the time of feeding as T0.

At T-96, all abalone in treatments to be tested were moved, along with their vertical culture racks to clean baskets housed in the general culture RAS, essentially removing animals from any contact with feed and initiating a fast period. Four abalone per sampling time were sampled at T-76, T-48 and T-24. In addition, at T-24, 36 abalone were randomly selected in equal proportion from the treatment baskets and transferred to the chambers in ES. The chambers were randomly allocated to receive feed (n = 20) or remain as unfed controls (n = 16) to allow postprandial sampling at T4, T8, T12, T16 and T32, according to the sampling protocol described in Appendix 4.

The baseline HGC value was determined at T0 as the mean of 96h fasted abalone sampled from the basket (n = 4) and experimental chamber (n = 4). At T0, 24 hours after being placed into chambers, abalone were fed the diet corresponding to their treatment. The carbohydrate content (non-nitrogen extract; NNE) and glycaemic carbohydrate proportion were estimated based on proximate analyses conducted during the growth trial (refer Chapter 3). Separate trials for each dietary treatment were run sequentially over a 12-day period in week 98 and week 99. The profile of postprandial TAN excretion was characterised per the variables defined in Table 18.

Table 17. Sampling protocol for the determination of haemolymph glucose concentration (HGC) in the abalone *H. midae* over a 128-hour period (96 h fasting; 32 h postprandial). Sample timing is centred on the introduction of feed at T0 (96 h post initiation of fast)

Time	Abalone habitat		
	Basket	Experimental chamber	
	Unfed control (n)	*Fed (n)*	*Unfed control (n)*
T-96	(initiation of fast)		
T-72	4		
T-48	4		
T-24	4		
T0	4		4
T4		4	3
T8		4	3
T12		4	2
T16		4	2
T32		4	2

Table 18. Variables employed to describe the postprandial haemolymph glucose concentration (HGC) response of the abalone *H. midae*.

Variable	Definition
Baseline HGC	Combined mean of animals (n = 8) fasted for 96 h.
Cumulative HGC response	Area under curve (AUC) of HGC plotted against time (h) bound by the baseline HGC over a set time-period (T0 – T32)
Peak	Maximum postprandial HGC measured.
Scope	HGC peak divided by baseline HGC.
Glycaemic carbohydrate coefficient	Cumulative HGC response (AUC) divided by total glycaemic carbohydrates consumed (% BW).

4.2.8 Statistical analyses.

If not otherwise stated, values presented are mean ± standard deviation. Linear regression was used to describe the relationship between fasting time (X) and haemolymph glucose concentration (Y). A student's t-test was used to test for differences when two independent groups were being compared, while multiple groups ($n > 2$) were compared using Analysis of Variance (ANOVA) with post-hoc analysis using Tukey's HSD test. In cases where Levene's test revealed heterogeneous variance, the non-parametric Mann-Whitney U-test was used to compare two groups and the Kruskal Wallis H-test to compare multiple groups, with post-hoc analysis using the multiple comparisons of mean ranks tests. All statistical tests were performed using Statistica 13 for Windows (StatSoft, 2016) at the 0.05 level of significance.

4.3 Results

4.3.1 Oxygen consumption

4.3.1.1 Standard metabolic rate

When the MLND method was employed to calculate the standard metabolic rate (SMR) of abalone, all $C.V._{MLND}$ values, bar one individual, were above the recommended upper limit of 5.4 recommended by Chabot et al. (2016b). As such, SMR values presented were calculated using the alternative quantile method ($p = 0.2$). The SMR of all abalone measured ranged from 19.36 – 31.97 mg O_2 kg^{-1} h^{-1} (Table 19). There was no significant difference between the SMR values for the FD and MD treatments ($P = 0.79$, $t = 0.26$). The two individuals from the CML treatment were located towards the lower end of the range.

Table 19. Apparent SDA response variables for abalone fed a mixed macroalgal diet (MD), a formulated pellet (FD) and a combination diet (CML) of macroalgae (25%) and formulated feed (75%).

Treatment	Mass[1] (g)	Ration[2] (% BW)	Ration energy[3] (Total KJ / KJ g^{-1})	Apparent Specific Dynamic Action (aSDA)						
				SMR[4] ($\dot{V}O_2$)	Peak ($\dot{V}O_2$)	Time to Peak (h)	Duration (h)	SDA[5] (mg O_2 Kg^{-1} / Total KJ)	SDA Coefficient (%)	SDA Scope
Formulated										
FD - 1	49.45	0.53	5.05 / 0.10	31.97	50.52	0	59.0	381.7 / 0.27	5.27	1.58
FD - 2	55.71	0.52	5.59 / 0.10	26.58	48.49	0	71.5	473.8 / 0.37	6.66	1.82
FD - 3	47.26	0.49	4.52 / 0.10	25.02	37.77	0	68.2	465.5 / 0.31	6.87	1.51
FD - 4	54.78	0.49	5.23 / 0.10	29.21	54.67	0	66.0	516.7 / 0.40	7.63	1.87
FD - 5	39.89	0.49	3.78 / 0.09	20.43	38.71	5.80	61.0	495.3 / 0.28	7.37	1.89
FD - 6	47.65	0.47	4.32 / 0.09	20.96	36.52	0	34.5	370.4 / 0.25	5.76	1.74
FD - 7	56.19	0.48	5.23 / 0.09	27.73	45.85	6.80	71.5*	640.0 / 0.51	9.69	1.65
FD - 8	53.88	0.50	5.23 / 0.10	21.18	37.00	7	69.5	554.9 / 0.42	8.06	1.75
FD - 9	46.62	0.52	4.69 / 0.10	24.56	37.93	0	70.0	434.6 / 0.29	6.09	1.54
FD - 10	47.32	0.47	4.32 / 0.09	23.13	44.38	26	70.0*	969.3 / 0.65	14.96	1.92
	49.87 ± 5.2[a]	**0.50 ± 0.02[a]**	**4.8 ± 0.56[a] / 0.10 ± 0.00[a]**	**25.08 ± 3.83[a]**	**43.18 ± 6.52[a]**	**4.45 ± 8.1[a]**	**64.1 ± 11.3[a]**	**530.2 ± 173.5[a] / 0.37 ± 0.13[a]**	**7.83 ± 2.80[a]**	**1.73 ± 0.2[a]**
Macroalgae										
MD – 1	82.98	0.50	5.01 / 0.06	20.54	49.23	6	71.5*	877.5 / 1.04	20.85	2.40
MD – 2	81.08	0.51	5.01 / 0.06	28.03	53.37	5.8	71.5*	712.2 / 0.83	16.54	1.90
MD – 3	68.83	0.60	5.32 / 0.08	24.10	52.58	5.5	69.5	858.9 / 0.85	15.92	2.18
MD – 4	71.36	0.59	5.51 / 0.08	27.80	56.18	0	66.5	576.9 / 0.59	10.71	2.02
MD – 5	94.50	0.63	5.32 / 0.06	30.48	60.42	0	70.8	704.2 / 0.95	17.92	1.98
MD – 6	95.98	0.64	5.51 / 0.06	22.60	46.04	0	71.5*	643.7 / 0.89	16.07	2.04
	82.45 ± 11.3[b]	**0.58 ± 0.1[b]**	**5.28 ± 0.23[a] / 0.07 ± 0.01[b]**	**25.59 ± 3.78[a]**	**52.97 ± 5.06[b]**	**2.9 ± 3.2[a]**	**70.2 ± 2.0[a]**	**728.9 ± 118.5[b] / 0.86 ± 0.15[b]**	**16.33 ± 3.31[b]**	**2.09 ± 0.2[b]**

Table 19 con.

Combination										
CML1	83.90	0.50	7.48 / 0.09	19.36	43.68	5.5	71.5*	920.0 / 1.09	14.60	2.26
CML2	69.74	0.49	6.21 / 0.09	21.93	47.93	0	71.5*	1058 / 1.04	16.80	2.19
	76.82 ± 10.0	**0.49 ± 0.0**	**6.85 ± 0.89 / 0.09 ± 0.00**	**20.65 ± 1.82**	**45.81 ± 3.01**	**3 ± 4.2**	**71.5**	**920.0 ± 97.6 1.07 ± 0.03**	**15.70 ± 1.55**	**2.22 ± 0.1**
Total Range	*39.89 – 95.98*	*0.47 – 0.64*	*3.78 – 7.48 / 0.06 – 0.10*	*19.36 – 31.97*	*36.52 – 60.42*	*0 - 26*	*34.5 – 71.5*	*370.4 – 1058.0 / 0.25 – 1.09*	*5.27 – 20.85*	*1.51 – 2.40*
Total Mean (± S.D.)	**63.73 ± 17.74**	**0.52 ± 0.05**	**5.19 ± 0.80 / 0.08 ± 0.02**	**24.76 ± 3.80**	**46.74 ± 7.20**	**3.80 ± 6.30**	**66.97 ± 8.9**	**647.4 ± 211.6 / 0.61 ± 0.31**	**11.54 ± 5.08**	**1.90 ± 0.25**

Values in bold are treatment mean ± S.D. unless otherwise stated. [1]Mean of wet weight values determined at the start and end of the trial; [2] dry weight of feed as a % of body weight; [3] Diet energy is sum of crude protein (23.6 KJ g^{-1}), lipid (39.5 KJ g^{-1}) and carbohydrate (NNE; 17.2 KJ g^{-1}) as proportional contribution of dry weight as determined by proximate analysis (see Chapter 3, section 3.1) (Bureau et al., 2002), ; [4] SMR calculated using the quantile method (p = 0.2) (Chabot et al., 2016b); [5] SDA (mg O_2 kg^{-1}) calculated as the area under the $\dot{V}O_2$ curve bounded by SMR and converted to SDA (KJ) using: (1) Diet-specific oxygen conversion factors (KJ per l of 0_2 consumed) calculated using the proportional contribution of protein (19.2 KJ l^{-1}), lipid (19.8 KJ l^{-1}) and carbohydrate (NNE; 20.9 KJ l^{-1}) in each diet (Gessaman & Nagy, 1988), and (2) a conversion factor for mg O_2 : ml O_2 of 0.700 based on a molar volume at STP of 22.391 and molar weight of oxygen of 31.998 g . * denotes SDA curves that did not reach the SMR within the postprandial measurement period.

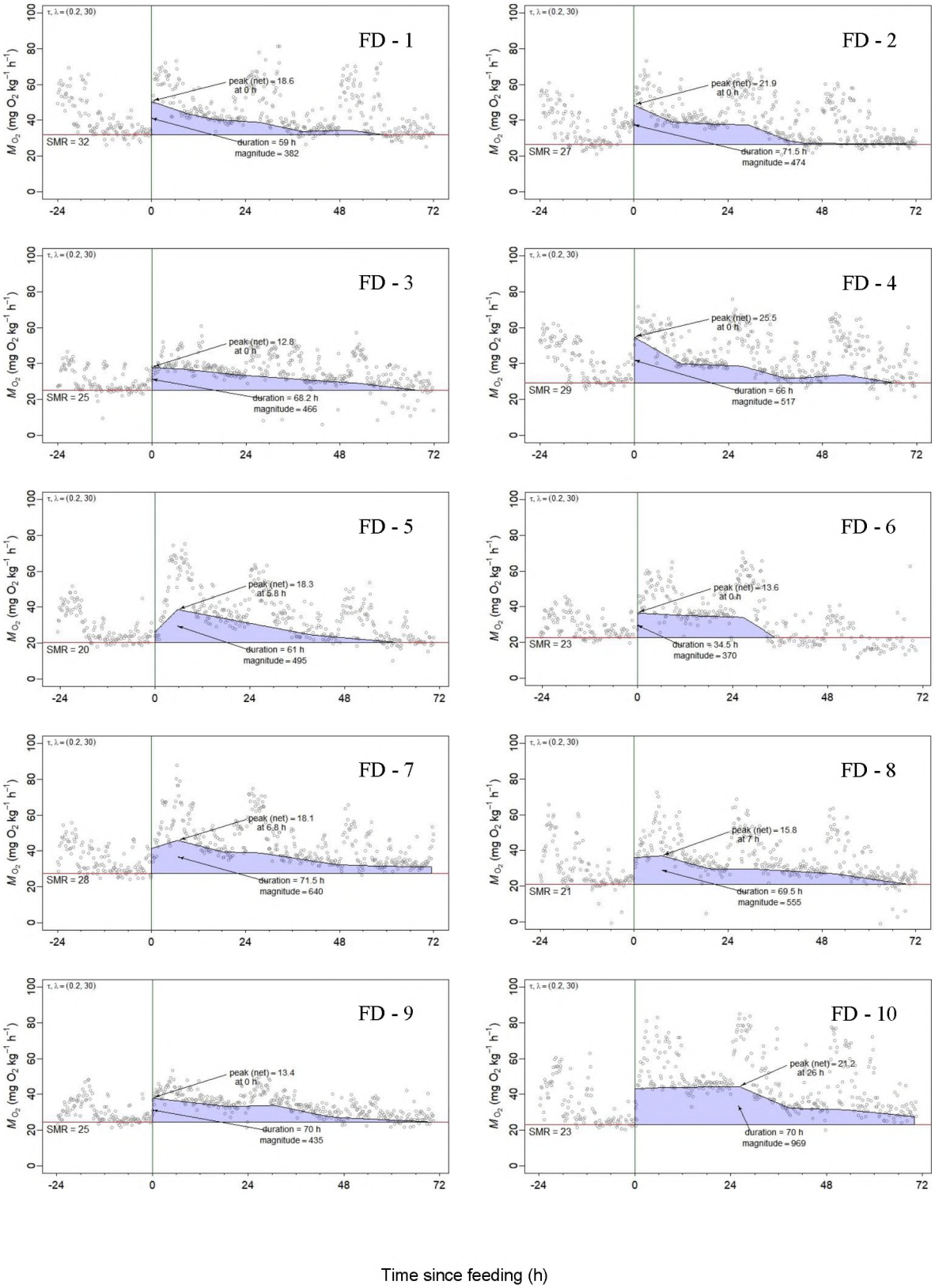


Figure 47. Apparent SDA response of abalone fed a formulated pellet (FD). Blue shaded areas define the area under the $\dot{V}O_2$ curve bounded by the baseline metabolism (i.e. the accumulated metabolic cost for the duration of the SDA response). The $\dot{V}O_2$ curve was determined using the quantile regression method ($\tau = 0.2$; $\lambda = 30$) described by Chabot et al. (2016b).

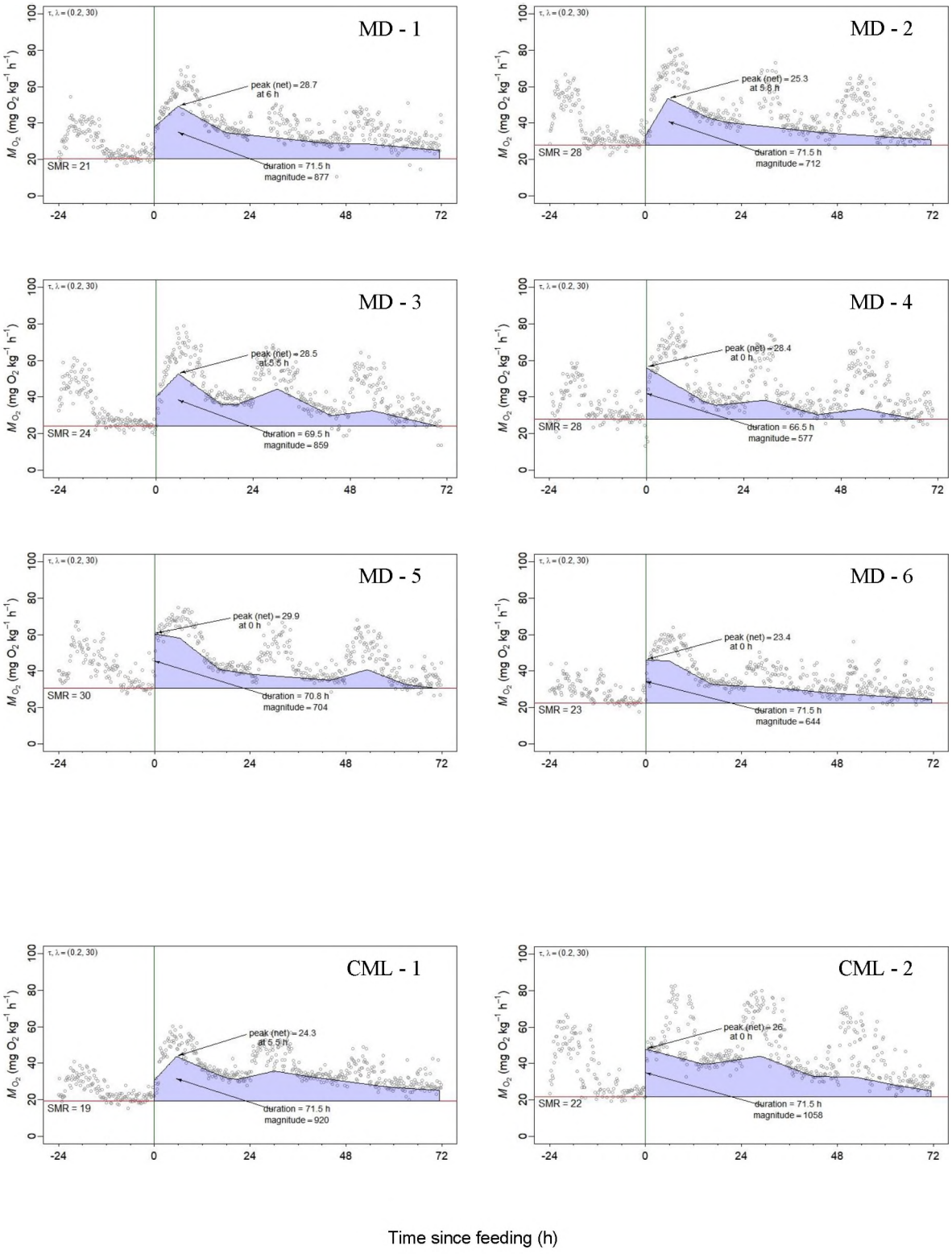


Figure 48. Apparent SDA response of abalone fed macroalgae (MD) and a combination diet (CML). Blue shaded areas define the area under the $\dot{V}O_2$ curve bounded by the baseline metabolism (i.e. the accumulated metabolic cost for the duration of the SDA response). The $\dot{V}O_2$ curve was determined using the quantile regression method ($\tau = 0.2$; $\lambda = 30$) described by Chabot et al. (2016b).

4.3.1.2 Apparent specific dynamic action (aSDA)

Key variables describing the SDA response of all animals are presented in Figure 47, Figure 48 and Table 19. Despite attempts to standardise the ration at 0.5% of body weight (BW) as dry weight feed, the mean ration of the MD treatment (0.58 ± 0.10) was significantly higher than the FD treatment (0.50 ± 0.02) ($P = 0.12$, $Z = -2.49$). This was attributed to discrepancies between the estimated moisture content as used to determine feeding ration, and the true moisture content determined from samples of the macroalgae fed used to calculate the true dry weight of the ration fed. Despite the MD treatment receiving a slightly larger ration as % BW, the mass-specific energy ration (KJ g^{-1}) was significantly larger for the FD treatment (0.10 ± 0.00) compared to the MD diet (0.07 ± 0.01) ($P = 0.001$, $Z = 3.20$) owing to the increased nutrient density of the FD diet (19.38 KJ g^{-1} as dry feed) compared to the FD diet (12.29 KJ g^{-1} as dry feed; equal proportions). The time to peak of the aSDA response was not significantly different between the FD and MD treatments, with the peak generally occurring within the first 5.5 – 7 hours. The total duration of the aSDA was also not significantly different between the FD (64.1 ± 11.3 h) and MD (70.2 ± 2.0 h) treatments. However, it should be noted that 50% of the duration values for the MD diet were limited by the duration of the trial compared to 20% of the FD diet's values; consequentially the true duration of the MD diet was likely underestimated (Table 19; Figure 47; Figure 48)

The mass-specific magnitude of the aSDA response was significantly higher for the MD (728.9 ± 118.5 mg O_2 kg-1) treatment compared to the FD treatment (530.2 ± 173.5 mg O_2 kg^{-1}) ($P = 0.03$, $t = 2.46$) and, given the larger mean abalone mass of the MD treatment, the absolute magnitude (as energy units; KJ) was also significantly larger for the MD treatment ($P < 0.001$, $t = 6.88$). The significantly larger magnitude of the aSDA for the MD treatment was driven partly by a significantly higher peak in the aSDA ($P = 0.007$, $t = 3.14$) for this treatment. This resulted in a significantly higher SDA scope for the MD diet ($P < 0.001$, $t = 4.33$), with abalone on average more than doubling their $\dot{V}O_2$ at the peak of their aSDA response (2.09 ± 0.2), compared to 1.73 ± 0.2 for the FD diet. The SDA coefficient was significantly higher for the FD treatment ($P = <0.001$, $t = 5.50$), with the mean SDA coefficient for the MD treatment (16.33 ± 3.31%) being more than double that of the FD treatment (7.83 ± 2.80%).

4.3.2 Ammonia excretion

4.3.2.1 Baseline TAN excretion

There was no significant difference between the light and dark phase preprandial TAN excretion measurements for the FD ($Z = 0.08$, $P = 0.94$), MD ($Z = 1.47$, $P = 0.14$) and CML ($Z = 0.69$, $P = 0.49$) treatments. As such, data from the light and dark phase were pooled for further analyses. The preprandial TAN excretion rate was significantly higher for the MD treatment ($H = 18.9$, $P < 0.001$), being 342% and 479% greater than the CML and FD treatments respectively (Table 20). The CML and FD treatment were not significantly different from each other.

4.3.2.2 Postprandial TAN excretion

Tan excretion results relating to feeding ration, feed intake and TAN excretion dynamics of *H. midae* fed macroalgal (MD), formulated pellet (FD) and a combination diet (CML) are summarised in Table 20. Due to differences between the expected dry-weight conversion factors used for the calculation of rations of macroalgal dietary components, and the true dry-weight conversion factors determined, treatments containing macroalgae received rations more than the targeted ration of 0.5% of body weight (BW) as dry-weight feed. The ration consumed by the MD treatment was significantly higher than the FD treatment, but not the CML treatment ($H = 10.0$, $P = 0.007$). A similar separation was evident for protein consumption relative to body weight, with the MD treatment consuming significantly less protein than the FD and CML treatments, which were no different from each other ($H = 13.4$, $P = 0.001$). Nitrogen (N) intake relative to body weight was not significantly different between treatments ($H = 3.8$, $P = 0.15$). In general, dietary treatments containing a formulated-pellet component (FD and CML) exhibited a marked net positive postprandial increase in TAN excretion compared to a small net negative decrease in the macroalgae only (MD) treatment. The cumulative TAN excretion ($H = 16.0$, $P < 0.001$), peak ($H = 7.4$, $P = 0.02$), scope ($H = 15.6$, $P < 0.001$) and nitrogen coefficient ($H = 16.4$, $P < 0.001$) were all significantly lower for the MD treatment compared to the FD and CML treatments which were generally not significantly different from each other.

Table 20. Total ammonia nitrogen (TAN) excretion dynamics of the abalone *H. midae* fed a mixed macroalgal diet (MD), a formulated pellet (FD) and a combination diet (CML) of macroalgae (25%) and formulated feed (75%).

Treatment	Mass (g)[1]	Baseline TAN excretion rate[2]	Ration fed [consumed[3]]	Protein consumed[4]	Nitrogen Consumed[5]	Cumulative TAN excretion[6]	Peak	Scope	Nitrogen coefficient[7]
		($\mu g TAN\ g^{-1}\ h^{-1}$)	*(% BW)*	*($\times 10^{-3}$ % BW)*	*($\times 10^{-4}$ % BW)*	*($\mu g\ TAN\ g^{-1}$)*	*($\mu g\ TAN\ g^{-1}\ h^{-1}$)*		*(%)*
Formulated diet (FD)									
Mean ± S.D.	65.64 ± 16.83[a]	0.16 ± 0.06[a]	0.51a [0.41±0.07][a]	1.5 ± 0.3a	2.4 ± 0.4[a]	23.1 ± 20.8[a]	1.22 ± 0.57[a]	8.77 ± 8.02[a]	6.7 ± 5.2[a]
Range	45.42 – 98.27	0.09 – 0.27	0.33 – 0.51	1.2 – 1.9	2.0 – 3.1	5.7- 60.1	0.74 – 2.13	2.8 – 22.7	2 – 15.3
Macroalgal diet (MD)									
Mean ± S.D.	109.18 ± 12.35[b]	0.78 ± 0.26[b]	0.80b [0.66 ± 0.12][b]	1.1 ± 0.1b	2.0 ± 0.2[a]	-22.9 ± 13.2[b]	0.65 ±0.17[b]	0.90 ± 0.31[b]	-7.9 ± 4.6[b]
Range	92.73 – 138.20	0.34 – 1.08	0.51 – 0.80	1.0 – 1.2	2.0 – 2.4	-36.6 – -2.7	0.38 – 0.86	0.6 ± 1.6	-14.4 – 1.1
Combination diet (CML)									
Mean ± S.D.	87.08 ± 8.46[c]	0.23 ± 0.12[a]	0.71a [0.54 ± 0.11][ab]	1.7 ± 0.4a	2.7 ± 0.7[a]	11.8 ± 6.2[a]	0.97 ± 0.34[ab]	5.91 ± 5.19[a]	3.7 ± 2.6[a]
Range	75.44 – 105.34	0.08 – 0.42	0.40 – 0.67	1.1 – 2.1	1.8 – 3.6	2.2 – 22.0	0.52 – 1.45	1.7 ± 16.5	0.6 – 9.3

Different superscript letters in the same column denote treatment means are significantly different ($P < 0.05$). [1]Mean of wet weight values determined at the start and end of the trial. [2]Mean of combined light and dark preprandial measurements for each treatment. [3]Uneaten feed was collected, oven dried at 100°C for 24 h and weighed; individuals that consumed less than 50% of the fed ration were excluded from analyses. [4]Protein content was calculated using mass spectrometry derived N values and mean N-Prot factors derived by Lourenço & Barbarino (2002) for the taxonomic groupings of red (n = 9, N-Prot 4.59), green (n = 6, N-Prot 5.13) and brown (n = 4, N-Prot 5.38) macroalgae were used. [5]Nitrogen content of feed components was determined using mass spectrometry. [6]Cumulative TAN excretion was determined by integrating the area under the curve bound by the baseline TAN excretion rate for the period T7.5 – T65.5. [7] Nitrogen coefficient calculated as total nitrogen excreted divided by meal N content.

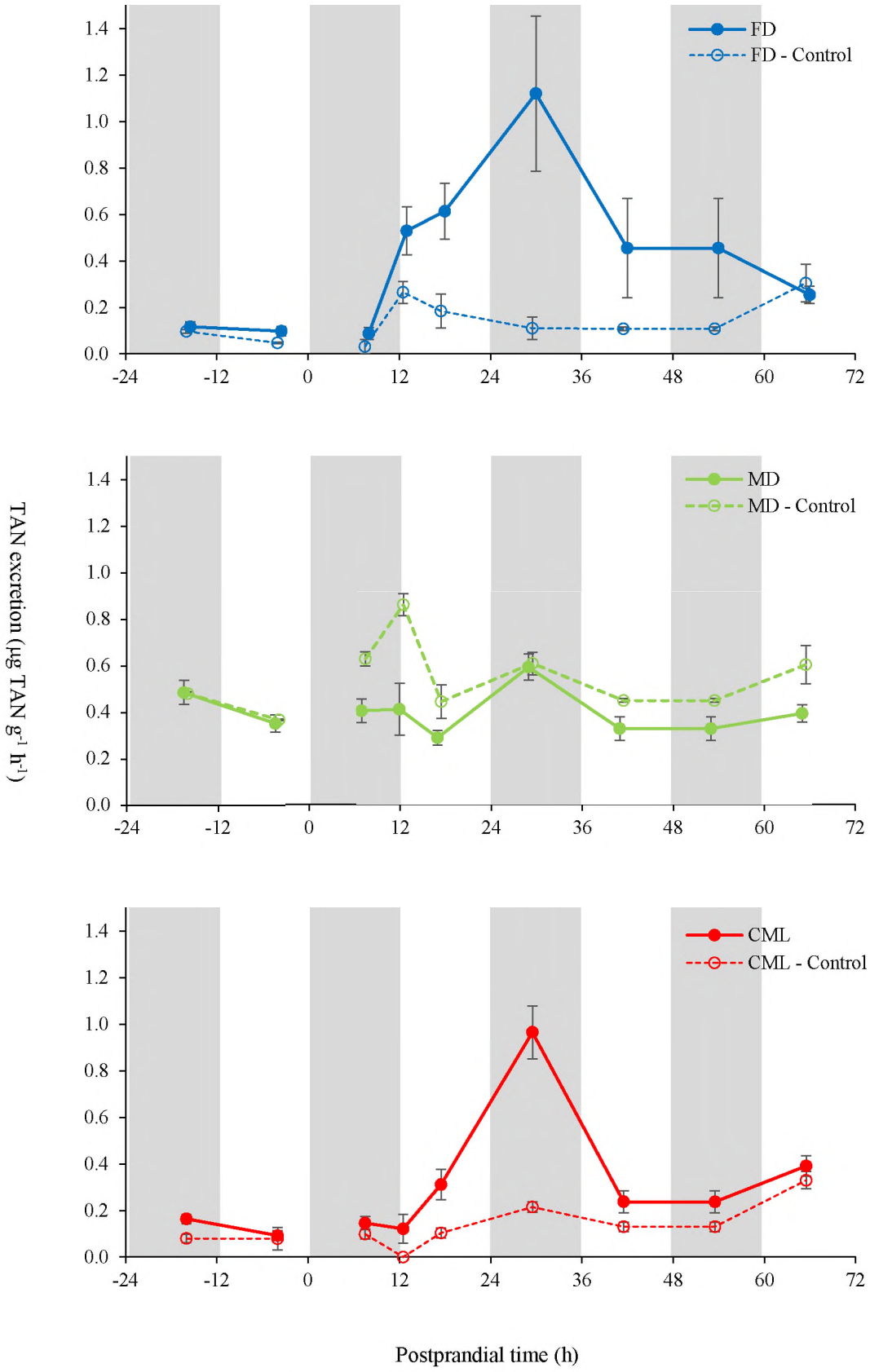


Figure 49. Mean total ammonia nitrogen (TAN) excretion rate (µg TAN g^{-1} h^{-1} ± S.E.) of individual abalone fed a formulated pellet (FD), a macroalgal diet (MD) and a combination diet (CML) of macroalgae (25%) and a formulated pellet (75%). Dotted lines indicate the mean TAN excretion rate for two unfed controls per treatment. Feed was placed into the chamber at postprandial time 0 hours.

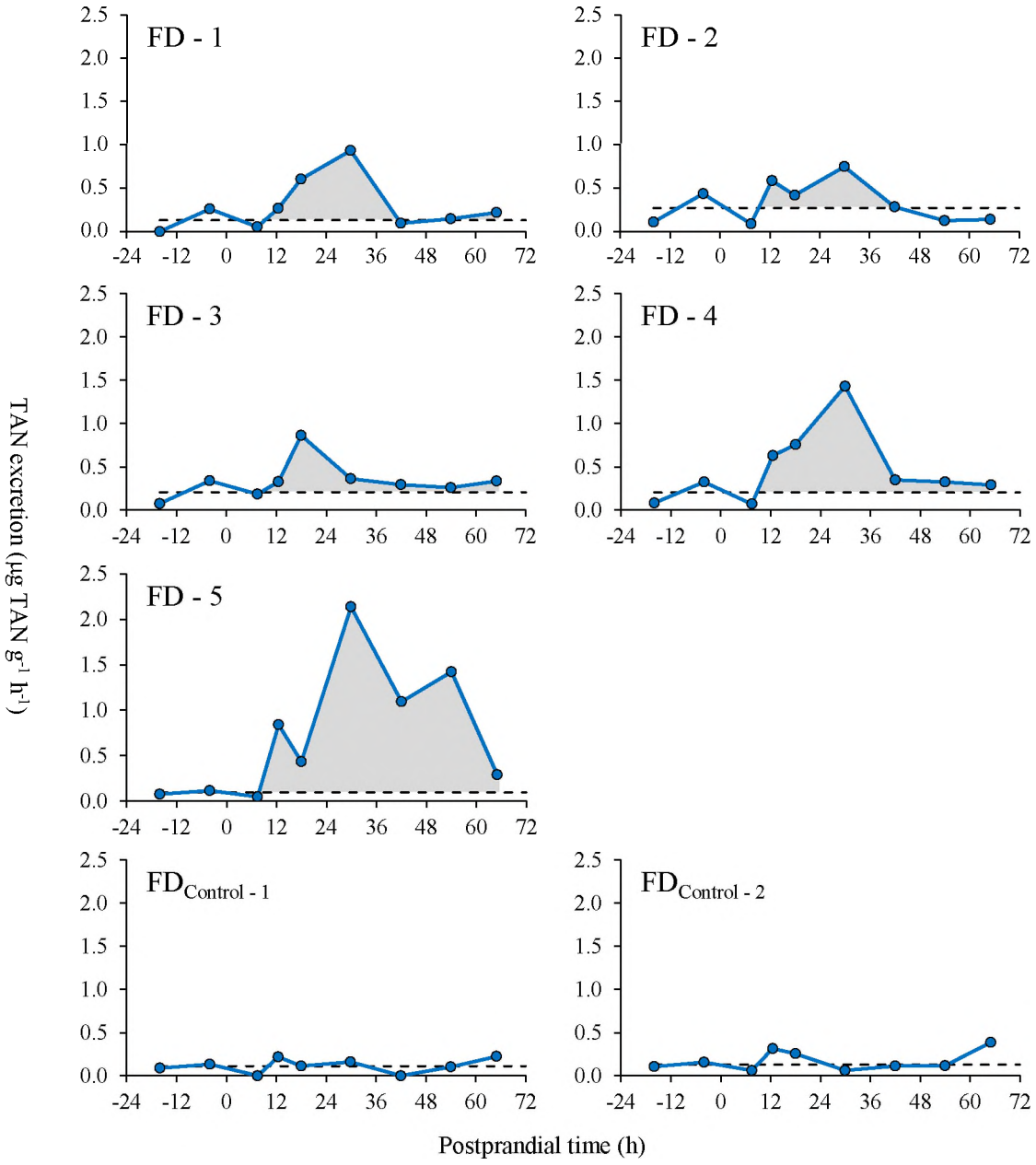


Figure 50. Total ammonia nitrogen (TAN) excretion rate (µg TAN g^{-1} h^{-1}) of individual abalone fed a formulated feed (FD; 1 – 5) and two unfed controls. Grey shaded areas define the area under the TAN curve bounded by the baseline excretion rate (– – –). Feed was placed into the chamber at postprandial time 0 hours.

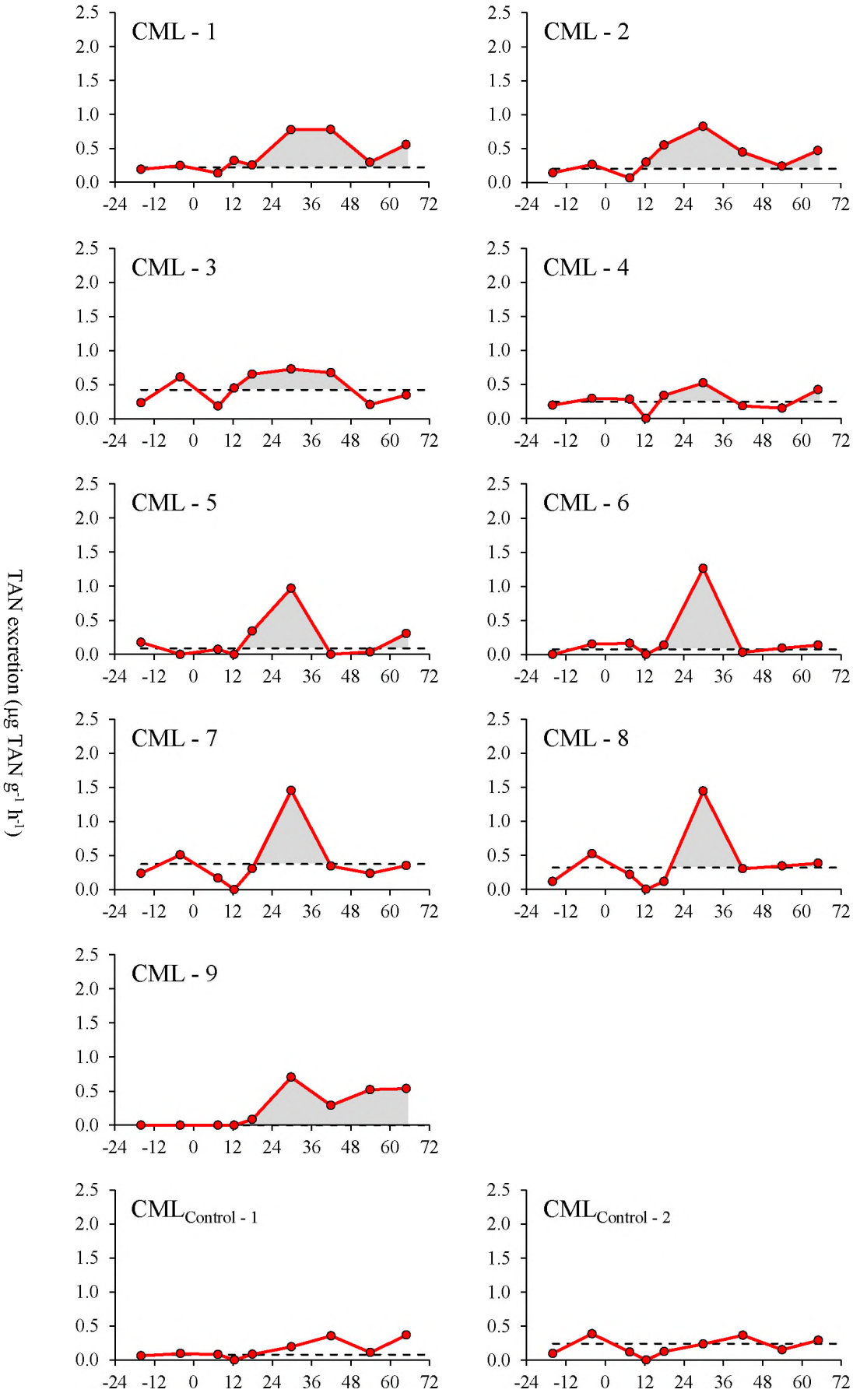


Figure 51. Total ammonia nitrogen (TAN) excretion rate (µg TAN g^{-1} h^{-1}) of abalone fed a combination diet (CML) of macroalgae (25%) and a formulated pellet (75%) (CML; 1 - 9) and two unfed controls. Grey shaded areas define the area under the TAN curve bounded by the baseline excretion rate (– – –). Feed was placed into the chamber at postprandial time 0 hours .

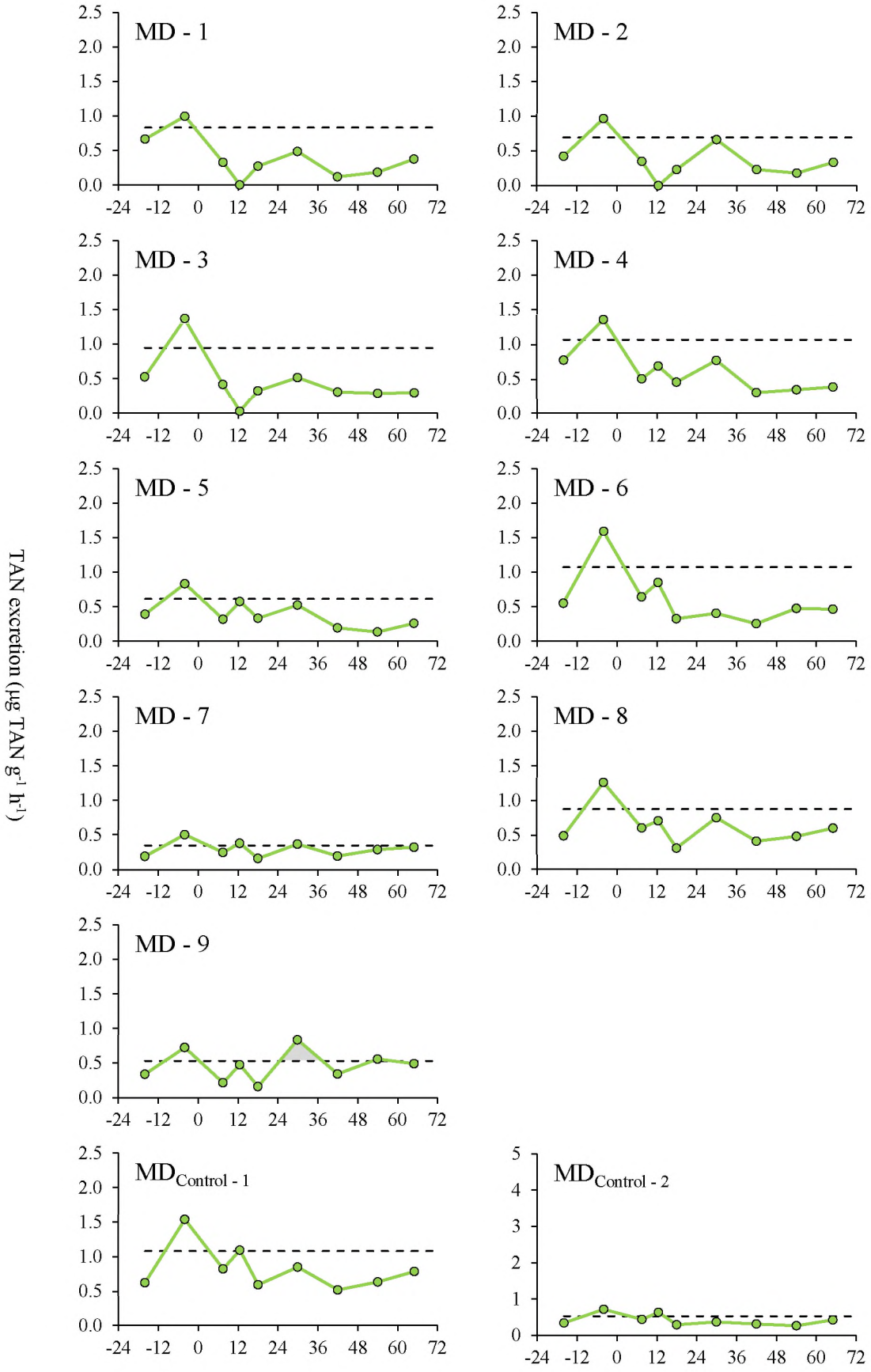


Figure 52. Total ammonia nitrogen (TAN) excretion rate (µg TAN g^{-1} h^{-1}) of abalone fed a macroalgae diet (MD; 1 - 9) and two unfed controls. Grey shaded areas define the area under the TAN curve bounded by the baseline excretion rate (– – –). Feed was placed into the chamber at 0 hours.

4.3.3 Haemolymph glucose concentration (HGC)

4.3.3.1 Baseline HGC

The baseline HGC after a 96-h fast period was significantly lower for the MD treatment ($F = 20.5$, $P < 0.001$) compared to the FD and CML treatments, which were not significantly different from each other (Table 21). Furthermore, the MD treatment exhibited a significant positive linear relationship between HGC and time, over a the 128-h fasting period ($y = 0.1675x + 35.235$; $R^2 = 0.6889$; $P = 0.006$). The HGC concentration of the FD and CML treatments showed no significant linear trend over the 128 h fast (Figure 53).

4.3.3.2 Postprandial HGC

All treatments exhibited a marked increase in HGC concentration following consumption of a rationed meal compared to an unfed control (Figure 54). Differences between the dry weight conversion factors used for the calculation of the proportional inclusion of macroalgal dietary components, and the true dry weight conversion factors determined, resulted in treatments containing macroalgae received rations greater than the targeted ration of 0.5% of body weight (BW) as dry-weight feed (Table 21). Furthermore, when uneaten feed was accounted for, this trend continued, with the true consumed ration being significantly higher for the MD and CML treatments ($F = 17.9$, $P < 0.001$). Due to the increased carbohydrate content of the FD diet when compared to the MD diet, carbohydrate consumption (% BW) was not significantly different between treatments FD and MD despite significantly lower total feed intake for treatment FD, although both were significantly lower than treatment CML ($F = 14.4$, $P < 0.001$). Glycaemic carbohydrate consumption was not significantly different between the FD and CML treatments, both of which were significantly higher than the FD treatment ($H == 84.7$, $P < 0.001$).

The postprandial response in HGC to feeding was markedly greater in terms of all measured parameters (i.e. area under the curve, peak, duration and scope) for the two treatments containing formulated feed (CML and FD) when compared to the MD treatment (Table 21). Interestingly, the glycaemic carbohydrate coefficient (GCC), a

measure of the magnitude of the HGC response relative to the intake of glycaemic carbohydrate (% BW), was an order of magnitude lower for the MD treatment when compared to the FD and CML treatments.

Table 21. Postprandial haemolymph glucose concentration (HGC) dynamics of the abalone *H. midae* fed a mixed macroalgal diet (MD), a formulated pellet (FD) and a combination diet (CML) of macroalgae (25%) and formulated feed (75%).

Treatment	Abalone mass[1]	Baseline HGC[2]	Ration fed [consumed[3]]	Carbohydrate consumed[4]	Glycaemic carbohydrate consumed[5]	Area under curve[6] (AUC)	Peak	Duration	Scope[7]	Glycaemic carbohydrate coefficient[8]
	(g)	*($\mu g\ ml^{-1}$)*	*(% BW)*	*($\times 10^{-3}$ % BW)*	*($\times 10^{-3}$ % BW)*	*($\mu g\ ml^{-1}$)*	*($\mu g\ ml^{-1}$)*	*(h)*		*($\times 10^{-6}$ %)*
Formulated diet (FD)										
Mean ± S.D.	58.7 ± 14.0[a]	50.8 ± 3.7[a]	0.54 [0.39 ± 0.08][a]	2.0 ± 0.4[a]	1.5 ± 0.3[a]	1258	158	[≈ 32][9]	3.1	1.22
Range	39.41 – 101.8	46.9 – 57.0	0.25 – 0.49	1.3 – 2.5	1.0 – 2.0					
Macroalgal diet (MD)										
Mean ± S.D.	113.4 ± 29.1[b]	33.6 ± 6.7[b]	0.63 [0.52 ± 0.11][b]	2.3 ± 0.5[a]	0.05 ± 0.01[b]	325	72.6	16	2.2	0.15
Range	70.9 – 187.8	23.7 – 40.7	0.33 – 0.63	1.5 – 2.8	0.03 – 0.06					
Combination diet (CML)										
Mean ± S.D.	101.9 ± 21.2[c]	52.2 ± 8.2[a]	0.67 [0.60 ± 0.09][b]	2.8 ± 0.4[b]	1.7 ± 0.3[a]	1519	197.5	32	3.8	1.11
Range	68.6 – 158.8	44.0 – 66.9	0.37 – 0.60	1.7 – 3.2	0.8 – 1.9					

[1]Wet weight values determined post removal of haemolymph sample. [2]Mean of abalone sampled (n = 8) at T0 (96 h fast). [3]Uneaten feed was collected, oven dried at 100°C for 24 h and weighed; individuals that consumed less than 50% of the fed ration were excluded from analyses. [4]Carbohydrate content was calculated as non-nitrogen extract (NNE; NNE = 100 – (% Protein + % Ash + % Lipid); formulated pellet – 48.7%, *Ulva sp.* – 51.0%, *Gracilaria sp.* 38.0%). [5]Glycaemic carbohydrate calculated as proportion of total NNE based on proximate analyses (formulated pellet – 78.0% of NNE, *Ulva sp.* – 1.7% of NNE, *Gracilaria sp.* 2.5% of NNE). [6]Area under the curve was determined by integrating the area under the curve for the period T0 – T32. [7]Scope calculated as Peak HGC ÷ Baseline HGC. [8]Glycaemic carbohydrate coefficient calculated as glycaemic carbohydrate consumed (% BW) ÷ AUC. [9]Mann-Whitney U-test could not be used to determine the point at which fed HGC was not significantly different from unfed HGC due to low sample size in this treatment, fed HGC values were still elevated at 16 h but on par with unfed HGC by 32 h post feeding.

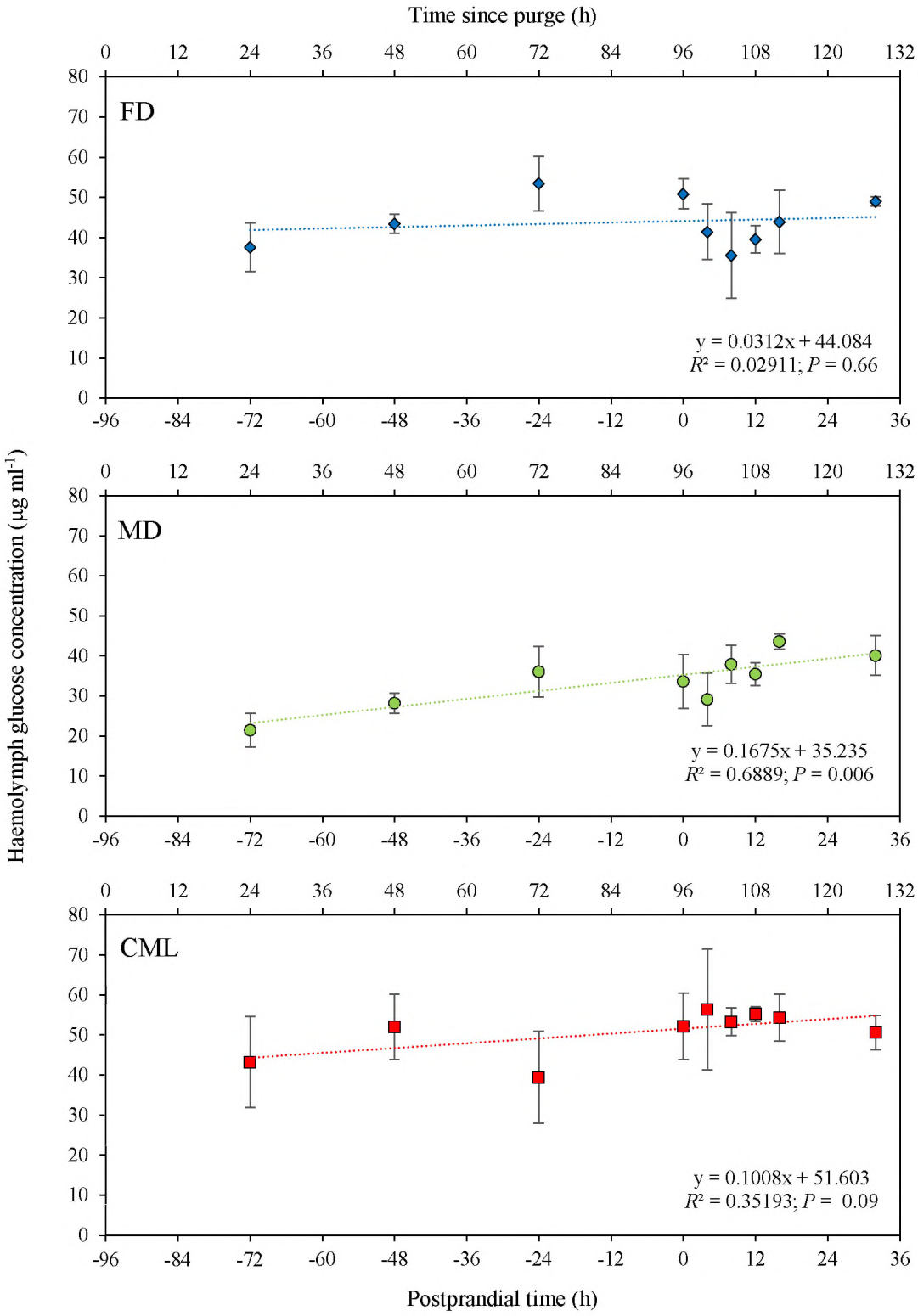


Figure 53. Mean haemolymph glucose concentration (HGC; µg ml^{-1}) of abalone over a 128-hour fasting period following culture for 97 weeks on formulated feed (FD), a macroalgal diet (MD) and a combination diet (CML) of macroalgae (25%) and a formulated pellet (75%). Each data point represents the mean of a minimum of two abalone.

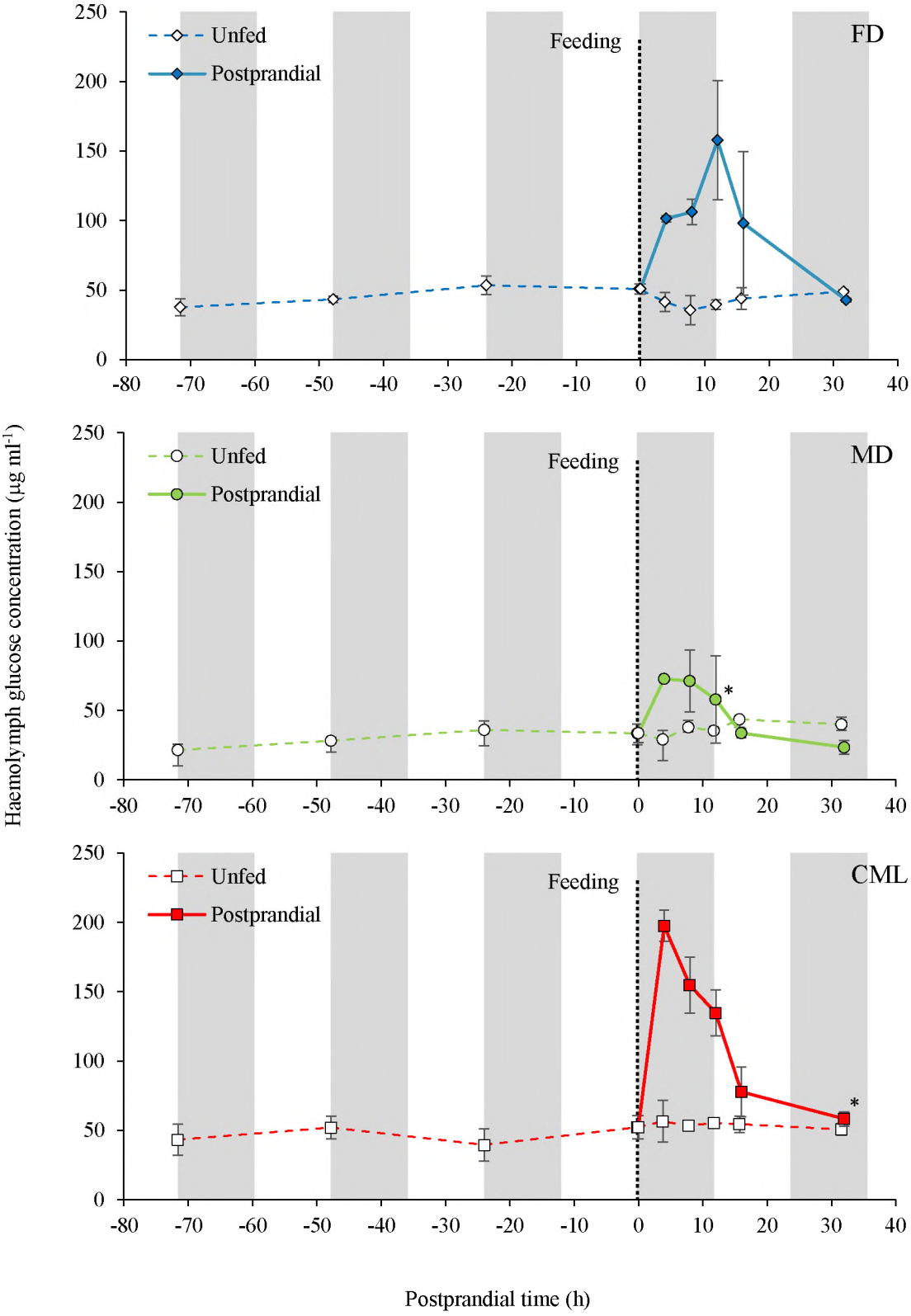


Figure 54. Mean haemolymph glucose concentration (HGC; µg ml^{-1}) of abalone fed a formulated feed (FD), a macroalgal diet (MD) and a combination diet (CML) of macroalgae (25%) and formulated feed (75%) at a target ration of 0.5% body weight as dry weight feed. Dotted lines indicate the mean HGC for unfed abalone (minimum of two abalone at each measurement time). * denotes the point at which fed HGC values were not significantly different from the baseline unfed HGC at T0.

4.4 Discussion

Oxygen consumption

Abalone exhibited marked changes to their oxygen consumption rate, ammonia excretion rate and haemolymph glucose concentration in response to feeding. Furthermore, a distinct diel cycle in oxygen consumption was evident, both pre- and postprandially. For abalone purged for 72 hours, nocturnal oxygen consumption rates ($\dot{V}O_2^N$; mg O_2 kg^{-1} h^{-1} ± *SD*) were significantly higher than diurnal values for abalone conditioned on both formulated feeds ($\dot{V}O_2^N$ – 36.9 ± 6.5; $\dot{V}O_2^D$ – 29.0 ± 3.8; t_{18} = -3.32, P = 0.003) and macroalgae ($\dot{V}O_2^D$ – 37.7 ± 6.8; $\dot{V}O_2^D$ – 28.6 ± 4.6; t_{10} = -2.27, P = 0.02), with nocturnal values 27.8 ± 17.3 and 32.8 ± 22.2% higher than daytime ($\dot{V}O_2^D$) values respectively. A similar diel activity pattern was still observable for 72 hours postprandially. Comparable diel patterns[1] in oxygen consumption have been reported for other abalone species. Chacon et al. (2003) reported $\dot{V}O_2^N$ to be significantly greater than $\dot{V}O_2^D$ by ca. 20% for *H. fulgens*, with a similarly elevated $\dot{V}O_2^N$ evident in *H. corrugata* (Montaño-vargas et al., 2005) and *H. discus hannai* (Uki & Kikuchi, 1975). Higher (ca. 10%), but not significantly different, $\dot{V}O_2^N$ values have also been reported for *H. tuberculata* (Peck et al., 1987). These results are, however, contrary to the two other studies that have investigated diurnal differences in oxygen consumption in *H. midae*, with both Barkai & Griffiths (1987) and Lyon (1996) reporting no significant differences between $\dot{V}O_2^N$ and $\dot{V}O_2^D$ values. Interestingly, Jan et al., (1981b) showed that the diel cycle of oxygen consumption in *H. diversicolor supertexta* under natural lighting conditions was not conserved under continuous light conditions, indicating the onset of darkness is associated with elevated oxygen consumption.

Abalone are regularly described as nocturnal feeders in their natural environment, as evidenced by full crops in animals sampled during the early light phase and limited feeding and foraging behaviour observed during daylight hours (Britz et al., 1996; Muller, 1984; Poore, 1972; Wells & Keesing, 1989; Wood & Buxton, 1996). It has been demonstrated that *H. discus hannai* exhibit significant tropism towards darkness, red and orange light, but a significant escape behaviour from blue, green and purple light (Gao et al., 2016b). Furthermore, light quality and duration can have significant effects on abalone health, growth and survival (Gao et al., 2016a). A nocturnal activity

pattern has been suggested as a means to avoid predators with diurnal activity patterns (Wood & Buxton, 1996). Given the increase in oxygen consumption associated with abalone being alert or undertaking locomotion (Donovan & Carefoot, 1998, 1997), it is likely that much of the elevated oxygen consumption observed at night is the result of activity related to exploratory foraging behaviour. Abalone in the current study were generally quiescent during the light phase and were observed actively exploring the respirometer during the night phase. In studies where no diel cycle was observed (Barkai & Griffiths, 1987; Lyon, 1995; Peck et al., 1987), it is possible that the experimental method employed may have affected the results. The use of non-automated closed respirometry involves manipulating the respirometry chamber at the start and end of each measurement. Given that oxygen consumption rates can increase when abalone are in an alert but non-locomotory state (Donovan & Carefoot, 1998), and the likely cessation of exploratory locomotion activity when disturbed, there is the potential for observing artificially elevated $\dot{V}O_2^D$ and depressed $\dot{V}O_2^N$ values using this method. In addition to the effect of handling stress and disturbance, excess food availability has been suggested as a cause of reduced $\dot{V}O_2^N$ due to reduced foraging behaviour (Peck et al., 1987).

The absolute $\dot{V}O_2$ values for quiescent abalone recorded in the current study were in the range of those of some studies previously conducted on *H. midae* (Barkai & Griffiths, 1987; Lyon, 1996). They were, however, markedly lower than those reported by Vosloo & Vosloo (2010) for *H. midae*, as well as being markedly lower than the respiration rates for a number of other species (Table 22). The only comparable values reported in the literature are for large individuals (115 – 300 g) of *H. iris* at 15 °C, *H. kamtschatkana* at low water temperatures (2 – 10 °C) and a single study on *H. laevigata* with a 72 hour purge time (Duong et al., 2016). The negative relationship between body size and $\dot{V}O_2$ (Barkai & Griffiths, 1987; Farías et al., 2003; Gaty & Wilson, 1986; Peck et al., 1987) and the positive relationship between water temperature and $\dot{V}O_2$ (Gaty & Wilson, 1986; Lyon, 1995; Paul & Paul, 1998) have been confirmed in abalone.

[1] These patterns are referred to as 'circadian rhythms' in the referenced works, although the term diel cycle is perhaps more appropriate, given that the authors do not provide evidence that the observed patterns are generated endogenously and conserved in the absence of external stimuli, such as light.

Table 22. Summary of studies investigating the oxygen consumption rate of abalone using closed, flow through and intermittent respirometry techniques.

Study	Species	Abalone size (*g / mm*)	Water temperature (*°C*)	Respirometry method	Purge period	Chamber acclimation period	Mass exponent reported	[3] $\dot{V}O_2$ ($mg\ O_2\ g^{-1}\ h^{-1}$)	Ammonia excretion ($ug\ g^{-1}\ h^{-1}$)
Chacon et al. (2003)	*H. fulgens*	25 - 35 mm	23	closed	24 h	24 h		57 - 100	
Montaño-vargas et al. (2005)	*H. corrugata*	< 1 g	21	closed	3 days	3 days		70 - 79	4.79 – 8.78
Paul & Paul (1998)	*H. kamtschatkana*	45 - 55 g	2 - 24	closed	56 - 72	56h		14 - 100	
Peck et al. (1987)	*h. tuberculata*	[1] 5 – 100 g	15	closed	[2] *NR*	*NR*	0.765		
Taylor & Ragg (2005)	*H. iris*	115 - 330 g	15	closed	12h	12h		15 - 16	
Gaty & Wilson (1986)	*H. tuberculata*	10 – 70 mm	8 – 24	closed	*NR*	*NR*	0.743		
Ahmed et al. (2008)	*H. discus*	4.8 g	20	closed	24h	0h		55	3.92
	H. gigantea	4.8 g						47	2.73
	H. mandaka	4.4 g						79	4.43
Carefoot et al. (1993)	*H. kamtschatkana*	100 - 150 g	10	closed	*NR*	10 min	0.62	11 - 33	
Viana et al. (2007a)	*H. fulgens*	0.6 g	20	closed	*NR*	*NR*			
Gómez-Montes et al. (2003)	*H. fulgens*	0.2 g	21	closed	*NR*	*NR*			
Farías et al. (2003)	*H. fulgens*	0.8 – 28 g	16	closed	*NR*	*NR*	0.739	57 - 172	2.87 – 5.79
Harris (1999)	*H. laevigata*	10.8 g	18	flow through	48hr	48hr		165	
Harris et al. (1998c)	*H. laevigata*	4.5 g	16.9	flow through	48hr	48hr		37	
Baldwin et al. (2007)	*h. asinina*	88 - 138 g	25	closed	*NR*	*NR*		65	
Barkai & Griffiths (1987)	*H. midae*	20 - 500 g	14 - 19	closed	*NR*	4-5hr	0.83 / 0.94	15 - 36	0.18 – 0.27
Vosloo & Vosloo (2010)	*H. midae*	Adult	1 6 - 22	closed	24hr	*NR*		48 - 96	2.0 – 8.8
Segawa (1991)	*H. diversicolor*	0.2 – 24 g	24	closed / manual	fed	*NR*		43 - 87	1.09 - 1.85
Lim et al. (2014)	*H. discus*	2 g	15 - 25	flow through	24hr	*NR*		110	
Lyon (1996)	*H. midae*	10 - 60 mm	16 - 23	intermittent flow	Unfed / *NR*	4-5 days		15 - 56	0.0141 – 0.1061
Duong et al. (2016)	*H. laevigata*	49 g	22 - 25	closed	24	72 hr		30 - 44	0.63 – 2.40
This study	*H. midae*	40 - 96	18	intermittent flow	96	36 hr	0.754	19 - 32	0.08 – 1.08

[1] Abalone dry weight. [2] *NR* = not reported. [3] Values reported are for baseline or control animals if treatments were applied or starvation initiated. The range reflects differences resulting from animal size or water temperature. When consumption rates were not reported as mg O_2 kg^{-1} l^{-1}, conversions were effected using the following factors: 1mg O_2 l^{-1} = 0.700 ml O_2 l^{-1}; 1 mole O_2 = 32 g O_2.

It is therefore likely that higher water temperatures combined with the use of small experimental animals may be partially responsible for higher $\dot{V}O_2$ values recorded in previous studies (Ahmed et al., 2008; Chacon et al., 2003; Montaño-vargas et al., 2005). However, given the general reliance on manually-operated, closed respirometry systems combined with variable (if reported) chamber acclimation protocols it is possible that operator disturbance may have influenced the recorded respiration rates. Furthermore, in the previous studies extended single incubations, combined with the lack of a methodology to account for spontaneous activity, may have confounded measurements further due to locomotory activity. In addition, the use of purging to control for digestion-related oxygen consumption is applied sporadically and variably. The current study shows quiescent postprandial $\dot{V}O_2$ values that peaked in the range of 73 ± 15 to 109 ± 18% of preprandial levels depending on diet, with active postprandial abalone having $\dot{V}O_2$ levels exceeding 350% of quiescent preprandial levels. While elevated levels of $\dot{V}O_2$ related to feeding generally returned to baseline levels by 72 hours, some individuals still exhibited raised $\dot{V}O_2$ at 72 hours. This would indicate that a purge period of at least 72 hours (3 dark cycles), but most likely 96 hours (4 dark cycles) is required to control for the SDA response in abalone, given a meal of 0.5% of body weight as dry feed. The SDA response has been shown to increase with meal size in other organisms (Secor, 2009; Secor & Boehm, 2006), although the ration of 0.5% BW used in the current study is similar to the predicted daily consumption of a formulated feed (0.43%) for a 60 mm abalone at 18 °C (Britz et al., 1997b). Only two studies to date report a purge time of 72 hours (Montaño-vargas et al., 2005; Paul & Paul, 1998), with most in the range of 24 – 48 hours or not reported (Table 22). Given these potential confounding factors, occurring either singly or in combination, it is likely that many of the oxygen consumption rates determined in abalone to date, especially where basal rates were intended, have been overestimated to a degree. However, the extent to which this affects experimental interpretations will be governed by whether all treatments were equally affected and the degree to which confounding factors may have masked treatment effects.

Abalone here exhibited a marked postprandial metabolic response, termed apparent specific dynamic action (aSDA) but comparable to the specific dynamic action (SDA) reported in other studies. Comparative data regarding the SDA response in haliotids are not available in the literature, with limited information on other marine gastropods and

bivalves (see Secor (2009) for a review). Within these studies, the factorial scope of the SDA averaged 2.98 ± 1.32, 1.56 ± 0.61, 2.22 ± 1.21 and 2.45 ± 012 respectively for gastropods, bivalves, gastropods and bivalves combined, and all invertebrates. Values for SDA scope in the current study ranged from 1.73 – 2.09. Information on other metrics such as the SDA duration and SDA coefficient are more limited, but invertebrate SDA appear to increase linearly with meal energy, and equations have been established based on available information for invertebrates to allow the prediction of SDA if meal energy is known (Secor, 2009). Based on meal energy values in this study, the predicted magnitude of the SDA response would be 0.37 ± 0.04 and 0.41 ± 0.02 kJ for formulated and macroalgae respectively. The observed SDA in the current study for formulated feed of 0.37 ± 0.13 was comparable to the model-predicted value, but the SDA magnitude of 0.86 ± 0.15 for macroalgae was more than double than that predicted. Perhaps more insightful than the comparison of experimental results to studies with high variation in terms of taxonomic class, meal type, body mass, body temperature and meal size is the exploration of differences between dietary treatments where many of these factors were controlled.

The type of food consumed resulted in a significant difference in the nature of the SDA response observed in this study. The peak $\dot{V}O_2$ of the aSDA for abalone consuming macroalgae was significantly greater than abalone consuming formulated feed, with abalone fed either diet generally reaching this peak within the first 7 hours post-feeding. The elevated peak, combined with the longer SDA duration for abalone fed macroalgae, resulted in a 1.4 times higher SDA response per unit body weight. The SDA coefficient, essentially the proportion of the ingested energy in a meal that is required to process that meal, provides an interesting metric to gauge the energetic costs associated with a certain feed type. The SDA coefficient for macroalgae was 2.1 times that observed for formulated feed. This would indicate that not only is macroalgae energetically costlier to digest than formulated feed in absolute terms, but it's low energy content per gram relative to formulated feed also means that the effect of this disparity on its proportional energy yield is greater than would be expected from the total magnitude of the aSDA response alone. The source and mechanisms that contribute to the SDA response have been extensively theorised (Garrow, 1973; James, 1992; Kleiber, 1961; Tandler & Beamish, 1979), but partitioning the contribution of different energy consuming processes remains difficult (Secor, 2009). Pre-absorptive components include the

energy involved with eating and swallowing, the mechanical and chemical gastric breakdown of food and intestinal peristalsis and absorption (Secor, 2009). The difference in water content between macroalgae (ca. 80 – 90%) and formulated feed (ca. 8 – 10%) translates into a substantially greater volume of feed that needs to be processed when feeding on macroalgae so as to attain a similar ingestion of dry matter compared to formulated feed. Furthermore, the complex interlinked cellular structure of algae would likely require greater effort to rasp into ingestible sizes, compared to the lightly bound particulate nature of rehydrated formulated pellets whose ingredient components have been pre-milled. Dietary related differences in the accumulative cost of eating have been reported for cattle and sheep, with pressed pellets requiring 0.44% of meal energy to process, compared to 1.3 – 19.9% for grass or hay (Adam et al., 1984; Christopherson & Webster, 1972; Young, 1966). The morphology of the abalone stomach, particularly the muscular style sac, gastric shield and ciliated sorting areas, indicate the important role of mechanical mechanisms for the breakdown of ingested food particles. It is possible that the presence of course algal fragments (Campbell, 1965) and the increased bulk associated with feeding on macroalgae stimulates style sac activity, which, when combined with the extended gastric evacuation time associated with macroalgal diets, may result in a greater energetic cost for mechanical breakdown compared to formulated diets. Given that the intestinal walls of abalone generally lack musculature, with the action of cilia and the style sac driving the movement of ingesta towards the anus (Owen, 1966), the contribution of intestinal peristalsis is likely to be negligible.

Post-absorptively, substrate catabolism and the direction of absorbed molecules to biosynthetic pathways represent the major contributors to SDA (Secor, 2009). The synthesis of the primary energy store in abalone is endergonic, requiring an estimated 2.1 ATP molecules for the addition of glucose to an existing glycogen molecule (Blaxter, 1989). In terms of protein, as the storage of amino acids post-absorption is limited, they are either rapidly catabolized or incorporated into new tissue, with their fate determined by an organism's specific amino acid requirement for protein synthesis at that time (Bender, 2012; Secor, 2009). The SDA cost of protein catabolism is a combination of relatively fixed costs such as oxidative deamination and ammonia synthesis and excretion, and variable costs associated with the amino-acid-specific end-pathway of the carbon skeleton (Secor, 2009). Depending on the amino acid structure and the pathway it enters post-deamination, the SDA response can vary greatly (Blaxter,

1989). Although ongoing in the non-feedings state, protein synthesis increases postprandially and can contribute 23 – 44% of SDA (Houlihan et al., 1990; Lyndon et al., 1992; Secor, 2009). Differences in the post-absorption amino acid profile, resulting from feeding on a specific diet, can therefore potentially affect the eventual use of both these molecules and their contribution to the SDA response. The elevated SDA in macroalgal-fed animals compared to formulated fed animals could be in part be the result of costs associated with protein synthesis, given the significantly higher growth rate observed in these animals. When amino acids are catabolized for energy, rather than being directed into protein synthesis, the liberated amine group forms ammonia, which must be rapidly excreted to prevent plasma concentrations from rising to toxic levels. (Randall, 2011). Measuring the excretion rate of this metabolite postprandially therefore provides a relative indication of the degree of protein catabolism occurring.

Ammonia excretion

Abalone exhibited marked differences in their total ammonia nitrogen (TAN) excretion dynamics, both pre- and postprandially, in response to diet. Baseline TAN excretion at 72 – 96 hr post-purge was significantly higher for abalone conditioned on macroalgae compared to formulated feed. Baseline TAN excretion rates ($\mu g\ g^{-1}\ h^{-1}$) in the current study (0.08 – 1.08) were comparable with those reported previously for *H. midae* by Lyon (1996) (0.014 – 0.106) and Barkai & Griffiths (1987) (0.18 – 0.27), particularly for abalone fed formulated feed (0.09 – 0.27). When peak postprandial rates from the current study are considered (0.08 – 2.13), the range observed is comparable to that reported (0.63 – 8.8) for a number of other abalone species (Ahmed et al., 2008; Duong et al., 2016; Farías et al., 2003; Montaño-vargas et al., 2005; Segawa, 1991; Vosloo & Vosloo, 2010) (Table 22). These previous studies only provide data obtained from incubations over a short time period, however, essentially providing snapshot views for abalone exposed to variable experimental protocols. The current study provides time-series data indicating that TAN excretion is not fixed, but can fluctuate over time, particularly in response to the ingestion of feed. Postprandial TAN excretion in abalone fed macroalgae remained fairly constant (mean scope 0.9 ± 0.3) while abalone fed a formulated feed showed a marked increase (mean scope 8.8 ± 8.0) in TAN excretion compared to baseline values, peaking 24 – 36 hours following the introduction of feed.

O:N ratio

The TAN excretion dynamics observed, particularly when integrated with previously determined SDA patterns (recorded under matching experimental protocols) to model representative O:N ratios (Figure 55), provide insight into the fate of ingested proteins under different dietary conditions. Given the generally poor utilization of lipids in abalone and low content in their diet, the O:N ratios for abalone feeding on macroalgae (63 – 128) were likely indicative of a combination of carbohydrate and protein substrates funding energy metabolism. In addition, there was also little indication of large postprandial fluctuations in the O:N ratio. The apparent partial reliance on protein in these animals may be an experimental artefact resulting from the purge period. Segawa (1991) showed that O:N ratios fell rapidly within the first 5 days of starvation in *H. diversicolor aquatilis*, from above 50 to under 20. Furthermore, Carefoot et al., (1993) reported that in *H. kamtschatkana*, both circulating haemolymph glucose and glycogen levels in abalone tissue dropped rapidly in the first 5 days of starvation. Given that muscle glycogen concentrations were observed to be low in abalone fed macroalgae (Figure 34), it is likely that a large proportion of the stored carbohydrate energy reserves may have been depleted by the time of measurement, necessitating a switch to protein substrates for energy metabolism. A single meal was not sufficient to facilitate a switch to a carbohydrate-substrate-based metabolism.

By contrast, for abalone given formulated feed there was a marked 11-fold increase in TAN excretion postprandially which coincided with a reduction in the O:N ratio from levels associated with a high contribution of carbohydrates as the metabolic substrate (243 – 511) to within the range indicative of a marked increase in the contribution of protein as the metabolic substrate (33 – 99) (Ikeda, 1977; Mayzaud & Conover, 1988) (Figure 55). This would indicate that the glycogen energy stores in abalone conditioned on formulated feed were sufficient to support energy metabolism up to 96 hours post-purge. Furthermore, the sharp decline in O:N ratio and rapid increase in TAN excretion peaking at 24 – 36 hours postprandially indicate a period of elevated amino acid catabolism and a shift towards protein-based substrates for energy metabolism. Given the stable high O:N ratios in the 36-hour period previous to this shift, it is unlikely that it is the result of a sudden depletion in carbohydrate energy stores. Rather, it likely represents an imbalance in the amino acid profile and/or absolute intake, relative to the metabolic requirements of the organism, which resulted in the end products of their deamination entering energy producing metabolic pathways. The utilization of a greater proportion of protein for catabolic rather than anabolic processes in animals fed

formulated feed is supported by the decreased protein efficiency (Table 9) and elevated nitrogen coefficient values (Figure 55) compared to animals fed macroalgae. Interestingly, the shift to an increased utilization of protein as the metabolic substrate occurred despite greatly elevated circulating haemolymph glucose concentrations, overlapping to a degree with the period of elevated TAN excretion.

Haemolymph glucose

Both the preprandial and postprandial haemolymph glucose concentrations (HGC) were affected by diet in the current study. Comparative data for absolute HGC in abalone are limited. Vosloo et al., (2013a, 2013b) report values between 41.4 and 99 μg ml^{-1} in *H. midae* for control treatments while HGC values in the region of 20 μg ml^{-1} have been reported for *H. diversicolor supertexta* (19.8 – 21.6) (Cheng et al., 2004) and *H. iris* (16 – 20) (Nollens et al., 2004). Carefoot et al. (1993) showed similar HGC levels of 23 – 25 μg ml^{-1} for fed *H. kamtschatkana*, with levels dropping rapidly over the first 6 days of starvation and stabilising at 14 – 15 μg ml^{-1} thereafter. Baseline values observed in the current study in the region of 20 – 55 μg ml^{-1} are comparable with these values, but markedly lower than the mean HGC value of 133.2 ± 100.8 μg ml^{-1} (n = 22) calculated for a mixed collection of gastropods (Livingstone & De Zwaan, 1983). Of interest is the wide range reported, spanning more than an order of magnitude, which Livingstone & De Zwaan (1983) attribute to not just interspecific differences and animal condition, but to both the large inter and intra-individual variation reported in studies. Similarly, HGC in abalone has been shown to vary considerably in response to a number of factors, including habitat (wave action) (Wells et al., 1998), starvation (Carefoot et al., 1993), toxin exposure (Zhou et al., 2010, 2015), disease (Nollens et al., 2004; Rosenblum et al., 2005; Viant et al., 2003), dissolved oxygen level (Cheng et al., 2004; Vosloo et al., 2013b) and water temperature (Vosloo et al., 2013b). This study provides possibly the first description of the effect of diet and feeding on the haemolymph glucose dynamics of an abalone species.

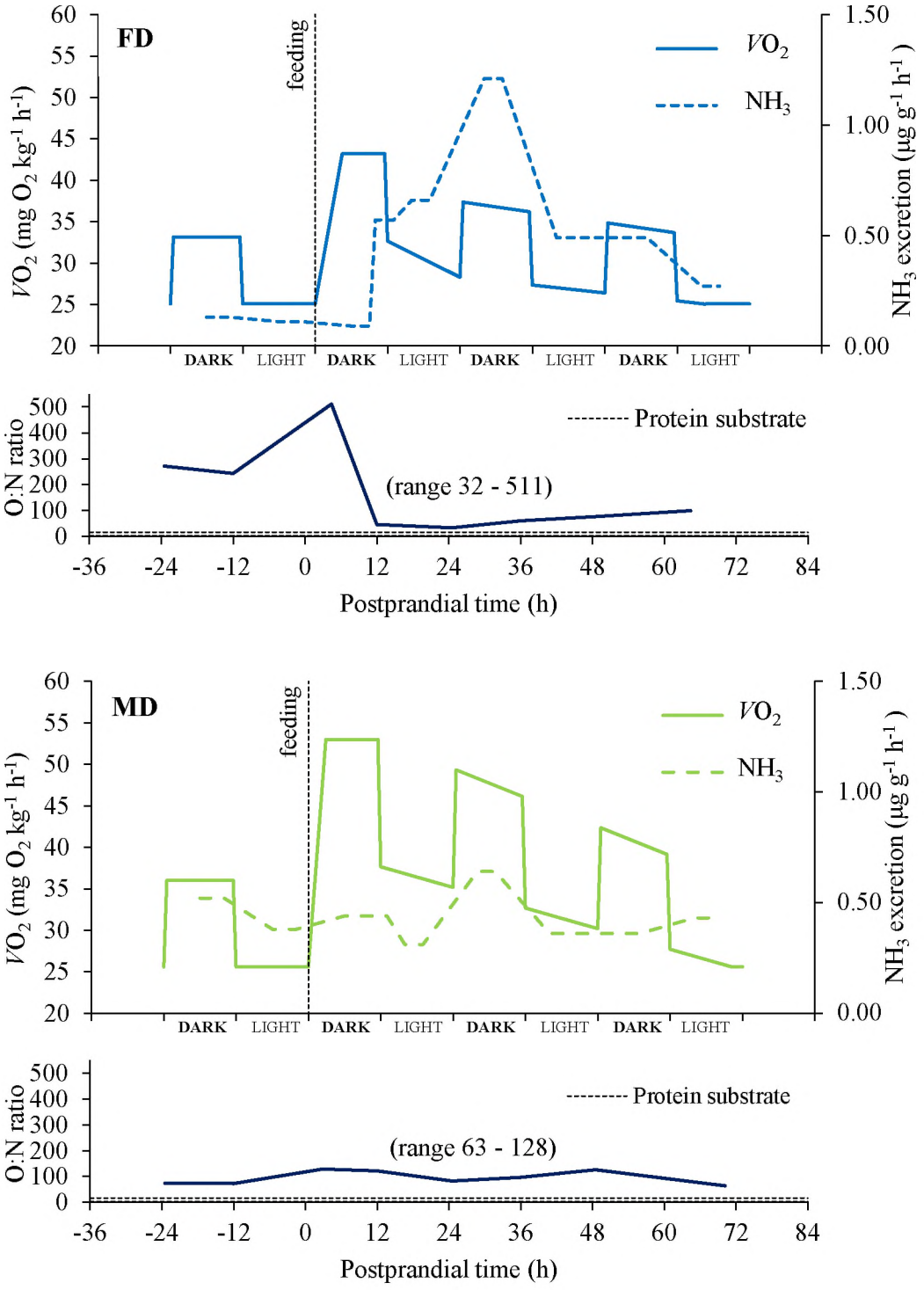


Figure 55. A representative model of the $\dot{V}O_2$, TAN excretion and O:N ratio of abalone fed a formulated feed and macroalgae at a target ration of 0.5% body weight as dry weight. $\dot{V}O_2$ data for the model were derived from mean treatment SMR and SDA values (Table 19), assuming a linear decline in $\dot{V}O_2$ following SDA peak and that the relative diel fluctuations observed preprandially could be applied postprandially. NH_3 data were derived from mean treatment values observed during the ammonia excretion trial (standardised to a mass of 63 g) (Figure 49). O:N ratios were calculated as O_2 consumed (moles g^{-1} h^{-1}) ÷ NH_3 excreted (moles g^{-1} h^{-1}).

At 96 hours post-feeding, HGC was significantly higher for abalone fed diets containing formulated feed (CML and FD) compared to those fed macroalgae. Diet related differences in HGC have not been assessed directly in abalone, although there are indirect indications that diet may have an effect. For example, control abalone in trials where animals were conditioned on macroalgae showed HGC in the range of 14 – 25 μg ml^{-1} (Carefoot et al., 1993; Cheng et al., 2004; Nollens et al., 2004) while those fed formulated feed showed higher values in the range of 41.4 – 99 μg ml^{-1} (Vosloo et al., 2013a, 2013b). The consumption of a carbohydrate-rich diet (Bemax), compared to a lettuce control diet, has been demonstrated to lead to a marked increase in haemolymph glucose concentration in the pond snail *Lymnaea stagnalis* (Plisetskaya & Joosse, 1985; Scheerboom, 1978; Scheerboom et al., 1978; Veldhuijzen, 1974; Veldhuijzen & van Beek, 1975). In these studies, lettuce-fed animals exhibited HGC of 15 – 30 μg ml^{-1}, compared to 86 – 700 μg ml^{-1} for those fed high carbohydrate diets. Similarly, in the terrestrial pulmonate mollusc *Megalobulimus oblongus*, HGC increased 46% when fed a high carbohydrate diet (77.9% carbohydrate) compared to lettuce (Rossi & Silva, 1993). Furthermore, while abalone conditioned on diets containing formulated feed exhibited fairly stable HGC up to 128 hours post-feeding, those conditioned on macroalgae showed a significant rise in HGC over the same period. This is in contrast to the findings of Carefoot et al. (1993), who reported a rapid drop in HGC within 6 days of initiating starvation, followed by stable levels for a further 25 days. However, the ability to maintain a stable HGC under short-term starvation has been reported in other gastropods such as *L. stagnalis* and *M. oblongus* (Veldhuijzen, 1974; Veldhuijzen & van Beek, 1975). Common among these studies is the observation that although HGC levels could be maintained under starvation, this was at the expense of tissue polysaccharide stores, with glycogen depletion evident during starvation whenever measured. In the case of *H. kamtschatkana*, the initial rapid drop in HGC corresponded with a parallel rapid depletion of glycogen in the digestive gland, while the depletion of glycogen in the foot muscle occurred more slowly (Carefoot et al., 1993). The authors suggest that this synchronicity could indicate that the regulation of haemolymph glucose levels in fed animals is related to the maintenance and turnover of these glycogen stores.

Despite the general consensus that abalone share the predominantly carbohydrate economy common to gastropods (Liebsch & Becker, 1990), the regulation of carbohydrate metabolism in the Haliotidae has received little research attention. Despite

this, a hypothetical functional model can be developed based on observations from other molluscs, particularly gastropods. It is increasingly apparent that mechanisms exist to regulate HGC and counter hypo- and hyperglycaemia in gastropods (Livingstone & De Zwaan, 1983). There is evidence to suggest that the endocrine control of carbohydrate metabolism exists in some molluscs. Studies on marine and freshwater bivalves, prosobranchs and terrestrial pulmonates have reported cells producing insulin-like substances (ILS) in a variety of tissues, including the gut epithelium, intestine, digestive gland, pyloris and central nervous system (see Joosse, 1984). However, attempts to elucidate the physiological effects of mammalian insulin and/or ILS on carbohydrate metabolism have revealed varied and often contradictory results (Livingstone & De Zwaan, 1983; Plisetskaya & Joosse, 1985). For example, in some freshwater and marine bivalve species, the injection of bovine insulin resulted in hypoglycaemia and increased glycogen synthetase activity and glycogen deposition (Plisetskaia et al., 1978; Plisetskaya et al., 1978a, 1978b; Plisetskaya & Joosse, 1985). Similar results using mammalian insulin have been reported for the gastropods *Laevicaulis alte* (Kulkarni, 1973), *Strophocheilus oblongus* (Marques & Falkmer, 1976) and *Cryptozonia belangeri* (Rajan & Sriramulu, 1978), as well as the slugs *Arion ater* and *Ariolimax columbianus* (Plisetskaya & Joosse, 1985), although artificial administration of a glucose load was at times required to elicit a response (Livingstone & De Zwaan, 1983). Conversely, *Helix pomatia* and *L. stagnalis* showed no response in HGC to mammalian insulin or an ILS fraction extracted from the intestine, despite this ILS showing high activity in a mammalian radioimmunoassay (RIA) system (Joosse, 1984; Plisetskaya & Joosse, 1985). Of interest are examples where an endogenously derived ILS affects HGC while mammalian insulin does not, which indicate possible molecular differences as has been reported for the bivalve *M. galloprovincialis* (Plisetskaia et al., 1978). Furthermore, HPLV analyses have shown that although the ILS molecule from *L. stagnalis* behaves similarly to mammalian insulin, it is not structurally identical (Joosse, 1984). It is evident that studies applying mammalian insulin should be interpreted with caution given the potential specificity issues associated with this approach.

Potentially functioning in parallel with endocrine regulation are glucostatic regulation mechanisms controlled directly by haemolymph glucose concentration. The possibility of elevated haemolymph glucose stimulating *in vivo* glycogen synthesis in the gastropod *L. stagnalis* has previously been proposed by Veldhuijzen & Cuperus (1975), Veldhuijzen & Dogterom (1974) and Veldhuijzen & van Beek (1975). This effect was

demonstrated in *M. oblongus*, whereby haemolymph glucose levels directly regulated the rate of glycogen synthesis in isolated tissue cultures derived from the mantle, hepatopancreas and muscle (Rossi & Silva, 1993). The primary mechanism likely involves the effect of glucose, or more specifically glucose-6-phosphate (G6P), on the enzyme glycogen synthase (GSase) responsible for glycogen synthesis. GSase activity is controlled through a combination of covalent modification, allosteric activation and enzymatic translocation (Greenberg, 2006). Initially, GSase was thought to exist in two interconvertible forms, the minimally phosphorylated active I form and phosphorylated D form (Hers et al., 1970; Shulman et al., 1995). It has since been shown to that GSase can exists in numerous intermediate forms, phosphorylated on up to nine serines by different kinases, resulting in progressive inactivation and reduced sensitivity (Greenberg, 2006; Jensen & Lai, 2009). Following transport into the cell and conversion to G6P by hexokinase, glucose can either enter glycolysis or be incorporated into glycogen. The binding of G6P to GSase triggers it to unfold, resulting in allosteric activation as the phosphorylation sites are exposed to phosphatase action (Greenberg, 2006; Shulman et al., 1995). Binding of G6P also promotes conformational changes that favour further dephosphorylation (Greenberg, 2006). In addition, phosphate compounds such as ATP and ADP can inhibit GSase, regardless of phosphorylation state, but this inhibition is reversed by G6P and thereby makes active forms of GSase G6P-dependent (Shulman et al., 1995). Essentially, the rate of glycogen synthesis is being controlled by the glucose-uptake steps through feed-forward allosteric control by G6*P*, with the change in phosphorylation state serving to adjust the kinetic properties of GSase to prevent an excessive build-up up G6P in the cell (Shulman et al., 1995). Insulin acts on this system by inducing dephosphorylation of GSase through the inhibition of kinases, such as PKA and activation of phosphorylases, as well as by the stimulation of glucose transport into the cell that thereby increase intracellular G6P concentrations (Greenberg, 2006; Saltiel & Kahn, 2001).

This theoretical framework potentially provides useful insight into the effect of the observed postprandial changes in HGC in the current study. Abalone consuming a diet with a formulated feed component exhibited a marked increase in their HGC postprandially, with levels 3.1 – 3.8 times the preprandial fasting levels. Total HGC peaked at 158 – 198 $\mu g\ ml^{-1}$ in these abalone. By contrast, HGC for abalone fed macroalgae was substantially lower at 2.2 times greater than preprandial levels, with a

peak of 72.6 μg ml^{-1}. Elevated blood glucose concentrations have been demonstrated to result in increased intracellular levels of glucose 6-phosphate in a number of tissues (Villar-Palasí & Guinovart, 1997). It is conceivable that the elevated postprandial HGC associated with feeding on formulated feeds results in elevated intracellular levels of G6P, thereby driving the formation of glycogen through the allosteric activation of GSase. The significant positive linear relationship between the proportion of formulated feed in the diet and the muscle glycogen content (Figure 34) would provide indirect evidence to support this hypothesis. Similarly, total tissue polysaccharides doubled in response to consumption of Bemax, a carbohydrate wheat-germ product, in *L. stagnalis* (Veldhuijzen & van Beek, 1975). Furthermore, there is evidence to suggest that elevated HGC may affect the neurosecretory response through the direct excitation of the central neurons producing insulin-related peptides (Kits et al., 1991). For example, glucose caused a dose-dependent excitation of the light green cells (LGCs), a group of 200 neuroendocrine neurons, in the gastropod *L. stagnalis* (Kits et al., 1991). The threshold concentration for depolarization was 0.2 mM (36 μg ml^{-1}) glucose, with slow polarization from 0.2 - 1 mM (36 – 180 μg ml^{-1}) and rapid depolarization after 1 mM. These concentrations fall within the physiological levels observed in the current study with the mean peak postprandial HGC of 158 – 198 μg ml^{-1} for treatments involving formulated feed showing potential for the rapid depolarization of MIP releasing neurons. Neurosecretory cells have been identified in the cerebral ganglia of abalone (Hahn, 1994; Upatham et al., 1998). However, the degree to which their role can be implied using a more complex pulmonate nervous system as a model remains to be demonstrated, particularly given that the abalone central nervous system exists in the primitive streptoneurous condition (Crofts, 1929; O'Brien & Degnan, 2000; Yahata, 1971). While the presence of both direct and endocrine-assisted glycostatic mechanisms is likely in the Haliotidae, the ability of these mechanisms to adapt to novel carbohydrate-rich formulated feeds remains largely unexplored.

There was a marked dietary difference in not only the magnitude, but also in the duration of the postprandial haemolymph glucose response. Abalone feeding on formulated feed took approximately twice as long (32 hours) to clear their postprandial glucose load compared to those feeding on macroalgae (16 hours) (Table 21). One explanation would be that the glycostatic mechanisms have a limited upper capacity in terms of the rate at which they can dispose of glucose from the haemolymph. This

would mean that as the total glucose load increases, so too would the duration required to return to preprandial levels. The significantly higher glycaemic carbohydrate intake per unit of total feed consumed for the diets containing formulated feed (Table 21) could directly determine the glucose clearance time. There is also evidence to suggest that the glycogen levels in the tissue provide a negative feedback mechanism to regulate the activity of glycogen synthase and thereby the excessive deposition of polysaccharides (Hers et al., 1970). An inverse relationship between muscle glycogen levels and GSase activity has been demonstrated in mammal models (Bogardus et al., 1983; Danforth, 1965; Furler et al., 1998; Munger et al., 1993; Nielsen et al., 2001), despite GSase binding directly to glycogen (Greenberg, 2006). It has been shown that GSase is primarily located in the membrane fraction of glycogen-rich muscle and in the cytoskeleton fraction of glycogen-depleted muscle. It was suggested that the specific subcellular location of GSase, is the mechanism regulating GSase activity as, determined by the inherent glycogen stores (Nielsen et al., 2001). Similar inverse relationships between tissue glycogen levels and glycogen synthesis have been observed in both gastropods and bivalves (Gabbott & Whittle, 1986; Hemminga et al., 1985; Rossi & Silva, 1993). It is therefore feasible that the ability of abalone to clear a postprandial glucose load may be partially determined by their nutritional history as reflected by their existing glycogen stores.

An extended period of elevated haemolymph glucose potentially results in a number of consequences for abalone feeding on formulated feeds. The first relates to the effect of HGC on food intake. Scheerboom et al., (1978) showed in *L. stagnalis* that food consumption was inhibited by HGC's above 120 $\mu g\ ml^{-1}$, although artificial infusion of glucose did not affect feeding behaviour (Scheerboom & Hemminga, 1978). It was suggested that a dual system may be regulating feed intake, through both the mechanical stimulation of mechanoreceptors resulting from gut dilation (Elliott & Susswein, 2002) and the HGC (Scheerboom & Hemminga, 1978). Recent work by Dyakonova et al. (2015) provides a possible mechanism for how a metabolic indicator of nutritional state (i.e. glucose) can directly mediate the hunger-related behavioural state. It was demonstrated in *L. stagnalis* that pedal serotonergic neurons (PeA) responsible for the control of locomotion changed their biophysical characteristics (i.e. reduced membrane potential and firing rate) in response to an elevated glucose concentration. The opposite feedback of glucose concentration has also been shown, with low glucose levels stimulating PeA neuron activity and excitatory response (Alania

et al., 2004; D'yakonova, 2014). If similar modulatory effects of HGC on the behavioural state of abalone are occurring, this might go some way to explaining the negative relationship between total feed intake and the proportion of formulated feed in the diet observed in the current growth trial (Table 9).

It has been proposed that the LGC system essentially combines the growth regulation (growth hormone and insulin growth factor) and the classic metabolic functions of insulin ascribed to mammalian models (Kits et al., 1991). Nutritional cues such as sustained elevated HGC can modulate the pattern of expression of molluscan insulin-related peptide (MIP) genes in the cerebral ganglia (Geraerts et al., 1992). In *L. stagnalis*, the change to a high carbohydrate diet (Bemax) has been shown to result in the decreases in the transcript levels of MIP II and III in the LGC's, compared to lettuce fed animals. This would indicate that the MIP gene family exhibits a differential pattern of stimulus-dependent expression. Furthermore, the switch to a high-carbohydrate diet resulted in the cessation of growth, massively increased glycogen stores and stimulation of reproduction (Geraerts et al., 1992). The observation of a nutritional-stimulus-driven alteration in gene expression in mature gastropods begs the question of whether this modulation is occurring at other life stages, and whether early exposure may play a role in the ability to metabolize novel diets later in their ontogeny. During early development, organisms have been shown to respond to environmental conditions with adaptations at the cellular, biochemical and biochemical level (Patel & Srinivasan, 2002). Permanent change to the physiology and metabolism of the animal that persist beyond the initiating stimulus has been termed metabolic programming (Lucas, 1991; Patel & Srinivasan, 2002). Much of the research investigating this phenomenon relates to studies using mammalian models, particularly rats and human (Lucas, 1991, 1998; Patel & Srinivasan, 2002). A number of mechanisms have been proposed to explain the retention of early programming. These including the immediate effects of nutritional stimulus on structural development (Bennis-Taleb et al., 1999; Berney et al., 1997), adaptive clonal selection or differential proliferation of tissue types (Lucas, 1998) and the epigenetic effect resulting from altered DNA methylation patterns (Lucas, 1998; Patel & Srinivasan, 2002). As altered DNA methylation patterns are transmitted to daughter cells during replication, this mechanism may potentially explain the intergenerational programming observed in some studies (Patel & Srinivasan, 2002).

Recently, the question of whether early hyperglucidic stimulus can metabolically program fish to deal with high carbohydrate diets in later life has been investigated in a number of species (Fang et al., 2014; Geurden et al., 2007, 2014; Gong et al., 2015; Rocha et al., 2014, 2015, 2016). Physiological plasticity to early programming of the glucose metabolism was most apparent in zebrafish and sturgeon (Fang et al., 2014; Gong et al., 2015), with digestive enzyme and intestinal fungal modification reported in rainbow trout (Geurden et al., 2007, 2014). A common observation amongst these studies is the importance of the nature of the stimulus (i.e. duration, source and magnitude) and the timing of application (i.e. the critical transition window) in determining the success of programming (Kamalam et al., 2017). The nutritional ontogeny of abalone would indicate two possible windows where imprinting could occur, firstly at commencement of feeding after yolk sac absorption and secondly when diet shifts from a benthic film and microalga to only macroalgae. As natural microalgal diets are generally mimicked under culture conditions, the period of weaning from microalgae to macroalgae or a formulated diet potentially provides a window of inherent plasticity where nutritional programming is possible. Abalone that are weaned directly onto formulated feed may be receiving a nutritional stimulus that modulates their glucose metabolism and provides an adaptive ability to utilise diets high in refined carbohydrates during later development. If metabolic programming is occurring, it may go some way to explaining the anecdotal observations on farm, supported by the growth data in the current study where abalone weaned onto macroalgae but then switched to formulated feed later in their development did not exhibit good growth rates. Furthermore, it is likely that any metabolic programming that may be occurring exists in parallel with other ongoing dietary-driven factors, such as bacterial faunal mediation (Nel et al., 2017b).

Furthermore, clear evidence of 'cross-talk' between peptidergic systems controlling growth and reproduction have been demonstrated in *Lymnaea* (Geraerts et al., 1992). The diversion to female reproduction has been demonstrated to play a major role in utilisation of excess haemolymph glucose in terrestrial and freshwater gastropods, with 60-80% of labelled glucose being incorporated into the albumen gland (Veldhuijzen & Cuperus, 1975). However, there is little indication of the role of galactogen in the development of gametes in abalone (Healy & Schulte, 2012), but a useful line of enquiry for future research would be to explore the degree to which the elevated HGC

levels, as observed for abalone in the current study, are being regulated by diversion to reproduction.

5 Conclusion

Our fundamental understanding of aspects of the nutritional physiology of members of the Haliotidae is enhanced by the data generated from the empirical studies, as documented in chapters 2 – 4 of this thesis. However, the context of this research is strongly rooted in the commercial culture of abalone and the desire to optimise nutritional approaches in the farming environment. This final concluding chapter reflects on key themes from the empirical chapters, discusses their potential commercial relevance and highlights future research directions. It is perhaps useful at this point to frame this synthesis briefly within the setting and observations that informed the research aims of the current study. In the last thirty years, the source of abalone for human consumption has shifted dramatically away from wild-capture fisheries to aquaculture, with 95% of the estimated global production in 2015 being farmed, compared to just 8% in 1989 (Cook, 2016). Farmers initially relied on the natural food of abalone as a culture feed in the form of fresh macroalgae, a practice that is still employed in many locations today. However, in regions where macroalgae were not available or faced harvest limits, the need to develop formulated feeds to facilitate long-term industry development was evident. Extensive research effort, embedded largely in the quantifying nutritionism paradigm, led to the development of a number of the commercial feed products currently used in the industry. Despite local trade-offs across divergent global locations between growth performance, cost, pellet stability, ingredient availability and manufacturing constraints, the resultant feeds are remarkably similar.

Central to this thesis is the observation that these formulated feeds differ markedly in their nutritional composition and structure when compared to the mixed macroalgal diets that abalone have evolved to utilise in their natural intertidal environment. Levels

of protein inclusion in formulated feeds generally exceed the protein content of macroalgae. Furthermore, although carbohydrates levels are comparable between macroalgae and formulated feeds, the structure of their carbohydrate contents differs. Macroalgal carbohydrates are primarily cell bound, composed of structural and mucilage polysaccharides, and exhibit a high ratio of soluble:insoluble fibre. By contrast, the carbohydrate component of formulated feeds is primarily composed of refined flours / starches of terrestrial origin, is gelatinised to some degree and has a low fibre content. This begs the question of the degree to which the nutritional physiology of abalone diverges in response to novel formulated diets, and whether the understanding gained from investigating this question can provide new insights into improving the efficiency of formulated feeds.

A number of more specific observations provided further focus to the specific research avenues that were pursued. Previous studies have shown that, despite lower protein levels and less energy-dense forms of carbohydrate, macroalgal diets regularly equal or outperform formulated diets in comparative studies. In addition, feeding combination diets (macroalgae and formulated feed together) results in equal or superior growth performance compared to feeding only the formulated diet component fed, despite offering a lower protein content. There are, therefore, several indications that macroalgal protein is utilised more efficiently than the protein supplied in formulated feeds. Furthermore, the differences in the form and availability of the carbohydrate component of formulated feeds and macroalgae may lead to divergent responses in the post-prandial glucose concentration of abalone. The biochemical and structural differences between formulated feeds and macroalgae also indicate that there may be differences in the metabolic cost of digesting these feeds, with direct implications for utilization efficiency. Finally, the expanding application of integrated feeding regimes, which combine formulated and macroalgal feed components (combination diets and/or macroalgal incorporation into formulated diets), generates complex nutritional scenarios beyond the dichotomous formulated-versus-macroalgae comparison, although little is understood regarding nutrient utilisation in these situations.

Within these contexts, the aims of the current study were therefore: (1) to assess the effect of feeding formulated, mixed macroalgal and combination diets on the growth

and indices for gross nutritional efficiency indices in two species of abalone (*H. rufescens* and *H. midae*), (2) to determine the contribution of different dietary components to the deposition of C and N into pedal muscle tissue under the aforementioned dietary regimes using stable isotopes as natural biomarkers, (3) to estimate the metabolic cost of digestion for macroalgal and formulated feeds by quantifying the SDA response, using high temporal resolution respirometry, (4) to quantify the postprandial dynamics of ammonia excretion and integrate these data with oxygen consumption data, to generate O:N ratios as a means of tracking the nature of the substrate funding metabolism and, (5) to determine the postprandial dynamics of haemolymph glucose concentration in response to ingestion of formulated, combination and macroalgal diets. A number of key themes, some with supporting evidence from multiple experimental approaches, have emerged from these studies.

Protein Utilisation

In the current study, data from a number of divergent sources support the hypothesis that abalone are able to utilise the protein component for growth from macroalgae more efficiently than the protein component of formulated feed. Firstly, independent growth trials returned a significantly higher protein efficiency ratio (PER) for macroalgae compared to formulated feeds for both *H. rufescens* (Table 5) and *H. midae* (Table 9). The data from the growth trial on *H. midae* are particularly robust in this regard, because it was possible to obtain accurate weekly estimates of the protein content of the three macroalgae used. In both cases, significantly higher PER for abalone fed macroalgae was also associated with significantly higher specific and linear growth rates (Table 11, Table 4). Secondly, by using stable isotope signatures as bio-markers, it was possible to trace the incorporation into pedal muscle tissue of individual dietary ingredients of the formulated feed consumed by *H. midae*. Results suggest that the poor efficiency of protein utilization could be traced largely to the low incorporation of the fishmeal component of protein in the diet. This was apparent from the large disparity between the model-estimated (Stable Isotope Analysis in R – SIAR) proportional contribution to pedal muscle tissue and the proportional contribution of dry feed intake in this dietary treatment (Figure 31) Thirdly, the marked drop in the O:N ratio observed soon after feeding indicated a postprandial shift to the utilisation of protein substrates

for energy metabolism. This would suggest that the low efficiency of protein utilization by abalone fed the formulated feed in the current study could be attributed, at least in part, to the diversion of protein into catabolic metabolic pathways rather than anabolic pathways that result in somatic growth. This would indicate that the digestibility of the protein components of the diet was not limiting but that poor protein utilization resulted from a mismatch between the protein requirement of the organism and the protein composition of the diet, particularly fishmeal.

From the perspective of commercial feed formulation, these data would indicate that there is still scope to refine the protein composition of the formulated diet. A number of options are available to this end, each requiring an increasing level of required input data. In the absence of a target amino acid profile, the simplest option would involve testing formulations that diversify the protein sources in formulated feeds, particularly using plant-based protein and production outcomes to gauge performance. This is essentially the "black box" approach. A more targeted approach would involve formulating diets to meet a defined amino acid profile. To date, the most common method for defining the amino acid profile of diets involves the ideal protein concept, using abalone soft tissue profiles as a reference to guide dietary protein inclusion. However, a number of factors indicate an alternative may be to benchmark the amino acid profile of diets against one derived from a representative mixed-macroalgal diet. These include: (1) the paucity of information on amino acid turnover, conversion or physiological roles unrelated to growth, (2) the good outcomes for growth under experimental conditions generally associated with a mixed-macroalgae diet, and (3) the propensity of abalone to consume this mixed-macroalgal diet in its natural environment.

This approach to guide the formulation of suitable diets, in the absence of a comprehensive knowledge base regarding the potentially diverse physiological roles of amino acids in abalone, relies on the evolutionary relationship between abalone and their natural food source. However, the selective pressures relevant to a commercial abalone farmer do not always align exactly with the selective pressures that have guided the evolution of abalone in its natural environment. Perhaps the most obvious example relates to the investment of nutritional resources into reproduction, a fundamental requirement in the natural environment but one which has been usurped in the culture

environment by the use of broodstock. Designing formulated diets that foster preferred outcomes, such as somatic growth, over undesirable outcomes, such as reproductive tissue growth, requires a fundamental understanding of the role of specific dietary components in each of these processes. Tracing nutrient utilisation using traditional stable isotope methods, as demonstrated in the current study, can provide useful information on a whole-ingredient basis in this regard. However, the chromatographic separation of sub-units of complex organic molecules preceding stable isotope analysis, termed compound specific stable isotope analysis (CSIA), provides the methodology to trace individual amino acids (Gamboa-Delgado, 2014; Le Vay & Gamboa-Delgado, 2011). This approach has been employed successfully to trace both amino acids and essential fatty acids in insects, crustaceans, rotifers and fish (Fantle et al., 1999; O'Brien et al., 2003, 2005; Parrish et al., 2007), and potentially provides an analytical technique to interpret individual amino acid utilisation for abalone and formulated diets accordingly.

Carbohydrate type and haemolymph glucose concentration

The dynamics of the postprandial haemolymph glucose concentration (HGC) in *H. midae* varied markedly in relation to diet. Abalone fed formulated feed exhibited a peak in HGC approximately twice that of abalone fed macroalgae. In addition, the duration of the response (i.e. the time taken to return to preprandial HGC levels) was greater for abalone fed the formulated diet. Taken together, these factors resulted in a total response for abalone fed formulated feed (as determined by the area under the curve bound by the preprandial HGC level) close to four times that of abalone fed macroalgae, despite a similar level of ingested carbohydrate (Table 21). The elevated HGC response observed is most likely the result of the form of the carbohydrate in the diet, with the gelatinised starch component of the formulated feed being both highly digestible and yielding a high proportion of free glucose per unit weight ingested. It is suggested that these elevated HGC levels provide conditions that stimulate the positive feed-forward allosteric control of glycogen synthase through glucose-6-phosphate, resulting in the high tissue glycogen levels observed in abalone fed formulated feed.

The implications of this proposed mechanism can be both positive and negative for abalone farmers. High pedal muscle glycogen levels hold advantages from the perspective of product quality and yield, particularly when abalone is canned. Simulated canning trials conducted on both *H midae* and *H. rufescens* provide supporting evidence to the anecdotal observations that abalone feeding on formulated feeds produce a higher canning yield compared to those consuming macroalgae (Figure 10, Figure 33). This effect is probably related to the displacement of collagen by glycogen in the muscle tissue in abalone fed formulated feed, and the resulting reduced loss of gelatinized collagen to cooking water under the high-temperature, submerged-heat treatment of canning. Abalone fed on formulated feed also exhibited a number of other positive traits in relation product quality. Glycogen concentration has been linked with increased palatability (Watanabe, 1992). Furthermore, studies comparing abalone fed formulated fed to those fed macroalgae report reduced foot-muscle pigmentation, increased taste-active components such as glycine, glutamate and AMP, and higher acceptability and preferred texture in tasting panels (Bewick et al., 1997; Brown et al., 2008; Chiou et al., 2001; Chiou & Lai, 2002; Preece, 2006).

However, any gains in product yield or quality need to be considered within the context of the growth rates achieved, as was highlighted by the disparity in growth rates between formulated feed and macroalgal diets in the current study. It seems probable that the high circulating levels of haemolymph glucose, which are driving the synthesis of glycogen, may also be affecting aspects of the abalone endocrine system and abalone behaviour. Assuming abalone resemble other gastropod models, there is evidence to suggest that elevated HGC, in combination with mechanoreceptors in the gut, acts as a metabolic signal of the fed state and can mediate hunger-related behaviour (Dyakonova et al., 2015; Elliott & Susswein, 2002), which may be occurring through the direct down-regulation of the pedal serotonergic neuron activity in response to elevated circulating glucose (Dyakonova et al., 2015). Essentially, the rapid increase in HGC associated with feeding formulated feeds may be influencing the signalling of satiation, resulting in a reduction of feeding activity and limitation of dietary intake of other dietary components such as protein. Behavioural studies investigating feeding periodicity and consumption rates in relation to diet would add valuable insight in this regard. There are also indications that a sustained elevated HGC may modulate the pattern of expression of molluscan insulin-related peptides (MIP) in the cerebral

ganglia. It has been suggested in gastropods models that the light-green cell system in the cerebral ganglia serves both the growth regulation (growth hormone and insulin growth factor) and the classic metabolic function of insulin in mammalian models, and that there is evidence of 'cross talk' between the peptidergic system that controls growth and reproduction. In *Lymnaea* models, certain peptides have been shown to have dual functions, such as stimulating reproduction while at the same time suppressing growth (Geraerts et al., 1992). Furthermore, feeding a high carbohydrate diet in *Lymnaea* resulted in cessation of growth and an associated increase in glycogen storage and reproductive stimulation. It is possible that in abalone the presence of high HGC stimulates an endocrine response that drives energy storage and reproduction while simultaneously supressing somatic growth. Information regarding the peptidergic systems in abalone is very limited. Initial research effort that focussed on isolating the neurological origin of the molluscan insulin-related peptides in abalone would facilitate the investigation of the effects of nutritional stimuli on the expression of the genes coding for these peptides, and their role in the nutritional physiology of abalone.

Inclusion of macroalgae

Feeding formulated feed in conjunction with fresh macroalgae to form combination diets resulted in increased growth and protein efficiency over abalone fed formulated feed in isolation. This trend was evident in the current study for growth trials conducted with both *H. midae* and *H. rufescens* and is line with findings from other studies in the literature. The potential roles played by macroalgae in these outcomes, include both nutritional and functional aspects, are discussed extensively in Chapter 2 and 3. From a nutritional viewpoint, macroalgae can compensate for deficiencies in the formulated diet by providing essential nutrients not available in the formulation. Alternatively, in terms of the functional nutrition, macroalgae may be providing a number of advantages, which include the provision of bioactive compounds that facilitate abalone health, action as a feeding stimulant, provision of dietary bulk that stimulates gut function and extends gut transit time, and action as a prebiotic to regulator of gut bacteria. It is unlikely that any of these drivers exist independently of each other, with the positive role of macroalgae in combination diets being multi-causal and the result of non-linear interactions between numerous factors. For abalone farmers currently utilising

formulated feed, but who wish to harness the positive growth and efficiency benefits of including macroalgae into their feeding regimes, at least three potential options are apparent.

Firstly, combination diets provide a method to integrate macroalgae into the diet of abalone without having to adjust any other parameters, assuming a source of fresh macroalgae (wild-harvested or cultured) is available. Growth trials indicated that a macroalgal contribution of 44% to total dry-feed intake resulted in a significant increase in both growth and protein utilisation efficiency for *H. midae*, while 32% was required to significantly improve protein efficiency and 76% for growth in *H. rufescens*. In outdoor culture systems, where the diatom film on the culture infrastructure is potentially already contributing to the combination diet, the required macroalgal inclusion levels could potentially be much lower. There is extensive scope to refine combination-diet protocols under commercial culture conditions, in terms of combinations of dietary components and inclusion levels. Given the complex nutritional environment resulting from theses regimes, it is indicated that methodologies for tracing nutrient utilization from individual components of the diet should be incorporated into these studies. Stable isotope analysis has been shown to be a viable option in this regard and the application of CSIA holds the potential to allow for even greater resolution in these studies.

Secondly, in the absence of a reliable source of macroalgae in site, the inclusion of macroalgae directly into a formulated feed during processing provides an alternative means to integrate macroalgae into dietary regimes. Initial trials investigating dried macroalgae meal as an ingredient in formulated feeds have yielded favourable results, but are not easily compared owing to their divergent experimental designs. A control diet of mixed fresh macroalgae (*U. lactuca* and *G. cornea*) resulted in significantly higher growth and protein efficiency in *H. tuberculata* than three formulated diets with inclusions of various combinations of macroalgae meal (*U. lactuca*, *G. cornea*, *P. palmate* and *L. digitata*) (Viera et al., 2015). In *H. discus hannai*, abalone fed formulated feeds with various combinations of macroalgal inclusion (*Laminaria digitata*, *Palmaria palmata* and *Ulva lactuca*) and control diet of fresh macroalgae (*L. digitate*) exhibited similar growth rate, although food conversion efficiency was

significantly higher for the formulated diet with a mixed macroalgal inclusion compared to the control (O'Mahoney et al., 2014). The experimental design of these studies however precludes making comparisons between formulated diets with and without macroalgae meal inclusion. In this regard, Nel et al. (2017b) investigated kelp (*E. maxima*) inclusion levels of 0 to 3.54% in formulated isonitrogenous diets for *H. midae*. Growth was significantly higher from a 0.44% inclusion level than a macroalgae-free control, with significantly higher protein efficiency and food conversion from 3.54%. Inclusion levels of 5% *Ulva* sp. or 10% *Gracilaria* sp. were reported to significantly increase growth in *H. laevigata* compared to a control diet (Bansemer et al., 2016a). In addition to growth outcomes, the inclusion of macroalgae meal into formulated feeds has been shown to have effects on digestive enzyme activity, the microbiome of the digestive tract and fatty acid concentrations in abalone tissue (Bansemer et al., 2016a; Nel et al., 2017a, 2017b; Viera et al., 2015). Given the paucity of knowledge surrounding dried macroalgae as a formulated feed ingredient, there is considerable scope to refine protocols around their use, particularly in terms of species diversity, pre-processing methods and inclusion levels. However, there are some technical considerations to take into account in this regard. The inclusion of macroalgae into formulated diets ultimately leads to the displacement of other dietary ingredients in the formulation. This can be problematic when the ingredients being replaced serve multiple purposes within the diet. For example, starches and gluten are often included in formulated feeds, both for their carbohydrate content and binding properties. In situations like this, the upper level of macroalgal inclusion may be limited by the ability to produce diets with suitable water-stability characteristics. Gel binding has been used to develop diets with macroalgal inclusion levels of 20% (Bansemer et al., 2016a) and may facilitate testing even higher inclusion levels at the experimental level. However, at the commercial scale, finding suitable and affordable non-starch binding solutions needs to be addressed if carbohydrate levels are to be adjusted below their minimum requirement for binding. The hydrocolloid binder complex of konjac glucomanna-xanthan gum (KX), with gelling concentrations as low as 0.02%, is a possible candidate in this regard (O'Mahoney et al., 2011, 2014).

The third option to include macroalgae into a formulated feed is, perhaps counterintuitively, to reduce or eliminate its inclusion by mimicking its potential benefits using derived or alternative ingredients. Sourcing sufficient macroalgae, from

either wild or cultured sources, can present a significant bottleneck in attempts to expand abalone production globally. The further development and refinement of formulated feeds to better match the nutritional requirements of abalone remains an important component in supporting future growth in the industry, both in terms of total output as well as expansion to alternative production sites. Using macroalgal amino acid profiles as a template for selecting alternative protein sources for inclusion in formulated feeds has been discussed previously. Two further potential areas of investigation stand out, relating to microalgae's role as a feed attractant and as a source of dietary fibre. The continuing development of algal extracts (Chojnacka & Kim, 2015), beyond the commoditised food and hydrocolloid industries, has the potential to produce concentrated algal products that may be included in formulated diets as a feed attractant. Furthermore, these products may also provide a concentrated source of bioactive compounds with associated health benefits. Research aimed at defining the specific chemical cues acting as feed attractants in macroalgae would provide valuable information for improving palatability of formulated feeds. However, results from the current study indicate that feeding activity may also be influenced by the metabolic state of abalone, with elevated HGC indicated as a potential driver of reduced feeding activity. Regulating HGC in abalone fed formulated diets would likely involve reducing the inclusion level of refined, high-density carbohydrate sources such as refined flours and starches by substitution with low-density, fibre-rich alternatives. Possible candidates such as corn fibre, fruit fibre and rice bran are often secondary by-products and may offer a price advantage compared to primary carbohydrate products. In addition to reducing overall carbohydrate density, these products provide other potential advantages in a formulated feed product. For example, fruit and vegetable fibres contain bioactive compounds such as flavonoids and carotenoids as well as water-binding properties that may assist in binding (de Godoy et al., 2013). Rice bran also contains several bioactive phytochemicals, including tocopherols, polyphenols, carotenoids and y-oryzanol, in addition to being a source of predominantly sulphur essential amino acids and micronutrient such as magnesium and B-vitamins (Ryan, 2011). A pair of studies related to incorporating alternative carbohydrate sources into formulated diets for abalone provided some promising results. In a formulated diet containing a 20% incorporation of *Laminaria japonicas* developed for H. discus, 80% of this *L. japonica* component could be substituted with rice bran without affecting growth rates or survival (Lee et al., 2015). Furthermore, in a later trial testing the inclusion of various carbohydrate sources into formulated diets for *H. discus*, the highly polymerized alpha-

cellulose resulted in significantly higher growth than simpler carbohydrates such as glucose, sucrose, maltose, wheat flour and corn starch (Lee et al., 2017). As discussed previously, any movement to adjust refined starch carbohydrate content in formulated diets may require a concomitant need to establish alternative feed binding. Macroalgal fibres have been shown to be fermented by bacteria in the gut of abalone, with indications that enzymes and other digestive products originating from bacteria may play an important role in the digestive physiology of abalone (Erasmus et al., 1997; Nel et al., 2017b; Sawabe et al., 2003). The specific requirement of abalone gut bacteria for macroalgal fibre and the degree to which non-algal sources of fibre may play a similar modulatory, prebiotic role remains largely unexplored. Culture-independent 16S rRNA gene microbiome sequencing and profiling analyses have been demonstrated to provide a useful method to investigate community composition and relative abundances of abalone gut bacteria (Nel et al., 2017b).

Nutritionally history and genetic considerations

There was a disparity between the growth rates in *H. midae* fed formulated feeds in the current study, and those reported for the same species grown on the same formulated feed under commercial cultures situations. One of the key differences between these two groups relates to their nutritional history, and particularly the weaning protocol employed. Under commercial culture conditions abalone are generally weaned directly from diatoms onto formulated feed, while the abalone used in the current study were exposed to a combination of two macroalgal species and formulated feed during weaning. Nutritional cues have been demonstrated to modulate the pattern of expression of genes associated with peptides for metabolic regulation in other gastropod species. Furthermore, early exposure to nutritional cues can lead to adaptations at the cellular, molecular and biochemical level that persist beyond the initiating stimulus in a phenomenon termed metabolic programming. One could postulate that exposure to formulated feeds during a critical transition period modulates the digestive and metabolic capacity of abalone to better utilise these diets later in their ontogeny. This has implications for commercial abalone farmers as early intervention could have marked effects on both the grow-out period required to reach market and the efficiency of nutrient utilisation, with knock on consequences for feed costs and the environmental

impact of both feed production and farm nutrient-waste production. Future research aimed at investigating the potential for metabolic programming would require the development of suitable indicators related to diet utilisation. Given that protein content and carbohydrate form are key differentiators between macroalgal and formulated feeds, exploring the transcriptomics of gene families associated with proteolytic enzyme production and carbohydrate metabolism would be logical starting points. In addition, the growing field of metabolomics provides a nonbiased system to identify and quantify the metabolites in abalone (Venter et al., 2016). Future development of formulated feeds for abalone likely lies at the interface between harnessing the positive characteristics of their natural macroalgal diet and exploiting their potential inherent digestive and metabolic plasticity, guided by -adoption of experimental approaches that generate a mechanistic understanding of their nutritional physiology.

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7 APPENDICES

APPENDIX 1 – A METHOD FOR PRODUCING THREE-DIMENSIONAL ACRYLIC RESIN CASTS OF THE DIGESTIVE TRACT OF ABALONE.

Current understanding of the morphology of the digestive tract of abalone is based primarily on dissection and histological studies (Bevelander, 1988; Campbell, 1965; Crofts, 1929; Harris et al., 1998b; McLean, 1970). The technique described here provides an alternative methodology to confirm the anatomical structures previously reported as well as provide information on their three-dimensional structure and relative spatial arrangement. The approach was developed based on insights gained from corrosion casting studies performed in other organisms, primarily to elucidate the layout of vascular systems (Lametschwandtner & Lametschwandtner, 1992; Ohtani & Murakami, 1992; Potter & Groom, 1992; Van Rij et al., 2004).

Casts were prepared as follows:

1) Healthy abalone with no outward indication of disease or shell damage were selected from culture baskets previously fed on both formulated feed and macroalgae. Abalone were maintained unfed for a period of 96 hours to allow the digestive tract to purge.
2) Animals were euthanised by an initial anaesthetic dose of 10% magnesium sulphate for 10 minutes, followed by a 20% solution for a further 20 minutes after which an incision to the cephalic region (to sever the cerebral ganglia) was administered (Schaefer et al., 2013; Upatham et al., 1998).
3) A low viscosity, acrylic-based resin was prepared using a Batson's #17 Anatomical Corrosion Kit (07349-1; Polysciences, Inc., 400 Valley Road, Warrington, PA 18976, USA) according to the manufacturer's instructions.
4) The catalysed resin was drawn into a syringe through a squared-off and smoothed 20G hypodermic needle. Initial attempts to introduce the resin into the digestive tract through the mouth were unsuccessful and resulted in considerable blow-back of the resin, likely due to the constriction/valve between the oesophagus and crop.
5) Injection of resin through the anus proved effective. This was accomplished by first removing the muscular tip of the section of the lower intestine that

protrudes between the gills. With abalone in a shell-down position, the adapted needle was then inserted a short way into the intestine and gentle pressure applied to the syringe plunger.

6) Resin was injected until resin could be seen emerging from the mouth region or blow-back was evident from the lower intestine injection area.
7) Abalone were placed in an ice slurry to cure for four (4) hours. The ice bath served to mitigate possible tissue damage from the ectothermic reaction associated with polymerisation.
8) Abalone were removed from the ice bath and placed into a maceration solution at 50 °C for 24 – 48 hours which dissolve all soft tissue and exposed the resin cast.
9) The cast was removed and rinsed in water before being stored.

Notes:

Obtaining complete casts of the entire digestive tract was extremely difficult as injection of the resin was undertaken blind with no outward indication of whether it had infiltrated all cavities in the system. The cast described in chapter 1 was the most complete cast produced and was taken from an animal cultured on formulated feed. The general presence and arrangement of structures was comparable to a number of partial casts obtained from animals previously fed both formulated feed and macroalgae.

APPENDIX 2 – A CHEMICAL METHOD FOR THE DETERMINATION OF GLYCOGEN CONTENT IN ABALONE ABDUCTOR MUSCLE TISSUE.

Abductor muscle glycogen concentrations were determined using a chemical assay method adapted from Woodcock & Benkendorff (2008) and Krisman (1962). A two-step procedure is employed consisting of the perchloric acid extraction of glycogen followed by iodine binding, where the intensity and hue of the glycogen-iodine complex produced by the addition of an iodine reagent represents a weighted average of total glycogen present (Dreiling et al., 1987).This method allows for the rapid estimation of tissue glycogen and provides results that are comparable with more complex enzyme-based assays that enzymatically digest glycogen using amyloglucosidase and then determine the resultant glucose (Dreiling et al., 1987).

1.1 Abductor muscle tissue samples

Following euthanasia and shucking, the rear portion of the abductor muscle tissue was removed by scalpel, placed into a 2 ml Eppendorf tube(s) and flash frozen in liquid nitrogen. Samples were then removed from the liquid nitrogen and transferred to a freezer for storage at -20 °C.

1.2 Preparation of reagents

1.2.1 0.06M Perchloric acid (PCA): Combine 16.82 ml 60% (w/v) PCA with deionised water to make up 1 L.

1.2.2 Iodine solution: Dissolve 10 ml 0.05M I_2KI solution in 90 ml H_20 to form a 10% solution.

1.3 Preparation of standards

A 5 mg.ml-1 glycogen standard (stock solution) was prepared by dissolving 2.5g purified oyster glycogen (Sigma Aldrich - G8751) in 100 ml deionised water and then diluted to a volume of 500 ml. Standard solution of 0.25; 0.5; 0.75; 1; 1.5; and 2 mg ml^{-1} were prepared by pipetting 50; 100; 150; 200; 300 and 400 µl into 2 ml Eppendorf tubes and making up each to 1ml with deionised water. A calibration curve was created from these standards and blanked with deionised water.

1.4 Sample analysis protocol

1.4.1 Remove frozen abductor muscle tissue from storage, dry with paper towel and, while still frozen, prepare a ~ 1g subsample.

1.4.2 Place tissue sample in a 50ml cellstar tube and homogenize (IKA Ultra-Turrax T25) with 20 ml cold 0.06M PCA. The resulting homogenate can be stored in a refrigerator for up to 6 months.

1.4.3 Pippete 1.5 ml of the solution into three separate 2ml Eppendorf tubes and centrifuge at 13 000 RPM for 5 minutes.

1.4.4 Pippete 0.5ml of supernatant from each tube into a single fresh tube.

1.4.5 Pippete 0.2 ml of combined supernatant into a fresh tube.

1.4.6 Add 1.3 ml 10% 0.05M iodine reagent

1.4.7 Allow to react for 30 min in the dark

1.4.8 Read the absorbance using a spectrophotometer at 460 nm against a blank of PCA and iodine reagent.

1.4.9 Calculate the glycogen content using the calibration curve.

1.4.10 Samples that fell outside the range of the calibration curve were diluted accordingly and re-analysed.

A new calibration curve was determined for each new batch of reagents. Figure 56 shows a typical calibration curve.

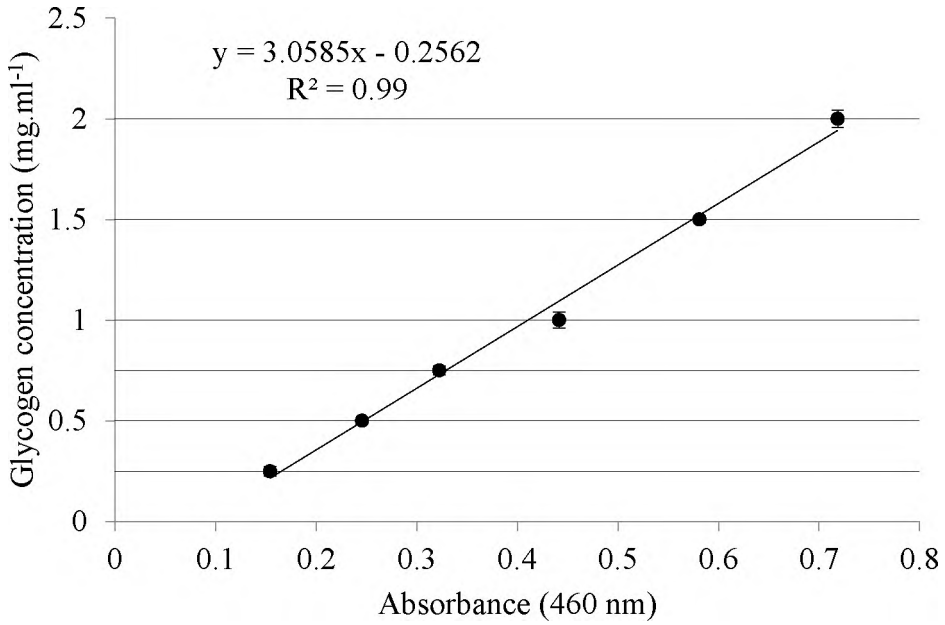

Figure 56. A typical calibration curve obtained for glycogen concentrations of 0.25 – 2 mg l^{-1} using purified oyster glycogen and a 10% 0.05M iodine reagent.

APPENDIX 3 – ANALYSIS PROTOCOL FOR THE DETERMINATION OF TAN IN SEAWATER.

The TAN concentration in the seawater samples was determined using the indophenol method utilised in the Merck Ammonium Test Reagent Set (Product code: 1.14752.0001/2). Briefly, ammonia in the sample reacts with a chlorinating agent to form monochloroamine, which in turn reacts with thymol to form a blue indophenol derivative that is measured spectrophotometrically. The test protocol used was as follows:

1. Collect 10 ml sample from incubation tank using syringe and place in a clean 50 ml PVC urine vial. Analyse immediately or place in freezer at -16 °C and analyse within 7 days.
2. To analyse a sample, remove a 5 ml subsample using a pipette and place in clean 10ml test tube previously cleaned using ammonia free detergent (1% solution of Liquinox Critical-Cleaning Laboratory Detergent; Alconox Inc. - http://www.alconox.com).
3. Add 0.60 ml of Reagent NH_4 – 1 and mix.
4. Add 0.1 ml of sodium hydroxide solution (5 mol l^{-1}) and mix.
5. Add 1 level microspoon of Reagent NH_4 – 2 and mix, completely dissolving the powder.
6. Leave to react for 5 minutes.
7. Add 4 drops of Reagent NH_4 – 3 and mix
8. Leave to react for 5 minutes.
9. Place in 10 mm cuvette cell and read concentration on a Merck Spectroquant Pharo 100 spectrophotometer using test-specific precalibrated program.

APPENDIX 4 – SAMPLING AND ANALYSIS PROTOCOL FOR THE DETERMINATION OF HAEMOLYMPH GLUCOSE CONCENTRATION (HGC) IN ABALONE.

Abalone to be sampled for the determination of HGC were removed from their culture container using a flat plastic paddle. A removal tool was used to remove abalone to prevent unnecessary stress and delay associated with abalone clamping onto a surface. Immediately following removal, abalone were placed ventral-surface up on a towel and the floor of the foot muscle patted dry using paper towel. A 21-gauge needle attached to a 5 ml sterile syringe was inserted centrally into the foot muscle on the anteroposterior axis, approximately ¼ of the distance from the anterior to the posterior end (Figure 57). The needle was inserted at a 45° angle to a depth of 5 - 10 mm and the plunger engaged to create negative pressure in the barrel. The needle was then slowly withdrawn until the needle tip reached the pedal sinus and haemolymph began flooding into the barrel. A sample of 2 - 3 ml of haemolymph was withdrawn from each abalone and transferred directly to an Eppendorf tube suspended in an ice slurry to prevent clotting. The complete capture and extraction process was undertaken in less than 60 seconds. Haemolymph samples were analysed immediately for their glucose concentration using an enzymatic method (Sigma-Aldrich Glucose (GO) Assay Kit GAGO-20).

The glucose present in a sample is oxidised to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a coloured product, which then reacts with sulphuric acid to form a more stable compound. The assay protocol is as follows:

1. Prepare assay reagent (oxidase/peroxidase/o-Dianisidine) per manufacturer's directions.

2. Prepare a 12 N sulphuric acid solution from 36 N sulfuric acid using deionized water.
3. Pipette 1 ml of haemolymph into a 10 ml test tube (dilute sample if glucose concentration exceeds 80 $\mu g\ ml^{-1}$).
4. Pipette 2 ml of assay reagent into test tube and mix.
5. Allow to react for 30 mins at 37 °C in a water bath.
6. At the end of the incubation period, stop the reaction by adding 2 ml of 12 N sulphuric acid and mixing the tube thoroughly.
7. Measure the absorbance of samples using a spectrophotometer against a reagent blank (deionized water) at 540 nm.

8. Concurrently, determine standard curve for each reagent batch as follows:
   8.1. Pipette 0.02, 0.04, 0.06 and 0.08 ml of a 1 $mg\ l^{-1}$ standard solution with 0.98, 0.96, 0.94 and 0.92 of deionized water to create glucose solutions of 20, 40, 60 and 80m $\mu g\ ml^{-1}$ respectively.
   8.2. Assay as per haemolymph samples and measure absorbance at 540 nm against a reagent blank of deionized water.
   8.3. Plot absorbance at 540 nm (y-axis) against glucose concentration (x-axis). Linear regression should show a strong linear relationship between absorbance and glucose concentration (R^2 values > 0.99). R^2 values of 0.997, 0.998, 0.997 and 0.995 were determined for the standard curves of the reagent batches used in this study.

9. Determine the glucose concentration from the standard curve. Multiply the glucose concentration by the dilution factor if applicable.

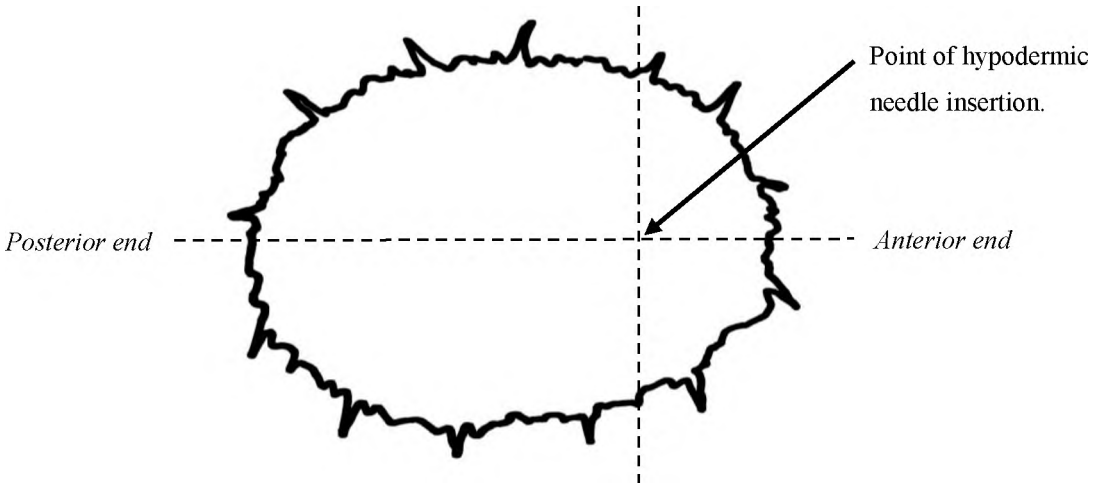

Figure 57. Ventral view of an abalone showing the point of insertion for a 21-gauge hypodermic needle to obtain a haemolymph sample from the pedal sinus.