Bioenergetics of Australian greenlip abalone (*Haliotis laevigata* Donovan)

DUONG NGOC DUONG

Presented for the degree of Doctor of Philosophy

School of Biological Sciences, Flinders University, South Australia

July 2016

Table of Contents

List of Tables

Thesis summary

The greenlip abalone, *Haliotis laevigata*, is a commercially important *Haliotis* species for aquaculture, mainly cultured in land-based systems, in southern Australia. In order to improve production and reduce culturing time, it is necessary to understand many aspects such as the production improvement the genetics of animals, the impact of major physical aspects, nutritional requirements and the influence of environmental factors such as water temperature. Studying bioenergetics, based on an energy balance, is one way to understand the basic biology of an animal and how the ingested food energy is partitioned and used for oxygen consumption, somatic growth, reproduction, excretion, mucus production and shell growth under different dietary and feeding scenarios or water temperatures.

In order to study the bioenergetics of abalone, five experimental trials (presented as Chapter 2, 3, 4, 5 and 6) were conducted to investigate the effects of diet and water temperature. Trial 1 (Chapter 2) was carried out to study the bioenergetics of greenlip abalone fed live macroalgae (*Gracilaria cliftonii* and *Ulva* sp. with or without enrichment) or commercial formulated diets. Ingested food, absorbed and somatic growth energy of greenlip abalone, fed live non-enriched *G. cliftonii* or commercial diets, improved compared to those fed live *Ulva* sp. The largest component of the energy budgets in abalone fed both live macroalgae diets and the commercial diets was somatic growth energy, except for abalone fed live *Ulva* sp. where it was the respiration energy. Nutrient enrichment increased the level of crude protein in live macroalgae and the amount of ammonia excretion energy in abalone.

In trial 2 (Chapter 3) the bioenergetics of abalone fed dried enriched *G. cliftonii* or *Ulva* sp. at graded levels at 5, 10 and 20% were investigated. The components of the greenlip abalone energy budget changed in response to dried macroalgae meal

types and inclusion levels. Improvements in ingested food energy, absorbed energy and somatic growth energy were observed in abalone fed inclusion of ≥ 10% dried *G. cliftonii* meal in the diet. A major portion of ingested food energy went to somatic growth energy and respiration energy.

In trial 3 (Chapter 4), the bioenergetics of abalone fed formulated diets of 27, 30, 33 and 36% crude protein levels at 14, 17 and 20 °C water temperatures were examined. Ingested food energy, somatic growth energy and respiration energy became significantly higher as water temperature rose from 14 to 20 °C, reflecting that up to 20 °C, greenlip abalone had not reached their upper tolerance limit. An increase in dietary crude protein (CP) levels had little influence on the components of energy budget. Thus, the dietary crude protein level of ~ 27 % is recommended for abalone at different seasonal water temperatures. Across treatments, the largest proportion of the abalone energy budget, fed graded levels of CP at different water temperatures, was respiration energy.

Trial 4 (Chapter 5) was conducted to evaluate the effects of inclusion of a commercial probiotic and prebiotic at 22 or 25 °C on each component of the energy budget and also to establish energy budgets of greenlip abalone in response to dietary manipulation at different water temperatures. The energy budget of greenlip abalone at 22 and 25 °C was not affected by dietary pro/prebiotic supplementation, but water temperature had a significant impact. Ingested food energy and absorbed energy were reduced, while ammonia excretion energy, egested faecal energy and pedal mucus production energy were increased when the abalone were under thermal stress (25 °C) compared to their optimal temperature for growth (22 °C). Abalone had available energy for somatic growth and reproduction at 22 °C, but they had negative somatic growth energy and showed no visible sign of gonad development at 25 °C.

Egested faecal energy was the main component of energy budget at both 22 and 25 °C water temperature.

In trial 5 (Chapter 6), the bioenergetics of abalone fed a variety of antioxidant additives, at high water temperature, was studied. Supplementation of 5.0% grape seed extract (GSE) had positive effects on ingested food energy, absorbed energy and somatic growth energy at 25 °C, whereas, green tea extract (GTE), Peanut skin extract (PE) and Vitamin C (Vit C) had no effect on the energy budget. A major portion of the ingested food energy was egested faecal energy as well as respiration energy during thermal stress.

Overall, the findings presented in this thesis will improve knowledge about the bioenergetics of greenlip abalone which are currently not well understood. This may help to choose more effective diets, systems, and even genetic selection in order to sattain efficiency where growth is the main energy target. Additionally, it also may assist in predicting ingested food energy, respiration energy, ammonia excretion energy, nutrient effluent levels, somatic growth energy and abalone culture management.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Duong Ngoc Duong

29th July 2016

Acknowledgments

I would firstly like to thank my principal supervisor Associate Professor James Harris for his patience, motivation, enthusiasm, vast knowledge, valuable guidance and great support throughout my research and writing this thesis. I would also like to acknowledge my co-supervisor Associate Professor David Stone for his encouragement, providing all experimental equipment and facilities needed for running the trials, useful advice and valuable comments on preparing manuscripts and all chapters of this thesis. I would also like to thank my co-supervisor Professor Jian Qin for his kindness, enthusiasm and quick responses to requests, insightful comments on preparing manuscripts and all chapters of this thesis.

I would like to thank Australian Awards Scholarships and the Thai Nguyen University of Agriculture and Forestry, Vietnam for their financial support throughout my PhD candidature. Most of this work was performed at the South Australian Research and Development Institute (SARDI) Aquatic Science Centre (West Beach), thanks for allowing me to use facilities and for financial support. This study is part of the Thriving Abalone Project (6251) and funding was provided by the Functional Food Focus Program being conducted by SARDI as part of the South Australian Government Primary Industries and Regions South Australia Agribusiness Accelerator Program. Additional funding was also provided by the Australian Abalone Growers Association. I would also like to thank Flinders University for the use of the laboratory and facilities.

I also wish to thank Joel Scanlon (Aquafeeds Australia), Dr Tom Coote and Kym Heidenreich (Eyre Peninsula Aquafeeds), Dr Nicole Ruff of Skretting Australia and Dr. Sasi Nayar (SARDI) for their technical and financial contributions to the supply and manufacture of the diets. I also wish to acknowledge Matthew Bansemer, Thanh Hoang, Krishna-Lee Currie and Jessica Buss of Flinders University, Nicole

Thomson of Adelaide University and Debasis De of Central Institute of Brackishwater Aquaculture, Kakdwip, West Bengai, India and SARDI Aquatic Sciences, for assisting experimental works.

Finally, I would like to thank my family and friends for their support spiritually throughout my PhD studies.

Chapter 1

General introduction

1.1 Introduction

Abalone is one of the highest priced marine gastropod molluscs cultured in many parts of the world (Freeman, 2001). There are over 100 species worldwide in the genus *Haliotis*, of which less than 10% are commercially important for fisheries or aquaculture (Freeman, 2001). As the worldwide catch from abalone fisheries has declined since the 1970s (from 19 720 metric ton (mt) in 1970 to 7486 mt in 2013), the interest in aquacultured abalone has increased significantly. In the 8 years immediately preceding 2010, farm production increased by more than 750% and by 2013, farm production had reached an estimated 103 464 mt (Cook, 2014). Thus, it is expected that production levels from the numerous existing or developing farms in several countries, including Australia, will be even more substantial as worldwide demand for abalone increases.

Limitations to wild caught abalone including minimum harvest sizes and total allowable capture numbers and increases in world abalone prices have made abalone aquaculture in Australia an increasingly viable proposition (Hart et al., 2013; Cook, 2014). Farm production gradually increased from 456 mt in 2010 to 910 mt in 2013 - 2014, and it was estimated to be around 965 mt in 2015 (Cook, 2014). Australia has anow become a major contributor to the global abalone market (Morash and Alter, 2015). Greenlip abalone, *H. laevigata*, blacklip, *Haliotis rubra* and their hybrids are currently the main focus of commercial aquaculture, being been grown intensively in both sea and land-based systems in South Australia, Victoria and Tasmania (Abare, 2011). To be successful and sustainable in the future, abalone farms will need to reduce production costs and improve profitability. For example, the implementation of improved feed quality regimes can boost abalone growth and reduce impacts on the environment.

Greenlip abalone have been cultured in Australia over the last three decades. This species is preferred for culture due to a faster growth rate compared to other species, and is currently farmed in South Australia, Victoria and Tasmania (Freeman, 2001; Stone et al., 2014). However, greenlip abalone normally require more than 3 years to attain a market size of 60-90 mm shell length and 35-90 g in weight (Freeman, 2001; Dang et al., 2011a). Along with the high cost of holding in culture systems and associated labour, naturally slow growth rates, dietary nutrition and high mortality during elevated water temperature (> 23 °C) are common factors which influence greenlip abalone aquaculture (Bansemer et al., 2014, 2015a, b, 2016 a, b, c; Lange et al., 2014; Stone et al., 2013, 2014a, b). Thus, more studies in these areas are required for sustainable greenlip abalone production. An ideal tool to investigate this is through the use of bioenergetics.

Bioenergetics is the balance between energy intake, in the form of food, and energy utilization by animals for life activities. Energy budgets provide a framework for the evaluation of various ways in which nutrients are utilized (Lawrence and Lane, 1982). The energy budget is composed of ingested food energy, egested faecal energy, somatic growth energy, reproduction energy, respiration energy, ammonia excretion energy and mucus production energy (Peck et al., 1987). An understanding of the balance between energy intake and outflow enables not only the potential evaluation of species for aquaculture but also for the prediction of growth patterns, reproductive strategies, waste production, mortality and population dynamics in cultured species (Donovan, 1998; Peck et al., 1987). The measurement of animals' physiological processes, such as respiration, excretion and mucus production, may allow feeding regimes, production rates and efficiencies of culture systems to be assessed when costs of food gathering, storage, and product returns are known (Peck et al., 1987). To date, the energy budgets have been published for a number of

haliotids such as the green ormer, *Haliotis tuberculata* (Lopez and Tyler, 2006; McBride et al., 2001; Peck et al., 1987), the South African abalone, *Haliotis midae* (Barkai and Griffiths, 1987, 1988), the Northern abalone, *Haliotis kamtschatkana* (Donovan and Carefoot, 1998), the green abalone, *Haliotis fulgens* (Farías et al., 2003; McBride et al., 2001) and the Thai abalone, *Haliotis asinina* (Ganmanee et al., 2010), but little information has been found relating to the bioenergetics of abalone fed nutritionally enriched live macroalgae, feed additives (dried macroalgae meals, pro/prebiotics and antioxidants) and the bioenergetics of abalone in response to water temperatures and increasing dietary protein levels. Since those above research gaps have been recognized, studies are needed to investigate the effects of those factors on the energy budget of abalone.

Abalone are herbivorous gastropods and mainly consume macroalgae in the wild (Mottet, 1978; Clarke, 1988). In aquaculture, feeding live macroalgae to abalone results in a number of benefits via improved health, marketability, water quality and feeding behaviour or reduced mortality and nutrient leaching (Bansemer et al., 2014; Kirkendale et al., 2010). Specifically, protein-enhanced seaweeds can provide significant gains in abalone growth rates (Bansemer et al., 2016c; Boarder and Shpigel, 2001; Naidoo et al., 2006; Shpigel et al., 1999; Viera et al., 2011). Although live macroalgae diets have been extensively used for the production of abalone in many countries including China, Korea, South Africa and Chile, feeding macroalgae to greenlip abalone was previously limited due to the prohibition of wild macroalgae collection on mainland Australia (Bansemer et al., 2016b, c; Clarke, 1988; Kirkendale et al., 2010). In addition, previous studies have been carried out to investigate the survival, health or growth of abalone fed macroalgae (Bansemer et al., 2016c; Lange et al., 2014; Stone et al., 2014b), but the current literature relating to

energy budget of greenlip abalone fed live macroalgae with or without nutrition enrichment is limited.

Land-based cultured, post-juvenile, abalone in Australia are fed almost exclusively with commercial formulated diets, even though live macroalgae are a food source of wild greenlip abalone (Bansemer et al., 2014, 2015a, b, 2016a, b, c; Stone et al., 2013, 2014a, b). Feeding formulated diets results in comparatively faster growth than macroalgae diets (Bansemer et al., 2016c; Bautista-Teruel and Millamena, 1999; Dang et al., 2011a; Viana et al., 1993). Although the protein content and energy ratios may be suitable in most formulated feeds, there are still nutrients or other factors present in seaweeds but missing in formulated feeds that may limit the optimal development of abalone (Kirkendale et al., 2010). Partial inclusion of dried macroalgae meal in formulated feed is one way to gain the nutritional benefits from macroalgae for aquatic animals including abalone (Bansemer et al., 2016b; O'Mahoney et al., 2014; Viera et al., 2005). Dried macroalgae meal has been used to partially replace fish meal in formulated feed for Japanese abalone, *Haliotis discus hannai* without compromising growth rate (O'Mahoney et al., 2014). Inclusion of dried macroalgae in the commercial diet improved health and enhances colour of abalone (Hoang et al., 2016; Lange et al., 2014; Lim and Lee, 2003). It has been reported that dietary type significantly influenced energy budget of abalone (Lopez and Tyler 2006). Thus, an evaluation of the components of the energy budget in greenlip abalone when fed dried macroalgae meal in the formulated diet is needed.

The growth of abalone is known to be influenced by the availability of a suitable quantity and quality of dietary protein (Britz, 1996a; Mai et al., 1995b; Ogino and Ohta, 1963). Determining the optimal protein level is necessary for maintaining growth of the animal as excess or insufficient protein levels can affect

metabolism and other bodily functions, impacts on the environment and decrease profitability in aquaculture (Fleming et al., 1996; Mai et al., 1995b). The response to dietary protein is known to depend on a number of factors including species, size, and water temperature (Bansemer et al., 2014, 2015a; Fleming et al., 1996; Mai et al., 1995b; Stone et al., 2013). Although some recent research has focused on optimal protein content in the diet for greenlip abalone in response to age and water temperature (Bansemer et al., 2015a, b, 2016a; Stone et al., 2013), there is limited knowledge on the energy budget of greenlip abalone relating to those factors. The information of this study can assist in identifying the suitable protein level through energy invested to somatic growth, calculate the effectivness of the diet and waste management through ingested faecal energy and ammonia excretion energy.

Temperature is known to significantly impact not only survival, but also food ingestion, somatic growth, reproduction, respiration and ammonia excretion, which are also the main components of the energy budget of abalone (Bansemer et al., 2015a, b; Barkai and Griffiths, 1987; Harris et al., 2005; Lange et al., 2014; Lopez and Tyler, 2006; Stone et al., 2013; 2014b). Since greenlip abalone are cultured in land-based systems, the energy requirements or energy allocation of the abalone are expected to change due to large seasonal fluctuations in water temperatures. Generally, within the optimum range, feed intake, somatic growth and metabolic rates, increase directly with temperature, whereas, outside the optimum range, they rapidly decrease (Bansemer et al., 2015a; Lange et al., 2014; Harris et al., 2005; Morash and Alter, 2015; Stone et al., 2013, 2014b). Water temperatures during grow-out of greenlip abalone oscillate seasonally from 10 to 25 °C (Stone et al., 2013). The optimal temperature varies depending upon sizes as well as location of abalone (Bansemer et al., 2015a; Stone et al., 2013). The optimal water temperature for the growth of greenlip abalone was 22 °C for 23 mm shell length (Stone et al., 2013).

The energy budgets of abalone in a range of seasons have been reported in some previous studies (Donovan and Carefoot, 1998; Lopez and Tyler, 2006; McBride et al., 2001), but no available report on the effects of fluctuating water temperatures on the energy budget of greenlip abalone is available.

As water temperatures exceeds 23 °C, thermal stress significantly impacts health, growth and mortality (up to 50%) of larger 3-year-old cultured abalone (≥ 60 mm shell length) in southern Australia (Dang et al., 2012; Lange et al., 2014; Stone et al., 2014b). Addittionally, feed intake significantly decreases during prolonged exposure to elevated water temperature (Lange et al., 2014; Stone et al., 2014b). It was predicted that a decline in the energy balance should occur in *H. midae* when temperatures increase from 14 °C to 19 °C because respiration energy increases more rapidly than the ingested food energy at the higher temperature (Barkai and Griffiths, 1987). During periods of prolonged exposure to high summer water temperatures, dietary or reserved energy will be partitioned preferentially to support essential metabolic functions such as respiration (McBride et al., 2001; Stone et al., 2014b). Some previous research has focused on dietary intervention to improve the survival of cultured greenlip abalone at high water temperature (Lange et al., 2014; Stone et al., 2014b), but other energy parameters such as absorption, respiration, ammonia excretion, egested faeces and pedal mucus of this species have not been studied.

In order to reduce mortality rates and optimise production efficiency, recent studies have focused on the effectiveness of adding various supplements to abalone diets (Dang et al., 2011a; Lange et al., 2014; Stone et al., 2014b). Probiotics and prebiotics have been applied in aquaculture due to many benefits such as disease control, increased growth, survival, water quality, immune response, decreasing the presence of intestinal pathogens and changing the production of health related

bacterial metabolites (Balcázar et al., 2006; Irianto and Austin, 2002; Ringø et al., 2010). Particularly, pro/prebiotics were able to assist in the digestion of seaweed in *H. midae*, and improve growth and survival rate of *H. midae* and *H. iris* (Erasmus et al., 1997; Hadi et al., 2014; Macey and Coyne, 2005). Thus, it is possible that supplementation of pro/prebiotics in the diet may improve the survival of greenlip abalone and also have positive effects on the energy budget of greenlip abalone at high water temperatures. Dietary intervention has also been shown to have positive effects on health and increase the survival of abalone (Stone et al., 2014b). Specifically, 5% Grape Seed Extract (GSE) significantly increased survival and feed intake of greenlip abalone at 25 °C (Duong et al., 2016) or 26 °C (Lange et al., 2014). However, incorporating GSE into abalone diets may not be economically viable, so alternatives need to be explored (Duong et al., 2016). Green tea extract (GTE), Peanut extract (PE) and Vitamin C (Vit C) have been recognized to have diverse health benefits including antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic properties (Perumalla and Hettiarachchy, 2011; Saito et al., 1998; Yu et al., 2005). Those antioxidant products have showed positive effects on health, survival and growth of aquatic animals. For example, dietary inclusion of GTE could significantly improve growth and feed utilisation, stress recovery, lowering serum lipoprotein cholesterol and glutamic oxaloacetic transaminase in olive flounder, *Paralichthys olivaceus* and black rockfish, *Sebastes schlegeli* (Cho et al., 2007; Hwang et al., 2013). Vitamin C has been reported to improve survival, stress resistance, immune response and disease resistance in shrimp, post larvae, European sea bass, *Dicentrarchus labrax*, turbot, *Scophthalmus maximus*, yellow croaker, *Pseudosciaena crocea*, and Indian major carp, *Labeo rohita* (Ai et al., 2006; Merchie et al., 1995; Merchie et al., 1996; Merchie et al., 1997; Misra et al., 2007). Similarly, it is expected that feeding abalone these antioxidant products would improve the

stress tolerance of greenlip abalone, immune response and disease resistance, and consequently result in higher survival and growth. Along with improving survival and growth, feed additives are also expected to have positive effects on the energy budget of abalone at high water temperature.

1.2 Overall thesis objectives

The main objectives of this study were to understand the bioenergetics of greenlip abalone fed different diets at different water temperatures and to establish energy budgets for greenlip abalone as useful tools to assist in abalone aquaculture management through predicting food consumption, oxygen consumption, ammonia production, nutrient effluent levels, somatic growth and biomass production.

1.3 Thesis outline

This thesis is presented in seven chapters; a general thesis introduction, five data chapters and a general discussion. Chapters 2 - 6 are currently in review or preparation for submission to peer-reviewed journals.

Chapter 1 is a general introduction to this thesis that outlines the important of greenlip abalone cultured in Australia, the main effects to abalone aquaculture, a description of the bioenergetics and the major gaps in knowledge bioenergetics for greenlip abalone aquaculture.

In Chapters 2 and 3, the energy budgets of greenlip abalone fed live macroalgae (with or without nutrient enrichment), the commercial formulated diets and inclusion of dried macroalgae meal in the formulated diet were measured. The aims of those studies were: 1) to examine whether components of the energy budget for juvenile greenlip abalone are affected by those above diets; 2) to compare each energy component of greenlip abalone fed live macroalgae against commercial formulated diets or diets of different dried macroalgae meal inclusion levels; and 3) to establish energy budgets for greenlip abalone fed different diets.

In Chapter 4, the bioenergetics of greenlip abalone fed different protein levels at seasonal temperature were determined. The aims of this study were: 1) to evaluate the effects of different protein levels at seasonal temperatures on each parameter of the energy budget of 6-month old greenlip abalone; and 2) to establish energy budgets for greenlip abalone fed the experimental diets.

In Chapter 5 and 6, the effects of antioxidant products and pro/prebiotic on the energy budgets of greenlip abalone at high water temperatures were investigated. Those chapters were also apart of a summer mortality project which was carried out in order to improve survival of greenlip abalone during summer months (Duong et al., 2016). The aims of the last two studies were to examine the effects of graded levels of GSE, GTE, PE and vitamin C or pro/prebiotic supplementation in a commercial abalone diet on the energy budgets of greenlip abalone at high water temperatures.

Chapter 7 is the general discussion, where all major research findings are summarised and discussed. Final recommendations to the abalone industry to improve production are given in this chapter. Further research is also recommended.

Overall, based on the literature review, energy budgets have been reported in several abalone species and most studies have focused on growth, nutrient intervention or health of greenlip abalone, no study has been undertaken on the bioenergetics of greenlip abalone responding to 1) diets of live macroalgae with or without nutrition enrichment, feed supplementation including dried macroalgae meal, antioxidants and pro/prebiotics; or 2) diets of different crude protein levels at seasonal water temperature. Thus, my original contribution to knowledge is to provide bioenergetic information of greenlip abalone fed different diets, at water temperature levels, which can help to choose more effective diets, systems or select better culture environments and management.

1.4 Publications

1.4.1 Co-authorship of chapters

Chapter 2, 3, 4, 5 and 6 are presented in stand-alone manuscript format suitable for the journal Aquaculture. As a result, there is some repetition between chapters, particularly in the methods and background sections. I am responsible for experimental design, running all experiments, doing sample analysis and writing all chapters, but each chapter is co-authored due to contributions from other people. Each chapter is co-authored by Associate Professor James O. Harris, Professor Jian Qin and Associate Professor David A.J. Stone, due to major contributions to experimental design, sample analysis and the review of each manuscript. Dr. Matthew S. Bansemer and Mrs. Thanh Hai Hoang are co-authors for Chapters 2, 3, 4, 5 and 6 due to their contribution towards running the experiments, sample analysis and review of manuscripts. Miss Krishna-Lee Currie is a co-author for Chapters 5 due to her contribution in designing, sample analysis, running the study and manuscript review.

I was involved in a larger project ("Thriving Abalone" project) at SARDI which ran a series of experiments. There were several students involved in each experiment, and each student investigated different aspects of abalone physiology. Particularly, I was involved in the running of three experiments in conjunction with Bansemer et al. (2015a; 2016b,c) which are Chapter 2, 3 and 4 in my thesis. We not only used the same experimental design and experiment, but we also shared raw growth data which measured at start and the end of each experiment. While Bansemer used raw data to analyse growth and feed utilisation patterns, I used the same raw data for calculating energy for somatic growth which were required a range of further analyses of energy in the tissue. Thus, my data, although derived from the same experimental animals, reported on completely different, although related

aspects of abalone physiology compared with Bansemer's. The method for measuring energy content in abalone tissue is explained in the method section in Chapter 2.

1.4.2 Thesis publications

Chapter 2: Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Thanh H. Hoang, Jian G. Qin and James O. Harris. Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed nutrient-enriched live macroalgae or formulated diets. In preparation.

Chapter 3: Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Thanh H. Hoang, Jian G. Qin and James O. Harris. Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed dried macroalgae meal inclusion in formulated diet. In preparation.

Chapter 4: Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Thanh H. Hoang, Jian G. Qin and James O. Harris. Seasonal bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed formulated diet with increasing dietary protein levels. In preparation.

Chapter 5: Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Krishna-Lee Currie, Thanh H. Hoang, Jian G. Qin and James O. Harris. Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed probiotic and prebiotic supplementation in formulated diet at different temperatures. In preparation.

Chapter 6: Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Thanh H. Hoang, Jian G. Qin and James O. Harris. Effects of dietary feed additives on bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) prolonged exposure elevated water temperature. In preparation.

Chapter 2

Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed nutrient-enriched live macroalgae or formulated diets

Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Thanh H. Hoang, Jian G. Qin and James O. Harris. Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed nutrient-enriched live macroalgae or formulated diets. In preparation.

2.1 Abstract

The Australian greenlip abalone, *Haliotis laevigata*, is predominantly cultured using formulated diets. Understanding the bioenergetics of abalone can be beneficial to improving the production of efficient and cost effective formulated diets, especially with reference to performance based on their natural algal diet. In this study, we investigated the energy budget of greenlip abalone (0.80 ± 0.01 g weight and 17.97 ± 0.04 mm shell length) fed live macroalgae (*Ulva* sp., *Gracilaria cliftonii* and an equal mix of *Ulva* sp. and *G. cliftonii*) with and without nutrient enrichment, or one of three control formulated commercial diets, at 22 °C for 93 days. Feeding nutrition enriched macroalgae has been reported to improve the growth rate of some abalone species, thus, these diets were tested in this study to see any effects on the energy budget. Among non-enriched treatments, abalone fed live *G. cliftonii* and mixed diet treatments had significantly higher ingested food energy and absorbed energy than those fed live *Ulva* sp. Abalone fed live *G. cliftonii* invested significantly more energy into somatic growth than those fed live *Ulva* sp. and the mixed diet treatment. For diets with nutrient enrichment, ingested food energy and absorbed energy of abalone fed live *G. cliftonii* and mixed diet treatments were significantly higher than those fed live *Ulva* sp., while they had similar somatic growth energy. Nutrient enrichment increased crude protein in live macroalgae and ammonia excretion energy of abalone. Respiration energy was not influenced by the macroalgae treatments or nutrient enrichment. Abalone fed the control commercial diets had higher ingested food energy, absorbed energy, somatic growth energy, respiration energy, ammonia excretion energy, egested faecal energy and shell growth energy compared to those fed the live macroalgae treatments. The major component of the energy budgets in abalone fed the commercial diets and live macroalgae diets was somatic growth energy, ranging from 25.5 to 37.7% of ingested

food energy, except for abalone fed live *Ulva* sp. where the major component was respiration energy (38.5%). Ammonia excretion energy (1.43 - 3.35%), pedal mucus production energy (0.81 - 1.97%), and shell growth energy (0.42 - 0.87%) accounted for small parts of the energy budgets. Based on the data from the bioenergetics and energy budgets in this study, abalone fed the commercial diet or live non-enriched *G. cliftonii* could increase ingested food energy, absorbed energy and somatic growth energy. This study established energy budgets for greenlip abalone under different feeding scenarios to predict ingested food energy, respiration energy, ammonia excretion energy, nutrient effluent levels and somatic growth energy.

Key words: Bioenergetics, *Haliotis laevigata*, live macroalgae, nutrient enrichment, energy budget.

2.2 Introduction

The development of abalone aquaculture has been accelerating rapidly in many countries due to increasing global demand (Cook and Gordon, 2010). The high value of this product has stimulated considerable effort into the development and optimisation of intensive abalone culture (Viera et al., 2011). The successful culture of any species relies on understanding and managing many aspects including reproduction (Freeman et al., 2006), nutrition (Britz, 1996a, b; Mai et al., 1994; Mai et al., 1995a, b; Mercer et al., 1993), dietary manipulation (Bansemer et al., 2015a, 2016a, c; Stone et al., 2013, 2014a, b), ecology and habitat (Shepherd, 1973; Shepherd and Turner, 1985) and environmental requirements (Freeman, 2001). Bioenergetics focuses on changes in ingested food energy, energy transformations and losses in relation to time (Jobling, 1993). The main components of the energy budget in abalone are ingested food energy, egested faecal energy, absorbed energy, somatic growth energy, reproduction energy, respiration energy, ammonia excretion energy and pedal mucus production energy (Peck et al., 1987). Thus, somatic growth, production as well as waste output can be measured using energy budgets. Understanding these factors can be beneficial to improving production efficiency and cost effective diets for abalone.

Abalone are herbivorous gastropods and mainly consume macroalgae in the wild (Clarke, 1988; Mottet, 1978). In aquaculture, live macroalgae diets are widely used for the production of abalone in countries including China, Korea, South Africa and Chile (Clarke, 1988; Kirkendale et al., 2010). Feeding live macroalgae to abalone results in a number of benefits via improved health, marketability, water quality, feeding behaviour, low mortality and reduction of nutrient leaching (Bansemer et al., 2014; Kirkendale et al., 2010). However, live macroalgae are relatively low in nutrient density and quality, particularly protein, which can impact

abalone growth (Bansemer et al., 2014; Bansemer et al., 2016c). Previous studies have shown that the culture of macroalgae in nutrient-rich waters can enhance the nutritional profile, specifically via increased protein content. Feeding nutrient-enriched macroalgae can improve growth rates of the green ormer, *Haliotis tuberculata coccinea* (Viera et al., 2011), Pacific abalone, *Haliotis discus hannai* (Shpigel et al., 1999), South African abalone, *Haliotis midae* (Naidoo et al., 2006), Roe's abalone, *Haliotis roei* (Boarder and Shpigel, 2001) and greenlip abalone, *Haliotis laevigata* (Bansemer et al., 2016a), compared to non-enriched equivalent treatments. However, in many countries, formulated diets are used in abalone culture promoting faster growth than macroalgae, such as in greenlip abalone (Bansemer et al., 2016a).

Greenlip abalone is currently one of the main species of abalone cultured in Australia (Freeman et al., 2006). Although live macroalgae are a food source of wild greenlip abalone, land-based cultured, post-juvenile abalone in Australia are fed almost exclusively with commercial diets. To date, the energy budgets have been published for different haliotids such as the green ormer, *H. tuberculata* (Lopez and Tyler 2006; Peck et al., 1987), the South African abalone, *H. midae* (Barkai and Griffiths, 1987, 1988), the northern abalone, *H. kamtschatkana* (Donovan and Carefoot, 1998), the green abalone, *H. fulgens* (Farías et al., 2003; Gómez-Montes et al., 2003) and the Thai or ass's-ear abalone, *H. asinina* (Ganmanee et al., 2010). However, there is limited data on the energy budgets of greenlip abalone. The aims of this study were: 1) to examine whether components of the energy budget for juvenile greenlip abalone are affected by the treatments of live macroalgae or nutrient enriched macroalgae; 2) to compare each energy component of greenlip abalone fed live macroalgae and commercial formulated diets; and 3) to establish an

energy budget for greenlip abalone fed either live macroalgae or commercial diets currently used in the culture of this species.

2.3 Materials and methods

2.3.1 Experimental animal and system

One-year old juvenile greenlip abalone (initial weight, 0.80 ± 0.01 g and shell length, 17.97 ± 0.03 mm; n = 540) were purchased from Kangaroo Island Abalone Pty Ltd (Smith Bay, SA, Australia). The abalone were acclimated in a 180 L tank provided in a flow-through seawater system at ambient water temperature (22 ± 1 ºC) at the South Australian Researchand Development Institute (SARDI) Aquatic Science Centre at West Beach, South Australia, prior to the experiment. A commercial 5 mm chip Abgrow diet (Eyre Peninsula Aquafeed Pty Ltd, Lonsdale, SA, Australia) was fed to the abalone at a rate of ~ 3% body weight d^{-1} during this period.

The experimental system was described by Stone et al. (2013). The photoperiod in the experimental system room was a continuous 12 h of low light (3.4 lux) followed by 12 h dark. The incoming seawater was UV treated (model 025120-2.120 W, Emperor Aquatics, Pottstown, PA, USA) and flowed into the experimental sytem comprising of a sump tank, an intermediate tank, a header tank (780 L) and twenty eight 12.5 L experimental culture tanks (39 × 29 × 11 cm). Water temperature was controlled at 22 ± 1 ºC within the system throughout the experiment using an immersion heater (3 KW, Austin & Cridland, Carlton, Australia). Screened standpipes (0.8 mm mesh size) on the outlet allowed a water depth of 3 cm and an effective water volume of 3.4 L in each tank. All experimental tanks were provided with single pass flow-through water from the reservoir by gravity at 300 mL min^{-1}.

2.3.2 Experimental design, diets, feeding and water management

The experimental design, diets and feeding are described fully in Bansemer et al. (2016a). In brief, there was a 3 × 2 factorial experiment, where three live macroalgae treatments (*Ulva* sp., *G. cliftonii*, and a mixed diet consisting of an equal mixture of both species) were fed as either non-enriched or nutrient (protein) enriched treatments. As greenlip abalone are usually fed commercial formulated diets under commercial conditions in Australia, three available commercial control diets (supplied by three feed companies: Eyre Peninsula Aquafeed (Lonsdale, S.A., Australia), Aquafeeds Australia (formally Adam and Amos, Mount Barker, S.A., Australia), and Skretting Australia (Cambridge, Tas., Australia) were used as a control to compare against experimental diets which are live macroalgae (*G. cliftonii* and *Ulva* sp., either with or without nutrition (nitrogen) enrichment). These commercial diets were used because they are the diets currently used by Australian greenlip abalone growers. The commercial diet formulations are relatively constant and have been used for several years prior to my study, although information on the actual ingredient composition of these diets was limited due to confidentiality issues. However, the comparison could be performed between treatments since the dietary analysis of the nutrients and energy was completed for all diets including the commercial diets. Diets were stored at -20 °C prior to feeding. Two species of live macroalgae (*Ulva* sp. and *G. cliftonii*) were collected from intertidal sand-flats at Outer Harbor, Gulf St Vincent, S.A., Australia and following ongrowing in parabolic tanks at SARDI Aquatic Sciences Centre. Those two macroalgae species were enriched fortnightly with 8 L of modified F2 nutrient media (Guillard and Ryther, 1962) since culturing macroalgae in a nutrient enrichment can improve the protein level of macroalga, abalone fed enriched macroalgae exhibited higher growth compared to those fed non-enriched treatment.

Eighteen abalone were stocked into each of 36 tanks (nine treatments; n = 4 replicates). Abalone were fed to *ad libitum* with a daily ration of 14% body weight (bw) day^{-1} for live macroalgae treatments and 4.0% bw day^{-1} for the commercial diet treatments for 93 days. The rations were adjusted based on the biomass at stocking and at monthly biomass checks by bulk weight. Feed was delivered once daily at 16:00 h and cleaning and collection of uneaten food were performed at 08:30 h daily by straining the entire tank contents through a fine mesh (500 µm). The uneaten feed were weighed, first stored frozen at - 20 °C and then dried in an oven at 105 °C and live macroalgae at 60 ºC for 16 h. The proportion of uneaten feed that was lost through leaching was estimated in a tank without animals and the correction factor was used to calculate the ingested food energy.

Water temperature (°C), dissolved oxygen (mg L^{-1} and % saturation), pH and salinity (g L^{-1}) were measured at 12:00 daily using an alcohol filled thermometer, an OxyGuard™Handygamma dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark), a pH meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, IL, USA) and a portable salinity refractometer (model RF20, Extech Instruments, Nashua, NH, USA). The dissolved oxygen meter was calibrated daily in air. Water quality was maintained at levels appropriate for greenlip abalone (Harris et al., 1999a, b; Stone et al., 2013).

2.3.3 Determination of the components of the energy budget

Energy budgets were calculated for greenlip abalone by measuring each component of the energy budget in the equation described by Peck et al. (1987) and Lopez and Tyler (2006), with several modifications: I - E = Ab = Pg + Pr + R + U + M + S, where I is the ingested food energy; E is the egested faecal energy; Ab is the absorbed; Pg is the somatic growth energy; Pr is the reproduction energy (as gonad tissue is laid down when abalone are ~ three years old (Wells and Mulvay, 1995), Pr of one year abalone was not investigated in this study); R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy. At the end of experiments, five animals were collected for determining components of energy budget.

2.3.3.1 Ingested food energy

Ingested food energy (J g abalone^{-1} h^{-1}) = [(amount of consumed food × energy content of food) / (initial weight + final weight) / 2] / time.

To determine amount of consumed food, the experimental diets were daily offered to abalone for 93 days. The uneaten feed was weighed, first stored frozen at -20 °C and then was dried in an oven at 105 °C and live macroalgae at 60 ºC for 16 h. The proportion of uneaten feed that was lost through leaching was estimated in a tank without animals and the correction factor was used to calculate the amount of consumed food.

To determine the energy content of food ingested, samples of feed were weighed, and then freeze dried for 48 h to constant mass. Samples of dried feed were combusted in a microbomb calorimeter to determine their energy content.

2.3.3.2 Somatic growth energy

At the beginning of experiment, initial abalone samples were collected and stored at - 80 °C. At the end of the experiment, abalone were weighed and removed

from their shells. Values for wet mass of the shell and tissue were recorded for each animal. The final weights of greenlip abalone (mean ± SE) were 5.48 ± 0.14 g (n = 12) in the control commercial diets; 1.09 ± 0.01 g (n = 4) in non-enriched *Ulva* sp.; 3.34 ± 0.12 g (n = 4) in non-enriched *G. cliftonii*; 2.79 ± 0.18 g (n = 4) in non-enriched mixed diet; 2.37 ± 0.18 g (n = 4) in enriched *Ulva* sp.; 2.81 ± 0.07 g (n = 4) in enriched *G. cliftonii* and 3.82 ± 0.16 g (n = 4) in enriched mixed diet (Bansemer et al., 2016a). The soft initial and final tissues from each treatment were freeze dried for 48 h to constant mass and then combusted in a microbomb calorimeter to determine energy content.

Somatic growth energy (J g abalone^{-1} h^{-1}) = [(energy content of soft final tissue energy - content of soft initial tissue) / (initial weight + final weight)/ 2]/ time.

2.3.3.3 Respiration energy

Oxygen consumption measurements were performed both in the morning (at 9: 00 h) and night (at 22: 00 h) because greenlip abalone show a circadian rhythm (Buss et al., 2015). The respiratory rate was determined as the difference between the initial and final oxygen levels measured over a 1 h period of incubation of five animals in a sealed respiratory chamber beginning at full oxygen saturation. This was done at the end of the growth experiment. Five abalone of each treatment were introduced into a 1 L chamber (n = 4 1 L chambers per treatment) and supplied with controlled seawater at 22 °C for three days to reduce the effects of handling on oxygen consumption. For the first two days, abalone were fed (4% bw day^{-1} for the control commercial treatments and 14% bw day^{-1} for live macroalgae treatments) then starved on the third day. At 9: 00 h or 22: 00 h on the fourth day, initial disolved oxygen concentrations in water in each chamber were measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). Subsequently, the water supply was cut off for 1 h of incubation. Oxygen levels in the chambers were

always above 70% oxygen saturation during the incubation, to avoid hypoxia depressing their metabolic rates (Harris et al., 1999b). After an hour, the chamber was partly opened one by one, with just enough space to allow the head of oxygen meter (probes) into the water. This step was performed as quickly as possible to limit the intrusion of oxygen in the air being dissolved in the water. The control chambers were run and sampled under the same condition as the other experimental chambers, but with no animals inside, for a comparison. The difference between initial and final value of oxygen in these control treatments could be the air oxygen dissolved in the water or consumed by bacteria present in the water. Thus, the actual oxygen value was adjusted. Addittionally, it is possible that dissolved oxygen can be enriched from the air, thus before conducting this experiment, some validation tests were done to be sure this method work well and accurately. To minimise the time lag at sampling, 4 people helped with oxygen sample collection.

Oxygen uptake rate (mg g abalone^{-1} h^{-1}) = [(initial levels of oxygen in the chamber - final levels of oxygen in the chamber) × volume of the chamber] / (biomass × time)

Since the oxygen consumption value of the animals is affected by relative propotions of carbohydrate, fat and protein in the diet, the rate of oxygen consumption was then transformed to energy equivalents by multiplying by 14.77 J mg O_2^{-1} for carbohydrate respiration, 13.72 J mg O_2^{-1} for lipid respiration and 13.39 J mg O_2^{-1} for protein respiration (Elliott and Davison, 1975).

2.3.3.4 Ammonia excretion energy

The ammonia excretion energy was measured as the difference between the initial and final ammonia concentration in water samples. Experimental conditions for ammonia production were the same as those for oxygen consumption and collection of ammonia samples was just right after dissolved oxygen measurement.

The water samples from incubation chambers were mixed in order to have equal ammonia distribution and then collected by using 5 mL syringes then transferred to 2 mL-tubes which were storaged at - 20 °C until analysing. The ammonia concentration in the water samples was analysed via the salicylate hypochlorite method (Bower and Holm-Hansen, 1980). Briefly, the water samples were defrosted at room temperature then 1 mL of the water sample was taken into 2.5mL eppendorf tube and mixed with 1.2 mL of salicylate catalyst solution (440g sodium salicylate and 0.28g Sodium intropruside in 1L of de-ionised water). After that, 0.2 mL Alkaline citrate solution (18.5g Sodium hydroxide plus 100g Sodium citrate in 1L of de-ionised water) was added and mix well. This final solution was kept in a dark room and after approximately 1h, the samples were analysed on a spectrophotometer at 640 nm.

Ammonia excretion (mg g abalone^{-1} h^{-1}) = [(final concentration of ammonia in the chamber - initial concentration of ammonia in the chamber)] × volume of the chamber / (biomass × time)

The ammonia excretion values were transformed to energy equivalents by multiplying by the conversion factor 5.94 cal mg^{-1} NH_3^{-1} or 24.85 J mg NH_3^{-1} (Elliott and Davison, 1975)

2.3.3.5 Egested faecal energy

Throughout the 93 days of the experiment, three times daily (11:00 h, 14:00 h and 17:00 h) faecal material from each tank was picked up with a plastic 10 ml pipette, placed into a fine mesh to drain water out. Feacal samples were then transferred to a 50 mL container and stored in a - 80 °C freezer. At the end of experiment, samples were freeze dried for 48 h to constant mass and then combusted in the microbomb calorimeter to determine energy content. The apparent digestibility coefficient (ADC) was also analysed in the freeze dried faecal material by using the

ash insoluble acid (AIA) method of Van Keulen and Young (1977) modified by Montaño-Vargas et al. (2002). Values for nutrient ADCs are reported in Bansemer et al. (2016c).

The apparent digestible energy (%) = $100 \times [1 - (F/D \times D_{AIA}/F_{AIA})]$, where F is the percent of nutrient or energy in faeces, D is the percent of nutrient or energy in diet, D_{AIA} is the percent of AIA in diet and F_{AIA} is the percent of AIA in faeces (Cho and Kaushik, 1990).

Absorbed energy (J g abalone^{-1} h^{-1}) = Ingested food energy × apparent digestible energy

Egested faecal energy (J g abalone^{-1} h^{-1}) = Ingested food energy - absorbed energy

2.3.3.6 Pedal mucus production energy and shell shell growth energy

Pedal mucus production energy

Although eighteen abalone (0.80 g) were stocked into each tank, only five abalone per tank were available for bioenergentic study at the end of the experiment (13 abalone went to other studies and abalone size was generally small (1.09 - 5.48 g)). Thus, we were unable to collect a sufficient amount of mucus from each individual treament to analyse energy content. In order to achieve the required quantity of mucus, the total number of 80 abalone (similar size and same batch which were used in this experiment) from the holding tank were placed into four previously weighed 250 mm diameter crystal plates and then immersed in a tank supplied with fresh seawater. Abalone were carefully removed after 10 min and the plates were rinsed using distilled water to remove faeces and seawater. The plates were then dried at 70 °C for 1 h, reweighed and mucus production was calculated by subtraction. The dried mucus was carefully scraped from the plate and its energy

content was finally analysed using combustion in a microbomb calorimeter (Davies, 1993; Donovan and Carefoot, 1998).

Pedal mucus production energy (J g abalone^{-1} h^{-1}) = (Mucus production × energy content of mucus) / (biomass × time)

Energy of mucus production (%) = Energy of mucus production rate / energy ingestion × 100.

Shell growth energy

The initial and final shells from each treatment were freeze dried for 48 h to constant mass, ground into fine powder and then combusted in a microbomb calorimeter to determine energy content. The incombustible material in the shell such as calcium was negligible.

Shell growth energy (J g abalone^{-1} h^{-1}) = [(Final shell energy content - initial energy content of shell) / (initial weight + final weight) / 2] / time.

2.3.4 Biochemical analysis

The biochemical compositions of the diets and test ingredients were analysed according to the methods of the AOAC (1995) and are displayed in Table 2.1. Crude protein (N × 6.25) was determined by the Kjeldahl method. Crude lipid was analysed with a Soxtherm rapid extraction system (Gerhardt GmbH and Co. KG, Konigswinter, Germany) with petroleum liquid (BP 100 °C) as the extracting solvent. Ash was determined using a muffle furnace at 550 °C for 16 h. Carbohydrate was calculated by difference.

Table 2.1 Nutrient composition of live non-enriched and enriched macroalgal diets and formulated commercial diets as g $100g^{-1}$ dry basis (reported by Bansemer et al., 2016c)

	Non-enriched macroalgae			Enriched macroalgae			Commercial formulated diets		
	Ulva sp.	*G. cliftonii*	Mixed[a]	*Ulva* sp.	*G. cliftonii*	Mixed[a]	Diet A	Diet B	Diet C
Proximate composition									
Moisture (%)	79.3	84.5	81.9	80.8	85.6	83.2	7.9	10.0	8.9
Crude protein (%)	5.3	12.9	9.1	27.7	38.1	32.9	36.9	34.0	36.7
Lipid (%)	1.6	1.8	1.7	1.8	1.6	1.7	5.2	5.0	6.7
Gross energy (MJ kg^{-1})	14.2	16.2	15.2	16.9	16.2	16.6	16.8	16.9	17.0
Ash (%)	27.7	27.7	27.7	24.3	28.9	26.6	7.3	6.9	8.3
Carbohydrate (%; calculated)[b]	65.4	57.6	61.5	46.2	31.4	38.8	50.6	54.1	48.3
Amino acids (g $100g^{-1}$ diet as fed)									
Arginine	0.20	0.53	0.37	2.06	3.25	2.66	1.77	1.83	1.98
Histidine	0.08	0.16	0.12	0.30	0.31	0.31	0.73	0.73	0.80
Isoleucine	0.17	0.52	0.35	0.74	0.91	0.83	1.29	1.26	1.26
Leucine	0.29	0.76	0.53	1.28	1.33	1.31	2.23	2.13	2.20
Lysine	0.17	0.51	0.34	1.03	0.95	0.99	1.99	1.96	1.75
Methionine	0.08	0.09	0.09	0.28	0.23	0.26	0.39	0.31	0.35
Phenylalanine	0.21	0.57	0.39	0.93	0.92	0.93	1.56	1.42	1.45
Threonine	0.18	0.54	0.36	0.84	0.94	0.89	1.14	1.08	1.11
Valine	0.26	0.63	0.45	1.12	1.06	1.09	1.39	1.39	1.44
Total Amino Acids[c]	3.47	9.14	6.31	18.58	19.47	19.03	29.23	26.99	27.54

[a] Proximate composition of mixed macroalgae diet is calculated based on feeding an equal mix of *Ulva* sp. and *Gracilaria cliftonii.*
[b] Carbohydrate = 100% - (protein % + lipid % + ash %)
[c] Total Amino Acids was measured using different assay to individual amino acids.

2.3.5 Statistical analysis

The statistical program IBM SPSS (Version 22 for Windows; IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. In order to ensure normal distribution, the data was transformed where appropriate, while Levene's test for equality of variance was used to assess the homogeneity of variance among means prior to running the ANOVA and the post-hoc comparisons. To assess the effects of nutrient enrichment (non-enriched and enriched) and macroalgae treatment (*Ulva* sp., *G. cliftonii* and mixed diet) on the energy budget of greenlip abalone, the data was analysed using a two-factor ANOVA. When significant main effects were observed, Tukey's HSD post-hoc test was used to detect significant differences between treatment means. When a significant interaction between macroalgae treatment and nutrient enrichment was observed, post-hoc test (Tukey's HSD test) was performed for macroalgae treatment within non-enriched or enriched diets. As there was no significant difference between different indices for the commercial diets, data for abalone fed the three commercial diets were pooled (n =12), and used as a control to compare to each of the six live macroalgae treatments (n = 4 replicates treatment^{-1}; one-factor ANOVA; Dunnett's post-hoc test). A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error of the mean, unless otherwise stated.

2.4 Results

2.4.1 General observation and water quality

The overall, the survival rate was 99.24 % and experimental abalone were healthy, showed no gross signs of disease and exhibited normal signs of feeding behaviour over period of study.

Water quality was maintained at levels appropriate for greenlip abalone: water temperature 21.9 ± 0.4 °C, dissolved oxygen 97 ± 4% saturation or 7.0 ± 0.5 mg L^{-1}, pH 8.2 ± 0.1 and salinity 35 ± 1 ppt (mean ± standard deviation (SD), n = 93; reported in Bansemer et al., 2016c).

2.4.2 Ingested food energy

The components of the energy budgets (J g abalone^{-1} h^{-1}) of greenlip abalone fed live macroalgae treatments with and without nutrient enrichment are displayed in Table 2.2. The ingested food energy was significantly affected by the macroalgae treatment ($P < 0.001$; two-factor ANOVA; Table 2.2), nutrient enrichment ($P = 0.047$) and the interaction between the two factors ($P < 0.001$). Abalone fed *G. cliftonii* (4.56 J g abalone^{-1} h^{-1}) and mixed diets (4.44 J g abalone^{-1} h^{-1}) had significantly higher ingested food energy than those fed *Ulva* sp. (2.93 J g abalone^{-1} h^{-1}) ($P < 0.001$). Ingested food energy of abalone was significantly higher in abalone fed nutrient enrichment diet treatments (4.06 J g abalone^{-1} h^{-1}) compared to non-nutrient enrichment diet treatments (3.90 J g abalone^{-1} h^{-1}) ($P = 0.047$). The interaction was due to a significant increase in ingested food energy for abalone fed live enriched *Ulva* sp. compared to those fed the corresponding diet without enrichment. However, there was a significant reduction in ingested food energy of abalone fed live enriched *G. cliftonii* compared to those fed live non-enriched *G. cliftonii*. In abalone fed non-enriched diets, the highest ingested food energy was found in live *G. cliftonii* and was significantly different from live *Ulv*a sp. ($P < 0.001$; one-factor ANOVA; Tukey's HSD test) but not from the mixed diet ($P = 0.070$). For enriched diets, abalone fed the live *Ulva* sp. diet had a significanly lower ingested food energy than those fed the other diets ($P < 0.001$). The ingested food energy of abalone fed the control commercial diets was significantly higher than those fed live macroalgae diets ($P < 0.001$; Dunnett's post-hoc test; Table 2.2).

2.4.3 Absorbed energy

Absorbed energy was significantly influenced by macroalgae treatment ($P < 0.001$; two-factor ANOVA; Table 2.2), nutrient enrichment ($P = 0.021$) and the interaction between the two factors ($P < 0.001$). Absorbed energy of abalone fed *G. cliftonii* (4.04 J g abalone^{-1} h^{-1}) and mixed diets (3.88 J g abalone^{-1} h^{-1}) was significantly higher than in those fed *Ulva* sp. (2.43 J g abalone^{-1} h^{-1}) ($P < 0.001$). Nutrient enrichment (3.57 J g abalone^{-1} h^{-1}) improved absorbed energy of abalone compared to non-nutrient enrichment (3.34 J g abalone^{-1} h^{-1}). The significant interaction was due to a significant increase in absorbed energy for abalone fed live enriched *Ulva* sp. compared to those fed the same diets without enrichment, whereas there was significant reduction in absorbed energy of abalone fed live enriched *G. cliftonii* compared to those fed live non-enriched *G. cliftonii*. For non-enriched diets, abalone fed live *Ulva* sp. had a significantly lower absorbed energy than those fed live *G. cliftonii* and the mixed diets ($P < 0.001$; one-factor ANOVA; Tukey's HSD test). Similarly, for diets with nutrient enrichment, the absorbed energy of abalone was significantly lower in live *Ulva* sp. than the mixed diet ($P = 0.001$) and live *G. cliftonii* ($P = 0.002$). Abalone fed the control commercial diets had a significantly higher absorbed energy than those fed macroalgae diets ($P < 0.001$; Dunnett's test; Table 2.2).

2.4.4 Somatic growth energy

Somatic growth energy was significantly affected by macroalgae treatment ($P < 0.001$; two-factor ANOVA; Table 2.2), nutrient enrichment ($P = 0.001$) and the interaction between the two factors ($P < 0.001$). Abalone fed *G. cliftonii* (1.64 J g abalone^{-1} h^{-1}) and mixed diet treatments (1.46 J g abalone^{-1} h^{-1}) invested significantly more energy in somatic growth than those fed *Ulva* sp. (0.77 J g abalone^{-1} h^{-1}) ($P < 0.001$). Nutrient enrichment (1.46 J g abalone^{-1} h^{-1}) improved somatic growth energy

of abalone compared to non-nutrient enrichment (1.12 J g abalone^{-1} h^{-1}). The interaction was due to a significantly higher somatic growth energy in abalone fed live enriched *Ulva* sp. than those fed live non-enriched *Ulva* sp., while the somatic growth energy was significantly lower in abalone fed live enriched *G. cliftonii* than those fed the same diets without enrichment. Abalone fed live non-enriched *G. cliftonii* invested the highest energy for somatic growth and was significantly different to those fed the other diets (live non-enriched *Ulva* sp., $P < 0.001$; non-mixed diet, $P = 0.007$; one-factor ANOVA; Tukey's HSD test). For diets with enrichment, there was no significant difference in the somatic growth energy of abalone ($P > 0.05$). Abalone fed the control commercial diets had higher somatic growth energy than those fed live non-enriched *Ulva* sp. ($P < 0.001$), non-enriched mixed diet ($P = 0.02$) and live enriched *Ulva* sp. ($P = 0.003$; Dunnett's test; Table 2.2). However, no significant difference was found in somatic growth energy of abalone fed the control commercial diets compared to those fed live non-enriched *G. cliftonii,* live enriched *G. cliftonii* and the enriched mixed diets ($P > 0.05$).

2.4.5 Respiration energy

The respiration energy of greenlip abalone was not affected by macroalgae treatment ($P = 0.331$), nutrient enrichment ($P = 0.070$) or the interaction between the two factors ($P = 0.626$; two-factor ANOVA; Table 2.2). Abalone fed the control commercial diets had a significantly higher respiration energy than those fed live macroalgae treaments ($P < 0.001$; Dunnett's test; Table 2.2).

2.4.6 Ammonia excretion energy

Nutrient enrichment significantly affected ammonia excretion energy ($P = 0.002$; two-factor ANOVA; Table 2.2), while macroalgae treatment ($P = 0.293$) and the interaction between the two factors ($P = 0.431$) did not. Abalone fed enriched diets (0.13 J g abalone^{-1} h^{-1}) had more ammonia excretion energy than those fed

corresponding non-enriched diets (0.08 J g abalone^{-1} h^{-1}) (P = 0.002). Ammonia excretion energy of abalone fed the control commercial diets was significantly higher than those fed live non-enriched *Ulva* sp. (P = 0.019; Dunnett's test; Table 2.2) and non-enriched mixed diets (P = 0.012), but was not significantly different from the rest (P > 0.05).

2.4.7 Egested faecal energy

The egested faecal energy was not significantly affected by macroalgae treatment (P = 0.794), nutrient enrichment (P = 0.283) or the interaction between the two factors (P = 0.250; two-factor ANOVA; Table 2.2). Egested faecal energy of abalone fed the control commercial diets was significantly higher than those fed live *G. cliftonii* and mixed diets with or without nutrient enrichment (P = 0.003) but not from those fed live *Ulva* sp. diets (P > 0.05; Dunnett's test; Table 2.2).

2.4.8 Mucus production energy and shell growth energy

The mucus production energy was 0.05 J g abalone^{-1} h^{-1} for all treatments in this study.

The shell growth energy was significantly affected by macroalgae treatment (P = 0.001; two-factor ANOVA; Table 2.2) and nutrient enrichment (P = 0.035), but was not influenced by an interaction between the two factors (P = 0.084). Shell growth energy was significantly lower in abalone fed live *Ulva* sp. (0.018 J g abalone^{-1} h^{-1}) compared to those fed live *G. cliftonii* (0.029 J g abalone^{-1} h^{-1}) (P = 0.021) and mixed diet (0.036 J g abalone^{-1} h^{-1}) (P < 0.001). Abalone fed live *G. cliftonii* and mixed diet had similar shell growth energy (P = 0.090). Abalone fed diets with nutrient enrichment (0.031 J g abalone^{-1} h^{-1}) had significantly higher shell growth energy than those fed the same diets without nutrient enrichment (0.024 J g abalone^{-1} h^{-1}). Shell growth energy in abalone fed the control commercial diets was significantly higher than those fed live *Ulva* sp. with or without nutrient enrichment

($P < 0.001$) but not from those fed the other diets ($P > 0.05$; Dunnett's test; Table 2.2).

Table 2.2 Energy budget components (J g abalone^{-1} h^{-1}) of greenlip abalone fed control commercial diet, non-enriched and enriched mono-and mixed-macroalgae diets [1].

Enrichment		Non-enriched macroalgae (NE)			Enriched macroalgae (E)			2 factor ANOVA (*P* value)[2]			Dunnett's test[3]
Diet	*Control commercial diets*	*Ulva* sp.	*G. cliftonii*.	Mixed	*Ulva* sp.	*G. cliftonii*	Mixed	Macroalgae treatment (A)	Enrichment (B)	A × B	*(P value)*
I	5.98 ± 0.18	2.44 ± 0.12^{a} *	4.77 ± 0.06^{b} *	4.48 ± 0.01^{b} *	3.43 ± 0.17^{a} *	4.35 ± 0.02^{b} *	4.39 ± 0.06^{b} *	< 0.001 (U<G=M)	0.047 (NE<E)	< 0.001	< 0.001
Ab	5.17 ± 0.15	1.83 ± 0.09^{a} *	4.28 ± 0.06^{b} *	3.90 ± 0.06^{b} *	3.03 ± 0.15^{a} *	3.81 ± 0.09^{b}	3.87 ± 0.06^{b} *	< 0.001 (U<G=M)	0.021 (NE<E)	< 0.001	< 0.001
Pg	1.75 ± 0.06	0.27 ± 0.06^{a} *	1.72 ± 0.10^{c}	1.37 ± 0.05^{b} *	1.27 ± 0.12 *	1.56 ± 0.18	1.55 ± 0.05	< 0.001 (U<G=M)	0.001 (NE<E)	< 0.001	< 0.001
R	1.63 ± 0.07	0.95 ± 0.25 *	1.03 ± 0.05 *	1.01 ± 0.07 *	0.72 ± 0.03 *	0.75 ± 0.13 *	0.94 ± 0.06 *	0.517	0.072	0.700	< 0.001
U	0.19 ± 0.03	0.07 ± 0.03 *	0.09 ± 0.02	0.06 ± 0.01 *	0.10 ± 0.03	0.15 ± 0.01	0.15 ± 0.01	0.293	0.002 (NE<E)	0.431	0.011
E	0.81 ± 0.06	0.61 ± 0.03	0.49 ± 0.11 *	0.58 ± 0.06	0.40 ± 0.05 *	0.54 ± 0.08 *	0.53 ± 0.10 *	0.794	0.283	0.250	0.003
M	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	NA	NA	NA	NA
S	0.04 ± 0.01	0.01 ± 0.01 *	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01 *	0.03 ± 0.01	0.04 ± 0.01	0.001 (U<G=M)	0.035 (NE<E)	0.084	< 0.001

U: *Ulva* sp.; G: *G. cliftonii*; M: mixed macroalgae diets.

[1] Data presented as mean ± SE; n = 4. SE less than 0.01 are reported as "0.01".

[2] A significance level of $P < 0.05$ was used for all statistical tests. Where significant main effects were detected, post-hoc tests were used to determine differences between means (Tukey's HSD test; $P < 0.05$). [a, b, c] For parameters with a significant interaction, differences in type of macroalgae are compared within non-enriched or enriched diets (one-factor ANOVA, Tukey's HSD test), values without a common superscript are different ($P < 0.05$).

[3] Abalone fed the three commercial diets were pooled (due to no significant differences in performance between abalone separately fed either one of the three commercial diets (one-factor ANOVA),n =12), and used as a control and compared to abalone fed fresh macroalgae (n = 4 treatment^{-1}; one-factor ANOVA; Dunnett's post-hoc test). * values without a common superscript compared to the control are significantly different ($P < 0.05$).

NA: not applicable

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Ab: Absorbed energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy.

2.4.9 Energy budgets

The energy budgets for abalone fed live macroalgae or the control commercial diets are displayed in Table 2.3. The major components of the energy budget varied depending on diets. The absorbed energy was the lowest in abalone fed live non-enriched *Ulva* sp. With the exception of live non-enriched *Ulva* sp., abalone fed all diets allocated the most energy to somatic growth, ranging from 25.5% for abalone fed the control commercial diets to 37.7% for abalone fed live enriched *Ulva* sp. The major components of the energy budget in abalone fed live non-enriched *Ulva* sp. were respiration energy (38.5%) and egested faecal energy (25.0%). Ammonia excretion energy formed a small part of energy budget and it was similar in all diets (from 1.43% in non-enriched mixed diet to 3.35 % in live enriched *G. cliftonii* and enriched-mixed diet). Mucus production and shell growth energy accounted for the smallest part of energy budgets, ranging from 0.81 to 1.97% and from 0.42% to 0.87%, respectively.

Table 2.3 Energy budgets (%) of greenlip abalone (*Haliotis laevigata*) fed the control commercial diets or live enriched and non-enriched macroalgae [1].

Diet	Control commercial diets	Non-enriched macroalgae			Enriched macroalgae		
		Ulva sp.	*G. cliftonii*	Mixed	*Ulva* sp.	*G. cliftonii*	Mixed
I	100	100	100	100	100	100	100
Ab	86.50 ± 0.77	75.00 ± 0.01	89.80 ± 2.54	87.00 ± 1.31	88.40 ± 1.21	87.50 ± 1.92	88.10 ± 2.09
Pg	25.50 ± 0.46	10.80 ± 2.39	36.00 ± 1.81	30.50 ± 1.07	37.70 ± 5.20	36.00 ± 4.25	30.50 ± 1.07
R	27.40 ± 1.21	38.50 ± 9.98	21.60± 1.31	22.60± 1.58	21.00 ± 0.78	17.30 ± 2.95	21.40 ± 1.63
U	3.11 ± 0.39	3.03 ± 1.34	1.89 ± 0.35	1.43 ± 0.33	3.05 ± 0.76	3.35 ± 0.20	3.35 ± 0.20
E	13.50 ± 0.77	25.0 ± 0.01	10.20 ± 2.55	13.00 ± 0.31	11.60 ± 1.21	12.50 ± 1.92	11.90 ± 2.09
M	0.81 ± 0.02	1.97 ± 0.10	1.00 ± 0.01	1.07 ± 0.01	1.41 ± 0.07	1.10 ± 0.01	1.09 ± 0.01
S	0.67 ± 0.04	0.42 ± 0.09	0.62 ± 0.10	0.74 ± 0.05	0.78 ± 0.13	0.66 ± 0.10	0.87 ± 0.08
Unexplained energy	29.00	20.30	28.70	30.70	24.50	29.10	30.90

[1] Data presented as mean ± SE; n = 4.

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy. Absorbed energy (Ab), somatic growth energy, respiration energy, ammonia excretion energy, pedal mucus production energy and shell growth energy calculated as a percentage of ingested food energy. Egested faecal energy obtained from 100% ingested food energy minus absorbed energy. Unexplained was calculated as 100% - (Pg + Pr + R + U + M + S + E)

Unavailable data for Pr as abalone showed not visible signs on gonad development.

Abalone fed the three commercial diets were pooled (n =12), fresh macroalgae diets (n = 4 treatment^{-1}).

2.5 Discussion

The somatic growth energy obtained in the current study demonstrates that abalone fed live *G. cliftonii* invested more energy in somatic growth than those fed live *Ulva* sp. The differences in the somatic growth energy of abalone fed live *G. cliftonii* and *Ulva* sp. may be attributed to feeding preference, digestibility and utilisation of the macroalgae. Similar to wild greenlip abalone, this cultured species' preference for red macroalgae resulted in higher ingested food energy (Bansemer., et al 2016c; Fleming, 1995; Shepherd, 1973; Stuart and Brown, 1994). Feed preference for abalone may reflect more effeciency in the ability to digest particular algae. Greenlip abalone are anatomically and biochemically adapted to digest and utilise unique carbohydrates found in live *G. cliftonii* such as agar, carrageenan and floridean starch, which may be an available energy source with spare protein for growth (Bansemer et al., 2016c; Stuart and Brown, 1994). According to Stuart and Brown (1994), *Haliotis iris* fed a diet containing *Gracilaria chilensis* assimilated all chemical components (protein, lipid, carbohydrate) more efficiently than those fed *Ulva lactuca*. Similarly, greenlip abalone fed live *G. cliftonii* had higher absorbed energy than those fed live *Ulva* sp. in the current study. Lower absorbed energy of abalone fed live *Ulva* sp. may be due to lower nutritional value, particularly in protein content or amino acid profiles. *Ulva* sp. is also known to contain some anti-nutrients such as saponins, tannins and phytic acid that may inhibit digestion (Azaza et al., 2008).

Nutrient enrichment of live macroalgal feed is an important economic consideration in haliotids as it can markedly influence the growth rate of abalone due to increased protein content and energy density of the feed (Bansemer et al., 2016c; Shpigel et al., 1999; Viera et al., 2011). For example, the protein contents of *Ulva rigida* and *Gracilaria cornea* cultured using waste water effluents in fishpond were

increased from 16.6 to 33.8% and 11.3 to 29.4%, respectively (Viera et al., 2011). Similarly, Bansemer et al. (2016c) reported that live *Ulva* sp. and *G. cliftonii* cultured in a nitrogen/protein-enriching medium increased protein levels from 5.3 to 27.7% and 12.9 to 38.1%, respectively. Abalone fed nutrient enriched macroalgae displayed higher growth rates compared with those fed non-enriched algae (Bansemer et al., 2016c; Shpigel et al., 1999; Viera et al., 2011). In the current study, nutrient enrichment significantly affected ingested food energy, somatic growth energy and ammonia excretion energy of greenlip abalone. The advantage of nutrient enrichment on somatic growth energy of greenlip abalone was dependent on the macroalgae treatment. Greenlip abalone fed enriched live *Ulva* sp. and the enriched mixed diet treatments expended more energy on somatic growth than those fed without enrichment, but no effect of nutrient enrichment was found in enriched *G. cliftonii* treatment. Ogino and Kato (1964) suggested the growth of abalone is influenced by the content of protein in the diet. Dietary protein deficiency may result in a reduction of growth rates, whereas those in excess may cause extra feed costs to the producer and negative effects to water quality (Coote et al., 2000). Similarly, Mai et al. (1995b) and Britz (1996b) reported that the availability of suitable quantity and quality of dietary protein is considered to be a prime factor that affects the growth of abalone fed natural diets. Thus, it is necessary to enrich *Ulva* sp. for culturing abalone. However, in some cases, energy of diets is an issue when growth of abalone is dietary protein independent.

The growth of abalone, apart of protein, was significantly affected by dietary digestible energy as these animals eat to satisfy their energy requirement (Green et al., 2011; Stone et al., 2013). In the present study, abalone fed live non-enriched *G. cliftonii* exhibited similar somatic growth energy to animals fed live enriched *G. cliftonii* even though the protein content was increased by 18.9% in the enriched diet

compared to the diet without enrichment. According to Stone et al. (2013), the growth rate of greenlip abalone was not affected by dietary protein levels when the dietary digestible energy (12.5 MJ/kg) was constant between diets. It is possible that in the current study the somatic growth energy of abalone fed live non-enriched and enriched *G. cliftonii* was not significantly different due to the similarity of dietary digestible energy levels.

In order to optimise the growth of abalone, commercial diets are formulated to contain highly palatable and digestible dietary ingredients such as fish meal, cereal grains, oilseeds and pulses as well as suitable dietary energy, lipid, protein and amino acid levels, and essential vitamins and minerals (Stone et al., 2013; Bansemer et al., 2014). In the present study, abalone fed the control commercial diets invested more energy for somatic growth than those fed *Ulva* sp. with or without nutrient enrichment. This improvement could be due to higher protein content, optimal protein to energy ratio or superior amino acid profiles, resulting in higher ingested food energy and absorbed energy. However, the somatic growth energy of abalone fed the control commercial diets was similar to those fed live enriched or non-enriched *G. cliftonii*, even though dietary crude protein was different. It is again possible that dietary digestible energy was similar between the commercial diet and *G. cliftonii* diets. Therefore, once the growth of abalone was protein-independent, the energy or protein energy ratio may be the next important component that affects somatic growth energy of abalone fed the commercial diet or *G. cliftonii* diets. Further study is required to clarify the role of somatic growth energy of greenlip abalone.

In this study, the components of the energy budgets for greenlip abalone varied greatly, and were dependent on macroalgae treatments and nutrient enrichment. One year-old greenlip abalone fed live non-enriched *Ulva* sp. expended most of their

energy into respiration or egested faecal, while those fed the rest channeled the major component of the ingested food energy into somatic growth. Lopez and Tyler (2006) used the female ormer abalone, *H. tuberculata* (~ 0.96 g) fed macroalgae (66% red macroalgae, *Palmaria palmata* and 34% green macroalgae, *Ulva lactuca*), at 22 ºC to determine the energy budget and reported that 37.3% of ingested food energy was invested into somatic growth. Peck et al. (1987) also reported that somatic growth energy accounted for the major proportion of the energy budget for the ormer fed *Ulva lactuca* at 15 ºC (37.5% of ingested food energy) in ~ 0.04g animals. The possible reasons for higher egested faecal energy in abalone fed non-enriched *Ulva* sp. in the current study may be due to poorly digestible green macroalgae components such as anti-nutritional factors including saponins, tannins and phytic acid, lower β-galactosidase and α-amylase activities (Bansemer et al., 2016b), sub-optimal amino acid profiles (Azaza et al., 2008; Wahbeh, 1997).

In the current study, ammonia excretion energy of greenlip abalone fed the control commercial diets and live macroalgae diet treatments only accounted for a small proportion of the energy budgets, ranging from 1.43 to 3.35% of ingested food energy. Previous studies also reported minimal energy losses due to ammonia excretion in the South African abalone (< 1%) (Barkai and Griffiths, 1988), ass's-ear abalone (~ 1.33%) (Ganmanee et al., 2010) and green ormer (~ 1.53%) (Lopez and Tyler, 2006). Additionally, ammonia excretion energy was significantly influenced by nutrient enrichment in this study. Ammonia is the major nitrogenous excretory product in abalone and excretion rates may be impacted by the quality and quantity of dietary protein (Bayne and Newell, 1983). Ammonia excretion was reported to increase with increasing protein and decreasing carbohydrate content in the diet in some abalone species (Rychly, 1980; Yang et al., 2002), reflecting what we observed with nutrient-enriched macroalgal treatments. Thus, the level of ammonia in the

water should be taken into account in a farming situation, especially when enriched macroalgae is given to abalone as enriched diets may foul the water more quickly.

Previous studies show that mucus production was affected by stress or fluctuations in water temperature. For example, large energy loss via mucus production energy (23.3 - 29.1% of ingested food energy) of *H. tuberculata* fed *Ulva lactuca* was found to be due to stress (Peck et al., 1987), while it increased from 4.0% in the winter to 16.0% in summer of ingested food energy in the Northern abalone, *Haliotis kamtschatkana* (Donovan and Carefoot, 1998). Other available publications showed that mucus production energy formed small portions of the energy budget and was independent of the diet. For example, Lopez and Tyler (2006) and Montaño-Vargas et al. (2005) reported that energy losses in the form of mucus production was not affected by diet and accounted for only 0.99% of the ingested food energy in the ormer, fed both seaweed and formulated diets, or 0.73 - 1.23% in the pink abalone, *H. corrugate,* fed formulated diets containing different protein content and starch: lipid ratios. It was also less than 1% of ingested food energy in the South African abalone (Barkai and Griffiths, 1988). In the present study, we were not able to measure mucus production energy corresponding to dietary treatments at the end of the experiment due to having not enough abalone available in the right size. The absolute value of pedal mucus production energy (from 80 animals) accounted for less than 2% of ingested food energy. However, it is also possible that the unknown component in the current study is most likely to be associated with mucus since it is only parameter which may have been underestimated. Mucus is used for number of processes in mollusca, however only pedal mucus production was measured in this energy budget. It was very difficult to collect dissolved mucus in the water that is used for other processes such as: locomotion, epithelial protection, egestion of faeces and cleaning the gills.

2.6 Conclusion

It appears that the control commercial diet or live non-enriched *G. cliftonii* can improve absorbed energy and somatic growth energy. The results of this study will assist in predicting food consumption, respiration, ammonia excretion, nutrient effluent levels and somatic growth at an optimal growth temperature (22 ºC) for these diets. Further research to understand these factors under sub-optimal conditions or different sizes is required to improve the knowledge of energy expenditure in greenlip abalone.

Chapter 3

Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed dried macroalgae meal inclusion in formulated diet

Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Thanh H. Hoang, Jian G. Qin and James O. Harris. Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed dried macroalgae meal inclusion in formulated diet. In preparation.

3.1 Abstract

Although formulated diets provide many advantages for abalone culture, further advances can be obtained by the inclusion of macroalgae meal. In this study, energy budgets were established for greenlip abalone, *Haliotis laevigata* (weight: 2.89 ± 0.00 g; shell length: 22.41 ± 0.06 mm), fed diets of dried macroalgae (*Ulva* sp. and *Gracilaria cliftonii*) at inclusion levels of 0 (basal diet), 5, 10 and 20%. The components of the energy budget were measured including ingested food energy, absorbed energy, egested faecal energy, somatic growth energy, ammonia excretion energy, respiration energy, pedal mucus production energy and shell growth energy over a 92 day study. Ingested food energy, absorbed energy and respiration energy were significantly influenced by macroalgae type and inclusion level while ammonia excretion energy, pedal mucus production energy and shell growth energy did not vary among treatments. Energy invested to somatic growth was influenced by the macroalgae type and the interaction with inclusion level, but not by inclusion level alone. Egested faecal energy was significantly higher in abalone fed the 20% dried *Ulva* sp. or 10% *G. cliftonii* than the basal diet. The ingested food energy of greenlip abalone fed 20% dried *Ulva* sp. and all inclusion levels of dried *G. cliftonii* significantly improved compared to the basal diet. Absorbed energy was significantly increased in 5 and 20% dried *Ulva* sp. treatments and all inclusion levels of dried *G. cliftonii* compared to the basal diet. Somatic growth energy of abalone fed 10 and 20% dried *G. cliftonii* increased significantly compared to the basal diet while it was not affected in the abalone fed dried *Ulva* sp. diets. Respiration energy of the abalone fed dried *G. cliftonii* was significantly higher than those fed dried *Ulva* sp. A major portion of the ingested food energy was somatic growth energy (31.5 - 44.9%) and respiration energy (17.6 - 29.2%), while ammonia excretion energy, pedal mucus production energy and shell growth energy accounted for only small proportions of

the energy budgets (1.42 - 2.62 %; 0.81 - 1.19% and 0.44 - 0.66%, respectively). We recommend an inclusion level of ≥ 10% dried *G. cliftonii* meal in the diet for greenlip abalone based on the improvement observed in the ingested food energy, absorbed energy and somatic growth energy. Information from this study will assist in commercial diet development for greenlip abalone.

Keywords: Greenlip abalone, bioenergetics, *Gracilaria cliftonii*, *Ulva* sp., inclusion.

3.2 Introduction

Marine macroalgae are harvested or cultured as food sources for farmed abalone in a number of countries, including China, Korea, South Africa and Chile (Bansemer et al., 2014; Britz et al., 1994; Kirkendale et al., 2010; Park and Kim, 2013). A large number of studies have demonstrated the potential benefits of feeding live macroalgae to abalone such as health, marketability, feeding activity and improved water quality by reducing leaching (Bansemer et al., 2014, 2016c; Buss et al., 2015; Kirkendale et al., 2010; Lange et al., 2014; Stone et al., 2014b). However, feeding live macroalgae is unsustainable due to supply problems, high moisture content and seasonally variable nutritional profiles (Bansemer et al., 2014, 2016c; Kirkendale et al., 2010). Even when a choice of seaweed is available, there is little chance of altering its nutritional components to improve the diet (Bautista-Teruel and Millamena, 1999). Additionally, feeding live macroalgae results in a high risk of introducing pests, predators and competitors to culture systems (Bansemer et al., 2014). Thus, for the development of substantial aquaculture, intensive abalone culture is becoming increasingly reliant upon practical formulated diets (Bansemer et al., 2016c; Bautista-Teruel and Millamena, 1999; Britz et al., 1994; Britz, 1996b).

Formulated feeds offer benefits over live macroalgae, such as convenience, low biosecurity risk, constant supply and reduced cost to farm management on land (Bansemer et al., 2014; Britz et al., 1994; Kirkendale et al., 2010). Research into developing formulated diets to replace algae in the diets of abalone began in the 1990s (Fleming et al., 1996), and has resulted in formulated diets currently being used in Japan, China, Australia, New Zealand and South Africa (Bautista-Teruel and Millamena, 1999; Britz, 1996b). Abalone fed formulated diets also grow better than those fed on macroalgae diets (Bansemer et al., 2016c; Britz, 1996b; Dang et al., 2011a, b). For example, greenlip abalone grew faster when fed the formulated diet

compared to either algae *Ulva lactuca* or *Spyridia filamentosa* (Dang et al., 2011a) or *Ulva lactuca* and *Gracilaria cliftonii* (Bansemer et al., 2016c). Since macroalgae are the main natural food sources for abalone in the wild, inclusion of dried macroalgae meal in formulated diets are advantageous due to abalone gaining nutritious benefits from both macroalgae and the formulated diet. For example, mixed species macrolgae meal has been used to partially replace fishmeal in formulated feed for Japanese abalone, *Haliotis discus hannai*, without compromising growth rates (O'Mahoney et al., 2014). Addittionally, Bansemer et al. (2016b) reported that the inclusion of 10 % *Gracilaria* sp. meal or 5 % *Ulva* sp. meal in formulated diets improved greenlip abalone growth. Dietary inclusion of macroalgae also improves the health (Lange et al., 2014; Stone et al., 2014b) and enhances the colour of greenlip abalone (Hoang et al., 2016; Lim and Lee, 2003).

Bioenergetics focuses on changes in energy intake, energy transformation and losses in relation to time (Jobling, 1993). Understanding the nutritional bioenergetics can provide an insight into growth patterns and assist in prediction of waste output and production. Therefore, it can be beneficial in improving the production of efficient and cost effective diets. Bioenergetics studies have been carried out on different Haliotids such as the green omer, *Haliotis tuberculata* (Lopez and Tyler 2006; Peck et al., 1987), South African abalone, *Haliotis midae* (Barkai and Griffiths, 1987; 1988), northern abalone, *Haliotis kamtschatkana* (Donovan and Carefoot, 1998), green abalone, *Haliotis fulgens* (Farías et al., 2003; Gómez-Montes et al., 2003), pink abalone, *Haliotis corrugata* (Montaño-Vargas et al., 2005) and Thai abalone, *Haliotis asinina* (Ganmanee et al., 2010). Greenlip abalone, *Haliotis laevigata*, is a high value species and has been cultured in land-based system fed a formulated diet in Australia. Most of the recent studies on greenlip abalone have focused on digestive physiology, feeding behaviour, improvement of growth or

survival, and identifying the optimum crude protein level at seasonal temperature (Bansemer et al., 2014, 2015b, 2016b, c; Buss et al., 2015; Lange et al., 2014; Stone et al., 2013, 2014b). However, there is no available data on the bioenergetics of greenlip abalone fed diets containing dried macroalgae. The aim of this study was to understand and develop energy budgets for greenlip abalone fed graded levels of dried macroalgae included in formulated diets by measuring individual parameters such as ingested food energy, egested faecal energy, somatic growth energy, respiration energy, ammonia excretion energy, pedal mucus production energy and shell growth energy.

3.3 Materials and methods

3.3.1 Experimental animal and system

The experiment was conducted at the South Australian Research and Development Institute (SARDI) South Australian Aquatic Sciences Centre (SAAS). One year old greenlip abalone were obtained from South Australian Mariculture (Boston Point, Port Lincoln, SA, Australia) and were held in holding tanks with a flow-through, UV-treated, seawater system. Abalone were fed a commercial abalone pellet Abgrow diet (Eyre Peninsula Aquafeed Pty Ltd (EPA), Lonsdale, SA, Australia) *ad libitum* daily (at a rate of ~ 3% body weight d^{-1}) until the energy budget experiment was started.

The experimental system was described in Chapter 2 (2.3.1)

3.3.2 Experimental design, diets, feeding and water management

The experimental design, diets, stocking and feeding are described fully in Bansemer et al. (2016b). In brief, eight diets were used in the experiment: 1) 0% inclusion of dried macroalgae (basal diet); 2) 5% dried *Ulva* sp.; 3) 10% dried *Ulva* sp.; 4) 20% dried *Ulva* sp.; 5) 5% dried *G. cliftonii*; 6) 10% dried *G. cliftonii*; 7) 20% dried *G. cliftonii*; and 8) The commercially available formulated diet (Abgrow

premium 5 mm chip; Eyre Peninsula Aquafeed Pty Ltd., Lonsdale, SA, Australia). As greenlip abalone is cultured mainly in land-based system and fed commercial formulated diets in Australia, the commercially available formulated diet was used as a reference to compare with experimental diets of dried macroalgae meal supplementation.

Enriched *Ulva* sp. meal was supplied by Venus Shell Systems (Narrawallee, NSW, Australia). To produce enriched *G. cliftonii* meal, live *G. cliftonii* was collected from Gulf St Vincent, SA, Australia, cultured in a 4000 L tank and was provided with gentle air supply under ambient sunlight. Fresh *G. cliftonii* was enriched with 8 L of modified F2 nutrient media for 7 days before harvesting (Bansemer et al., 2016a). After being harvested and washed, enriched *G. cliftonii* was sun-dried for about 4 h and then oven-dried at 45 °C for 72 h until moisture content was <10%. Dried *G. cliftonii* was then blended into fine powder using a laboratory blender (model HGBTWT53, Waring Commercial, Torrington, CT, USA) and stored at -20 °C until diets were made.

To produce the experimental diets, *Ulva* sp. or *G. cliftonii* meal was added into a basal diet at inclusion levels of 5, 10 and 20% by reducing solvent extracted soybean meal, wheat flour and de-hulled lupins levels. Diets were formulated to contain a 35% crude protein, 5% crude lipid and a gross energy content of 17.5 MJ kg^{-1} (suggested by Bansemer et al. (2015b) and Stone et al. (2013) as being optimal for greenlip abalone growth). All ingredients including abalone mash, dried macroalgae meals, fish oil, sodium alginate and calcium sulphate were accurately weighed and mixed in a Hobart mixer (Hobart Corp., Troy, OH, USA) for 5 min. Water (30%) was then added to the diet mash and mixed for a further 3 min. The diets were made using a TR110 pasta machine (Machine Per Pasta SRL, Molina Di

Malo, VI, Italy). All diets were produced with a size of 4 × 3 × 2 mm. Diets were oven-dried at 45 °C for 48 h and stored at - 20 °C until fed.

There is little information about the commercial dietary mash (basal diet) supplied by feed company, due to confidentiality. However, the dietary nutrient and energy data were still analysed and the nutrient availability was calculated. Therefore, the results of this study were not affected as the significant different between the experimental diets were absolute due to adding tested dried macroalgae meal.

Nutrient analysis of the test ingredients and experimental diets are displayed in Table 3.1.

Stocking

Fifteen greenlip abalone (initial weight, 2.89 ± 0.00 g and shell length, 22.41 ± 0.06 mm; n = 480) were weighed, measured and stocked into one of four replicate culture units per dietary treatment. Dead abalone were measured, weighed, recorded, and replaced with abalone of a similar weight.

Feeding

Abalone were fed to excess at 16:00 h daily (4% biomass day^{-1}) at 8.30 am. The amount feed offered was weighed daily and uneaten food was removed at 08:30 h daily by straining the entire tank contents through a fine mesh (500 µm). The uneaten feed was weighed, stored frozen at - 20 °C and then dried in an oven at 105 °C for 16 h. The proportion of uneaten feed that was lost through leaching was estimated in a tank without animals and the correction factor was used to calculate the apparent feed intake and ingested feed energy (Stone et al., 2013).

Calulation of leaching loss are described in Bansemer et al. (2016b). In brief, diets were immersed in water at 22 °C in experimental tanks for 16.5 h, and sieved through a fine mesh net (500µm), and dried to constant weight.

Water management was previously described in Chapter 2 (2.3.2).

Table 3.1 Proximate composition and stability of experimental ingredients and diets (reported by Bansemer et al., 2016b)

	Ingredients (Dried)		Diet							
Macroalgal species	*Ulva* sp.	*G. cliftonii*	Non		*Ulva* sp. meal			*G. cliftonii* meal		
Inclusion level (%)			EPA	0 (Basal)	5	10	20	5	10	20
Proximate composition (g 100g^{-1} diet as fed)										
Moisture	10.6	3.8	12.2	12.7	11.9	12.0	11.9	12.1	11.8	11.3
Crude protein	33.6	25.2	29.7	34.6	35.0	34.8	34.7	35.1	35.0	35.2
Lipid	4.7	1.1	4.2	5.1	5.4	5.2	5.1	5.4	5.4	5.5
Ash	16.9	30.9	5.8	4.8	5.9	6.9	8.8	6.5	8.0	10.9
Carbohydrate [1]	34.2	39.0	48.1	42.8	41.8	41.1	39.5	40.9	39.8	37.1
Gross energy (MJ kg^{-1})	15.7	13.1	16.94	17.5	17.6	17.3	17.0	17.5	17.2	16.9
Diet stability (%)	NA	NA	80.65	79.75	78.75	77.56	75.62	55.03	46.34	33.61

EPA, Eyre Peninsula Aquafeed Pty Ltd

[1] Calculated by difference, carbohydrate = 100 g g^{-1} - (protein g g^{-1} + lipid g g^{-1} + ash g g^{-1} + moisture g g^{-1})

Non: without algae inclusion

NA: not applicable

3.3.3 Determination of the components of the energy budget

Energy budgets were calculated for greenlip abalone by measuring each component of the energy budget in the equation was described in Chapter 2 (2.3.3).

3.3.3.1 Ingested food energy

The determination of ingested food energy was described in Chapter 2 (2.3.3.1).

3.3.3.2 Somatic growth energy

The determination of somatic growth energy was described in Chapter 2 (2.3.3.2). The final weights of greenlip abalone (mean ± SE) were 11.49g (n=4) in 0% macroalgae meal inclusion; 11.84g in 5% dried *Ulva* sp. meal; 11.63g in 10% dried *Ulva* sp. meal; 11.73g in 20% dried *Ulva* sp. meal; 12.38g in 5% dried *Gracilaria* sp. meal; 13.48g in 10% dried *Gracilaria* sp. meal and 13.33g in 20% dried *Gracilaria* sp. meal (Bansemer et al. 2016b).

3.3.3.3 Respiration energy

The determination of respiration energy was described in Chapter 2 (2.3.3.3).

3.3.3.4 Ammonia excretion energy

The determination of ammonia excretion energy was described in Chapter 2 (2.3.3.4).

3.3.3.5 Egested faecal energy

The determination of egested faecal energy was described in Chapter 2 (2.3.3.5).

3.3.3.6 Pedal mucus production energy and shell growth energy

As only 5 animals were available for this bioenegetic study per replicate and abalone available were small (11.49 - 13.33 g) at the end of experiment, there was insufficient mucus for analysis. Thus, in order to provide a value for pedal mucus production energy independent of treatments, 40 abalone, of a similar size were

introduced into four containers (600 mL beaker) that had been previously weighed and immersed in a tank supplied with fresh seawater. Abalone were carefully removed after 6 h and the containers were rinsed using distilled water to remove faeces and seawater. The containers were then dried at 70 °C for 1 h, reweighed. Pedal mucus production was calculated by subtraction. The dried mucus was carefully scraped and its energy content was analysed using combustion in a microbomb calorimeter (Lopez and Tyler, 2006). The pedal mucus production energy here was assumed similar among of abalone across treaments due to the difficulty of collecting enough mucus corresponding to dietary treatments.

The calculation of energy content for pedal mucus production energy was described in Chapter 2 (2.3.3.6)

Shell growth energy was described in Chapter 2 (2.3.3.6).

3.3.4 Statistical analysis

Data was analysed using the statistical program IBM SPSS (Version 22 for Windows; IBM SPSS Inc., Chicago, IL, USA). Two-factor ANOVA was used to assess the effects of macroalgae type (*Ulva* sp. and *G. cliftonii*) and inclusion level (0, 5, 10 and 20%) on each component of the energy budget for greenlip abalone. In order to ensure normal distribution, data was transformed where appropriate, while Levene's test for equality of variance was used to assess the homogeneity of variance among means prior running the ANOVA and post-hoc comparisons. When significant main effects were observed, Tukey's HSD post-hoc tests were used to detect significant differences between treatment means. When significant interactions between macroalgae type and inclusion level were observed, differences in level of inclusion are compared within *Ulva* sp. meal or *G. cliftonii* meal (one-factor ANOVA; Tukey's HSD test). The basal diet treatment (0% macroalgal meal) served

as control for both macroalgal species. The data for abalone fed the commercial diet was used as an reference diet to compare to each dried macroalgae inclusion treatment using one-factor ANOVA and Dunnett's post-hoc test. A significance level of $P < 0.05$ was used for all statistical tests.

3.4 Results

3.4.1 General observation and water quality

Mortalities were experienced during the first two weeks and at the end of experiment, the survival was 94.37%. Abalone were in good condition and fed actively on all diets.

Water quality were maintained at levels appropriate for greenlip abalone: water temperature 21.9 ± 0.3 °C, dissolved oxygen 94 ± 4, 85% saturation or 6.8 ± 0.3 mg L^{-1}, pH 8.15 ± 0.05 and salinity 36 ± 1 ppt (mean ± SD, n = 92; reported in Bansemer et al., 2016b).

3.4.2 Ingested food energy

The components of the energy budget of greenlip abalone fed different inclusion levels of two macroalgae species are shown in Table 3.2. The ingested food energy was significantly affected by macroalgae type ($P < 0.001$; two-factor ANOVA; Table 3.2), inclusion level ($P < 0.001$) and interaction between these two factors ($P < 0.001$). Abalone fed the dried *G. cliftonii* meal inclusion had significantly higher ingested food energy (5.97 J g abalone^{-1} h^{-1}) than those fed the dried *Ulva* sp. meal inclusion (4.87 J g abalone^{-1} h^{-1}). An inclusion of dried macroalgae improved the ingested food energy of greenlip abalone compared to those fed basal diet (0% macroalgae meal inclusion) ($P < 0.001$), but there was no significant difference in ingested food energy across all macroalgae diets ($P > 0.05$). The interactive effects of macroalgae type and inclusion level on ingested food

energy were due to a significant increase in ingested food energy for abalone fed 5 and 10% dried *G. cliftonii* meal compared to 0%, whereas abalone fed 5 and 10% dried *Ulva* sp. meal had similar ingested food energy to those fed 0%. Within the dried *Ulva* sp. meal inclusion treatments, ingested food energy of abalone fed 20% macroalgae was significantly higher than abalone fed the lower level inclusion diets ($P < 0.05$). For the dried *G. cliftonii* meal treatments, abalone fed the 5 and 10% inclusion diet had significantly higher ingested food energy than those fed 0 or 20% ($P < 0.001$; one-factor ANOVA, Tukey's HSD test; Table 3.2).

The ingested food energy of abalone fed the control commercial formulated diet was significantly lower than those fed all inclusion levels of *G. cliftonii* meal and 20% dried *Ulva* sp. meal ($P < 0.001$; one-factor ANOVA; Dunnett's post-hoc test; Table 3.2), but similar to fed 0, 5 and 10% dried *Ulva* sp. ($P > 0.05$).

3.4.3 Absorbed energy

Absorbed energy was significantly influenced by macroalgae type ($P < 0.001$; two-factor ANOVA; Table 3.2), inclusion level ($P < 0.001$), and the interaction between these two factors ($P = 0.001$). The inclusion of dried *G. cliftonii* meal (5.11 J g abalone^{-1} h^{-1}) improved the absorbed energy rate of abalone compared to those fed dried *Ulva* sp. meal inclusions (4.17 J g abalone^{-1} h^{-1}). Absorbed energy of abalone fed 5, 10 and 20% dried macroalgae meal inclusions (5.10, 4.79 and 4.82 J g abalone^{-1} h^{-1}, respectively) was significantly higher than that of abalone fed 0% dried macroalgae meal (3.87 J g abalone^{-1} h^{-1}). The interaction was due to the different responses to the diets with an inclusion compared to the basal diet. Abalone fed 5, 10 and 20% dried *G. cliftonii* meal had significantly higher absorbed energy than those fed the 0% diet ($P < 0.05$; one-factor ANOVA; Tukey's HSD test). However, there was no significant difference in absorbed energy among abalone fed the diet with 5, 10 and 20% dried *G. cliftonii* meal inclusion levels ($P > 0.05$). For abalone fed dried

Ulva sp. meal diets, there were significant higher absorbed energy rate in abalone fed 5% ($P = 0.010$) and 20% ($P = 0.033$) compared to those fed 0%, whereas, no significant differences were found in absorbed energy among abalone fed 5, 10 and 20% ($P > 0.05$).

Abalone fed the control commercial diet had lower absorbed energy than those fed dried *G. cliftoii* meal ($P < 0.001$; one-factor ANOVA; Dunnett's post-hoc test; Table 3.2), while it had similar absorbed energy to those fed 0% and all levels of dried *Ulva* sp. meal ($P > 0.05$).

3.4.4 Somatic growth energy

Macroalgae type ($P = 0.031$; two-factor ANOVA; Table 3.2), and the interaction between the macroalgae type and inclusion level ($P = 0.024$) significantly affected somatic growth energy, but there was no effect of inclusion level ($P = 0.169$). Abalone fed an inclusion of dried *G. cliftonii* meal (2.16 J g abalone^{-1} h^{-1}) invested significantly higher energy to somatic growth than those fed dried *Ulva* sp. meal inclusions (2.05 J g abalone^{-1} h^{-1}). The interaction was due to a significant increase in somatic growth energy of abalone fed 10 and 20% inclusions of dried *G. cliftonii* meal compared to those fed 0% whereas it was similar between abalone fed 0, 10 and 20% inclusions of dried *Ulva* sp. meal. Abalone fed all levels of dried *Ulva* sp. meal inclusions had similar somatic growth energy ($P > 0.05$; one-factor ANOVA; Tukey's HSD test). Abalone fed 10% ($P = 0.024$) and 20% ($P = 0.016$) dried *G. cliftonii* meal invested significantly higher energy in somatic growth than those fed 0% dried *G. cliftonii* meal. No significant differences were found in somatic growth energy between abalone fed 0 and 5%, and between 5, 10 and 20% dried *G. cliftonii* meal ($P > 0.05$).

Somatic growth energy of abalone fed the commercial control diet was significantly lower than those fed 10% ($P = 0.046$; one-factor ANOVA; Dunnett's

post-hoc test; Table 3.2) and 20% (P = 0.040) dried *G. cliftonii* meal, but was similar to abalone fed all other treatments (P > 0.05).

3.4.5 Respiration energy

Respiration energy was significantly influenced by macroalgae type (P = 0.020; two-factor ANOVA; Table 3.2), inclusion level (P = 0.005), but not by the interaction between the two factors (P = 0.101). Abalone fed with dried *G. cliftonii* meal inclusions had significantly higher respiration energy (1.31 J g abalone^{-1} h^{-1}) than those fed dried *Ulva* sp. meal inclusions (1.15 J g abalone^{-1} h^{-1}) (P = 0.020). Respiration energy of abalone fed 0% (1.32 J g abalone^{-1} h^{-1}) and 5% (1.39 J g abalone^{-1} h^{-1}) macroalgae meal inclusions were significantly higher than those fed a 20% dried macroalgae meal inclusion (1.06 J g abalone^{-1} h^{-1}). However, there was no significant diference between abalone fed 0, 5 and 10% dried macroalgae meal inclusion or the 10 (1.15 J g abalone^{-1} h^{-1}) and 20% dried macroalgae meal inclusion.

Abalone fed the commercial diets had significantly lower respiration energy than those fed 5% dried *G. clifftonii* meal inclusion (P = 0.001), but were similar to the rest (P > 0.05; one-factor ANOVA; Dunnett's post-hoc test; Table 3.2).

3.4.6 Ammonia excretion energy

Ammonia excretion energy was not significantly affected by macroalgae type (P = 0.205; two-factor ANOVA; Table 3.2), inclusion level (P = 0.998) or the interaction between these two factors (P = 0.702). Ammonia excretion energy ranged from 0.09 to 0.12 (J g abalone^{-1} h^{-1}) across the treaments.

Abalone fed the commercial diets had similar ammonia excretion energy compared to those fed the dried macroalgae meal inclusions (P = 0.876; one-factor ANOVA; Dunnett's post-hoc test; Table 3.2).

3.4.7 Egested faecal energy

Egested faecal energy was significantly influenced by the interaction between macroalgae type and inclusion level (P = 0.004; two-factor ANOVA; Table 3.2), but not by macroalgae type (P = 0.058) or inclusion level (P = 0.066) alone. The interaction was due to a significant increase in egested faecal energy in abalone fed the 10% inclusion of dried *G. cliftonii* meal compared to those fed 0%, whereas, it was similar between abalone fed the 0 and 10% inclusion of dried *Ulva* sp. meal. Egested faecal energy of abalone fed dried *G. cliftonii* meal was 0.86 J g abalone^{-1} h^{-1}, while those fed dried Ulva sp. meal was 0.70 J g abalone^{-1} h^{-1}. Among *Ulva sp*. meal treatments, abalone fed 20% inclusion diets had significantly higher egested faecal energy than those fed 0, 5 and 10% inclusions. However, there was similar egested faecal energy in abalone fed 0, 5 and 10% dried *Ulva sp*. meal inclusions (P > 0.05; one-factor ANOVA; Tukey's HSD test). For dried *G. cliftonii* meal inclusions, only the abalone fed 10% macroalgae meal lost significantly more egested faecal energy than those fed 0% (P < 0.01). No significant difference was found in egested faecal energy for among abalone fed 5, 10 and 20% dried *G. cliftonii* inclusions (P > 0.05). Egested faecal energy showed values ranging from 0.53 to 1.06 (J g abalone^{-1} h^{-1}) for abalone fed dried *Ulva* sp. meal and from 0.64 to 1.18 (J g abalone^{-1} h^{-1}) for abalone fed dried *G. cliftonii* meal inclusions.

There were no significant differences in egested faecal energy between abalone fed the commercial diet and all other diets (P > 0.05; one-factor ANOVA; Tukey's HSD test; Table 3.2).

3.4.8 Pedal mucus production energy and shell growth energy

The average pedal mucus production energy of all treatments in this study was 0.054 J g abalone^{-1} h^{-1}. The shell growth energy was not significantly affected by macroalgae type (P = 0.784), inclusion level (P = 0.054) or the interaction between

these two factors (P = 0.072; two-factor ANOVA; Table 3.2). Shell growth energy of abalone fed dried *G. cliftonii* meal or dried Ulva sp. meal was 0.03 J g abalone^{-1} h^{-1}.

There were no significant differences in shell growth energy between abalone fed the commercial diet and diets with dried macroalgae meal inclusions (P > 0.05; one-factor ANOVA; Tukey's HSD test; Table 3.2). Shell growth energy ranged from 0.029 to 0.031 (J g abalone^{-1} h^{-1}) across the treatments.

Table 3.2 Energy components (J g abalone^{-1} h^{-1}) of greenlip abalone (*Haliotis laevigata*) fed the control commercial, basal diets and diets containing dried macroalgae inclusions [1].

Diet			Dried *Ulva* sp. meal (%)			Dried *G. cliftonii* meal (%)			Two-factor ANOVA (*P* value)			Dunnett's test
	The control commercial	0 [2]	5	10	20	5	10	20	Species (A)	Inclusion level (B) 0 5 10 20	A × B	*P* value
I	4.74 ± 0.05	4.51 ± 0.07$^{a\,x}$	4.89 ± 0.14^{a}	4.69 ± 0.06^{a}	5.38 ± 0.11^{b} *	6.67 ± 0.09^{z} *	6.61 ± 0.13^{z} *	6.09 ± 0.07^{y} *	< 0.001 (U < G)	< 0.001 (X Y Y Y)	< 0.001	< 0.001
Ab	3.94 ± 0.16	3.87 ± 0.11$^{a\,x}$	4.37 ± 0.09^{b}	4.14 ± 0.10^{ab}	4.32 ± 0.09^{b}	5.83 ± 0.21^{y} *	5.43 ± 0.06^{y} *	5.32 ± 0.11^{y} *	< 0.001 (U < G)	< 0.001 (X Y Y Y)	0.001	< 0.001
Pg	2.06 ± 0.04	2.02 ± 0.05^{x}	2.18 ± 0.07	1.92 ± 0.07	2.07 ± 0.09	2.10 ± 0.03^{xy}	2.24 ± 0.08^{y} *	2.26 ± 0.06^{y} *	0.031 (U < G)	0.169	0.024	0.018
R	1.12 ± 0.06	1.32 ± 0.11	1.18 ± 0.05	1.14 ± 0.04	0.96 ± 0.02	1.60 ± 0.15 *	1.16 ± 0.10	1.15 ± 0.07	0.020 (U < G)	0.005 (Y Y XY X)	0.101	0.001
U	0.11 ± 0.01	0.11 ± 0.00	0.12 ± 0.03	0.12 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.205	0.998	0.702	0.876
E	0.80 ± 0.13	0.64 ± 0.06$^{a\,x}$	0.53 ± 0.09^{a}	0.55 ± 0.09^{a}	1.06 ± 0.19^{b}	0.84 ± 0.20^{xy}	1.18 ± 0.04^{y}	0.78 ± 0.08^{xy}	0.058	0.066	0.004	0.052
M	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	NA	NA	NA	NA
S	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.031 ± 0.01	0.031 ± 0.01	0.03 ± 0.01	0.784	0.054	0.072	0.100

[1] Data presented as mean ± SE; n = 4. Where a significant main effects were detected, post-hoc tests were used to determine differences between means (Tukey's HSD test; $P < 0.05$).

[2] The basal diet treatment (0% dried macroalgae) served for both macroalgal species for the two-factor ANOVA.

[a, b, c] For parameters with a significant interaction, differences in inclusion levels are compared within *Ulva* sp. meal group (one-factor ANOVA, Tukey's HSD test), values without a common superscript are different ($P < 0.05$).

[x, y, z] For parameters with a significant interaction, differences in inclusion levels are compared within *G. cliftonii* meal group (one-factor ANOVA, Tukey's HSD test), values without a common superscript are different ($P < 0.05$).

X, Y, Z For parameters with a significant difference in inclusion levels are compared among macroalgae inclusion level diets (two-factor ANOVA, Tukey's HSD test) (X indicates the smallest value).

Abalone fed the commercial diet compared to abalone fed dried macroalgae using one-factor ANOVA; Dunnett's post-hoc test; $n = 4$ treatment^{-1}. * values without a common superscript compared to the commercial diet are significantly different ($P < 0.05$).

NA: not applicable

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Ab: Absorbed energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy.

3.4.9 Energy budgets of abalone fed dried macroalgae meal inclusion or the commercial diet

Energy budgets for greenlip abalone fed different macroalgae species at each level of macroalgae inclusion and the commercial diet are shown in Table 3.3. Absorbed energy was similar across treatments and ranged from 80.4% of ingested food energy in the 20% dried *Ulva* sp. meal diet to 89.2% in the 5% dried *Ulva* sp. meal inclusion diet. The major portions of ingested food energy were somatic growth energy (ranging from 31.5% in the 5% dried *G. cliftonii* meal diet to 44.9% in the 0% dried macroalgae meal diet) and respiration energy (ranging from 17.6% in the 10% dried *G. cliftonii* meal diet to 29.2% in the 0% dried macroalgae meal diet). Ammonia excretion energy was similar in all treatments and accounted for 1.42 - 2.62% of ingested food energy. Egested faecal energy ranged from 10.7 % in the 5% dried *Ulva* sp. meal diet to 19.6% in 20% dried *Ulva* sp. meal diet. Pedal mucus production energy accounted for a small portion of ingested food energy, ranging from 0.81% to 1.19%. Shell growth energy accounted the smallest component of ingested food energy, ranging from 0.44 to 0.66% (Table 3.3).

Table 3.3 Energy budgets (%) of greenlip abalone (*Haliotis laevigata*) fed the control commercial, basal diets and diets containing dried macroalgae inclusions [1].

Diets	The control commercial	0 [2]	Dried *Ulva* sp. meal (%)			Dried *G. cliftonii* meal (%)		
			5	10	20	5	10	20
I	100	100	100	100	100	100	100	100
Ab	83.10 ± 2.82	85.80 ± 1.44	89.20 ± 1.54	88.30 ± 1.92	80.40 ± 3.16	87.40 ± 3.01	82.20 ± 0.29	87.30 ± 1.41
Pg	43.40 ± 0.89	44.90 ± 0.88	44.80 ± 2.62	41.00 ± 1.60	38.50 ± 1.78	31.50 ± 0.56	33.90 ± 1.14	37.20 ± 1.28
R	23.60 ± 1.58	29.20 ± 2.72	24.20 ± 1.78	24.20 ± 0.91	17.90 ± 0.83	24.10 ± 2.90	17.60 ± 2.12	18.90 ± 1.23
U	2.39 ± 0.26	2.47 ± 0.08	2.41 ± 0.48	2.62 ± 0.49	2.06 ± 0.15	1.49 ± 0.15	1.42 ± 0.15	1.79 ± 0.19
E	16.90 ± 2.83	14.20 ± 1.44	10.70 ± 0.53	11.70 ± 1.92	19.60 ± 3.16	12.60 ± 3.01	17.80 ± 0.30	12.70 ± 1.42
M	1.13 ± 0.01	1.19 ± 0.02	1.10 ± 0.03	1.14 ± 0.01	1.00 ± 0.02	0.81 ± 0.01	0.81 ± 0.01	0.88 ± 0.01
S	0.66 ± 0.02	0.65 ± 0.01	0.63 ± 0.02	0.63 ± 0.01	0.55 ± 0.01	0.44 ± 0.01	0.46 ± 0.01	0.50 ± 0.01
Unexplained	11.92	7.39	16.16	18.71	20.39	29.06	28.01	28.03

[1] Data presented as mean ± SE; n = 4.
[2] The basal diet treatment (0% dried macroalgae) served for both macroalgal species.
Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy. Absorbed energy (Ab), somatic growth energy, respiration energy, ammonia excretion energy, pedal mucus production energy and shell growth energy calculated as a percentage of ingested food energy. Egested faecal energy obtained from 100% ingested food energy minus absorbed energy. Unexplained was calculated as 100% - (Pg + Pr + R + U + M + S + E)
Unavailable data for Pr as abalone showed not visible signs on gonad development.

3.5 Discussion

The results of the present study showed that the components of the energy budget in greenlip abalone changed in response to different macroalgae types and inclusion levels. Generally, an inclusion of dried *Ulva* sp. or *G. cliftonii* meal improved ingested food energy of greenlip abalone compared to the basal diet. This agrees well with previous studies where macroalgae act as feeding stimulants or attractants for abalone and adding macroalgae to the diet enhances food intake and growth rate of abalone (Allen et al., 2006; Angell et al., 2012; Bansemer et al., 2015b, 2016b). However, ingested food energy was dependent on macroalgae species. In the present study, the ingested food energy of abalone fed diet including dried *G. cliftonii* meal was higher than those fed the basal diet and dried *Ulva* sp. inclusions. Our data supports previous observations that greenlip abalone show a preference for red macroalgae over brown or green macroalgae (Shepherd, 1973). The energy for ingestion increased proportionally with increasing inclusion levels in dried *Ulva* sp. meal treatments. Since animals were fed to apparent satiation, it is possible that greenlip abalone increased energy intake as an offset to satisfy nutrient requirement when fed the *Ulva* sp. meal diet.

Greenlip abalone fed dried *G. cliftonii* meal inclusion diets invested more energy to somatic growth than those fed dried *Ulva* sp. inclusion diets. Since all diets were formulated to similar protein (34.6 - 35.2g $100g^{-1}$ diet) and energy (~ 17.5 MJ kg^{-1}) levels, the differences in somatic growth energy response may be attributed to the difference in composition between red and green macroalgae such as amino acid composition, proportions of fatty acids, amount of fatty acids and other micro nutrients (Bansemer et al., 2016b; Bautista-Teruel et al., 2011; Kirkendale et al., 2010; Mai et al., 1995a; Mai et al., 1996; Viera et al., 2005). Furthermore, it has been reported that faster growth in abalone is associated with both quantity and quality of

the protein used in the feed (Fleming, 1995; Viana et al., 1993). Therefore, the higher energy invested to somatic growth in greenlip abalone fed dried *G. cliftonii* meal diet may be due to better protein quality which is associated with high digestible nitrogen and energy content or a balance between high intake of digestible nitrogen and high intake of digestible energy (Fleming, 1995; Gómez-Montes et al., 2003).

In the present study, there was no significant adverse effect of feeding with a high inclusion level of dried *G. cliftonii* meal on somatic growth energy, but diet stability decreased (Table 1). Addittionally, somatic growth energy was similar among of abalone fed 5, 10 and 20% dried *G. cliftonii* meal and only abalone fed with a diet of >10% *G. cliftonii* meal inclusion invested significantly more energy for somatic growth than those fed 0% (the basal diet). Therefore, a 10% inclusion of dried *G. cliftonii* is recommended, but the strategy of improving diet stability is required for the inclusion of *G. cliftonii* meal in formulated diets.

Respiration energy in abalone has been reported to be affected by several factors such as temperature, size of the animal and dietary nutrient composition (Barkai and Griffiths 1987; Ganmanee et al., 2010; Lopez and Tyler, 2006; Peck et al., 1987). In the current study, the respiration energy of abalone fed dried *G. cliftonii* meal was significantly higher than those fed the dried *Ulva* sp. meal diet. Since the food consumption rate was also a major factor affecting the feeding respiration of aquatic animals (Bayne and Newell, 1983; Cui and Liu, 1990), the higher respiration energy in abalone fed diets including dried *G. cliftonii* meal may be due to higher ingested food energy and absorbed energy.

Egested faecal energy of is influenced by differences in diet nutrient composition and consumption rates (Currie et al., 2015b; Lopez and Tyler, 2006; Jobling, 1993). In the current study, the egested faecal energy of greenlip abalone fed dried macroalgae meal was affected by the interaction between dried macroalgae

type and inclusion level. Abalone fed a 20% dried *Ulva* sp. meal inclusion had lost significantly more energy via faecal egestion than the rest, while egested faecal energy of abalone fed the 10% dried *G. cliftonii* meal inclusion was significantly higher than those fed the 0% diet only. Since absorbed energy was similar among of all dried *Ulva* sp. meal treatments or among dried *G. cliftonii* meal diets, those treatments with higher egested faecal energy may due to the higher ingested food energy. These findings are consistent with Lopez and Tyler (2006) where egested faecal energy of the ormer varied greatly depending on diet type. But despite having similar absorbed energy, *H. tuberculata* fed a fishmeal diet lost significantly less energy through faecal egestion than those fed the commercial diet due to lower ingested food energy at 15 °C. The authors also reported that the lower egested faecal energy in the ormer fed macroalgae diet compared to the formulated diet was influenced by both lower ingested food energy and absorbed energy at 15, 18 or 20 °C.

The energy budgets of *Haliotis* spp. have been published for some species fed either macroalgae diets or formulated diets (Barkai and Griffiths, 1987; Barkai and Griffiths, 1988; Donovan and Carefoot, 1998; Farías et al., 2003; Ganmanee et al., 2010; Lopez and Tyler, 2006; Peck et al., 1987). However, prior to this study, there were no available reports on energy budgets for greenlip abalone fed diets of dried macroalgae meal inclusion. Since in most studies including our study, all components of energy budgets were calculated as percentage of ingested food energy, the proportion of each component of the energy budgets were dependent on ingested food energy (Barkai and Griffiths, 1987; Farías et al., 2003; Ganmanee et al., 2010). Across treatments, the energy invested in somatic growth accounted for the major proportion of energy budgets in greenlip abalone (ranging from 31.5 to 44.9% of ingested food energy) and were not markedly different from other species

fed formulated diets. For example, somatic growth energy was 38.8% for the green abalone 10 to 60 mm shell length and 0.1 to 2.5 g dry weight (Farías et al., 2003), 24.2% for the ormer 16.36 mm shell length and 0.24 g dry weight (Lopez and Tyler, 2006) and 37.8 - 45.8% for Thai abalone 2.22 to 4.16 mm shell length (Ganmanee et al., 2010). However, only 7 - 10% of the ingested food energy was allocated to somatic growth in pink abalone, *H. corrugata*,10.7 mm shell length and 0.15 g dried weight, fed a formulated diet, which may due to have been an overestimation of ingested food energy (Montaño-Vargas et al., 2005).

Ammonia is the end product of protein catabolism and its release is likely to be affected by quality and quantity of dietary protein (Bayne and Newell, 1983; Randall and Wright, 1987; Lopez and Tyler, 2006). Ammonia excretion is known to increase with increasing protein and decreasing carbohydrate content in fish diets (Rychly, 1980; Yang et al., 2002). Lopez and Tyler (2006) reported ammonia excretion energy increased in the ormer fed formulated diets with a high protein content (31%) as opposed to a low-protein seaweed diet (15.5%). In the present study, all diets were formulated to contain similar protein levels (~ 35%), thus ammonia excretion energy was not greatly influenced by the inclusion of dried macroalgae. Ammonia excretion energy of abalone fed diets with macroalgae meal inclusions was similar to those fed the commercial diet and only accounted for a small proportion of energy budget (1.42 - 2.62 %).

Mucus production energy has been considered as an important part of the energy budget of abalone since it was responsible for significant excretory losses in ormer (23.3 - 21.9%) and the northern abalone (4 - 16%) (Peck et al., 1987; Barkai and Griffiths, 1988; Donovan and Carefoot, 1998). However, pedal mucus production energy is very low, between 0.81 - 1.19% of ingested food energy in the current study. Since all other components have been measured, the large unknown

component is likely to be where the mucus production energy truly lies. In addition, mucus production appears to be a sensitive measurement. A part from the challenge of collecting sufficient pedal mucus, many problems arise when assessing mucus production as it also is used in other processes such as egested faecal production and cleaning the gills (Peck et al., 1987). Thus, it is possible that the mucus production energy was underestimated and accounted for some of the unexplained energy loss in the energy budget for the different dietary treatments.

3.6 Conclusion

Dietary dried macroalgae meal inclusion improved ingested food energy and absorbed energy. Greenlip abalone fed >10% dried *G. cliftonii* meal partitioned more energy into somatic growth. However, a high inclusion level of this dried macralgae meal was associated with reduced diet stability. The major proportion of ingested food energy was allocated to somatic growth as well as respiration, while a small part of ingested food energy was used as ammonia excretion energy, pedal mucus production energy and shell growth energy. The data in this study increases the information available on the energy budgets of greenlip abalone and can be useful in developing formulated diets with suitable macroalgae species at optimal inclusion levels for cultured abalone as well as managing environmental impacts through predicting waste output.

Chapter 4

Seasonal bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed formulated diet with increasing dietary protein levels

Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Thanh H. Hoang, Jian G. Qin and James O. Harris. Seasonal bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed formulated diet with increasing dietary protein levels. In preparation.

4.1 Abstract

Understanding energy budgets of cultured animals at different water temperatures gives us the ability to maximise the efficiency of the culturing process. Manipulating the ingredients in formulated diets is one way of enhancing dietary nutrition. In this study we investigated the effects of graded levels of crude protein (CP) at different seasonal temperatures on components of the energy budget of greenlip abalone, *Haliotis laevigata*. Four diets containing graded crude protein levels (27, 30, 33 and 36%) were fed to 6-month-old greenlip abalone at three water temperatures (14, 17 and 20 °C) for 91 days. Ingested food energy, somatic growth energy and respiration energy significantly increased as water temperature rose from 14 to 20 °C, irrespective of dietary CP. Absorbed energy was significantly higher in abalone fed the diet of 36% CP compared to those fed 27 or 30% CP in abalone cultured at 20 °C compared to those at 14 or 17 °C. Absorption energy was similar among abalone fed different CP levels at 17 or 20 °C, but it was significantly higher in abalone fed 36% CP at 14 °C. Abalone significantly allocated more energy for somatic growth and respiration as water temperature increased, but those energy allocations were similar among abalone fed different dietary CP levels at each temperature. Ammonia excretion energy was proportional to dietary CP levels. Although egested faecal energy did not fall in a trend relating to CP levels, abalone cultured at 14 °C lost significantly more energy through faecal egestion than those at 17 or 20 °C. Pedal mucus production energy remained relative constant at different temperatures. The largest portion of energy budget was respiration energy (34.1 - 43.9% of ingested food energy), whereas only small portions were ammonia excretion energy (0.28 - 1.40%), pedal mucus production energy (4.12 - 6.20%) and shell growth energy (0.22 - 0.46%). Somatic growth energy (16.80 - 28.10%) also accounted for a major part of energy budget. The proportion of the ingested food

energy egested as faecal energy was 8.2 - 17.9%. Up to 20 °C, greenlip abalone did not reach their tolerance limit and the dietary CP level of ~ 27 % is recommended for the diet of 6-month-old abalone at these tested temperatures. The increase in egested faecal energy at 14 °C or ammonia excretion energy at high dietary CP should be taken into account in managing water quality and waste production in abalone farms. These findings provide a better understanding of energy budget of greenlip abalone in the seasonally variable land-based culture environment.

Keywords: Bioenergetics, greenlip abalone, *Haliotis laevigata*, protein, temperature.

4.2 Introduction

Aquaculture of abalone began in Australia in the early 1980s (Hone and Fleming, 1998). Although seaweed is a main food source of wild abalone and currently being used in a number of countries, abalone culture in Australia is predominantly land-based and uses formulated feeds due to unavailable and unreliable quantities and quality of seaweed (Bansemer et al., 2016b; Kirkendale et al., 2010). Since food production makes up a major part of operating costs in aquaculture, a number of studies have been performed to develop economically and nutritionally formulated diets for abalone in cultivation systems in combination with environmental issues (Bansemer et al., 2015a; Britz and Hecht, 1997; Stone et al., 2013). Protein has traditionally received the most attention in nutritional studies and feed research as it is the most expensive nutrient ingredient and is essential for soft tissue growth (Fleming et al., 1996; Mai et al., 1995). Abalone growth is influenced by the availability of the suitable quantity and quality of dietary protein (Britz, 1996b; Mai et al., 1995b; Ogino and Ohta, 1963). Knowing the optimal crude protein (CP) level is necessary for the growth of abalone as an excess or insufficient level of protein supply will negatively affect metabolism, growth efficiency or water quality (Fleming et al., 1996; Mai et al., 1995b). The response to dietary protein depends on a number of factors including species, size, and water temperature (Bansemer et al., 2014, 2015a; Fleming et al., 1996; Mai et al., 1995b; Stone et al., 2013). Since the growth of cultured abalone relies on the quality and quantity of protein, diet formulations have been continually modified to obtain the optimal CP level for different species, sizes and temperatures. Protein requirements for some important commercial species have been well documented but are highly species-specific. For example, the optimal CP level is 30% for the northern abalone, *H. kamtschatkana* (Taylor, 1992), 22.3 - 32.3% and 23.3 - 35.6% for the green ormer, *H. tuberculata*

and *H. discus hannai* (Mai et al., 1995b), 27% for the ass's ear abalone, *H. asinina* (Bautista-Teruel and Millamena, 1999), 20 - 36% for South African abalone, *H. midae* (Sales et al., 2003) and 24 - 35% for *H. laevigata* (Bansemer et al., 2015a; Stone et al., 2013).

Temperature has been considered one of the most important environmental factors to govern the physiological processes of aquatic organisms (García-Esquivel et al., 2007) and it has significant effects on metabolic rate and energy expenditure on somatic growth, reproductive investment, excretion and food utilisation (Lopez and Tyler, 2006). Haliotids are classified as thermoconformers meaning their body temperature varies depending to the surrounding environment (Prosser, 1991). Understanding the effects of water temperature on the physiology of abalone is important to the management of abalone aquaculture as well as fisheries. The optimal temperature for the growth of abalone varies depending on a number of factors such as species and age (Bansemer et al., 2015a; Gilroy and Edwards, 1998. Stone et al., 2003). It is also likely that the optimal temperature for growth varies within a species, according to strains, as earlier work modelled a temperature of 18.3 °C for Tasmanian greenlip abalone (Gilroy and Edwards, 1998) whereas more recent research reported an optimum of 22 °C for South Australia greenlip abalone (Stone et al., 2013).

Temperature regulates protein turnover and growth efficiency and the requirement is often species-specific (Green et al., 2011; Jobling, 1994; Tung and Alfaro, 2011). For example, according to Tung and Alfaro (2011), the protein requirements were higher when the environmental temperatures were low in juvenile New Zealand black-footed abalone, *Haliotis iris*, fed diets of 0, 10, 20, 30, 40 and 45% CP at two ranges of water temperature (13 - 21 and 8 - 16 °C). However, in greenlip abalone, the protein requirement for juvenile (0.55 - 0.94 g) abalone was

27% CP at 20 °C (Coote et al., 2000). Recently, Stone et al. (2013) reported that the optimum CP levels increased from 29 to 35% for one-year-old (1.8 g) greenlip abalone and from 24 to 34% for two-year-old (22.9 g) greenlip abalone as temperature increased from 14 to 22 °C. A previous study in our lab showed that the CP requirement at seasonal water temperature (20 °C) for 6-month old juvenile greenlip abalone was ~ 29% (Bansemer et al., 2015a).

Bioenergetics addresses the transformation of energy in living organisms by investigating the balance between ingested food energy, usage energy and output energy. The major components of an energy budget such as somatic growth energy and respiration energy are significantly affected by water temperatures and dietary CP (Harris et al., 2005; Tung and Alfaro., 2011; Stone et al., 2013; Bansemer et al., 2015a; Lopez and Tyler, 2006). However, the effects of temperature and dietary CP levels on the bioenergetics of greenlip abalone remain largely unknown. Using post-weaned greenlip abalone, the aims of the current study were to: 1) establish energy budgets for abalone fed the experimental diets; and 2) evaluate the effects of different CP levels at seasonal temperatures on each component of the energy budgets.

The understanding of the energy budget in abalone can be useful to farming especially for diet formulation by adjusting dietary CP level at different farming temperatures. Furthermore, results from egested faecal energy and ammonia excretion energy can be used to manage waste production and improve abalone culture efficiency.

4.3 Materials and methods

4.3.1 Experimental design and system

The 91-day study was carried out to investigate the effects of four dietary CP levels (27, 30, 33 and 36%) on each component of the energy budget of greenlip abalone at three temperatures (14, 17 and 20 °C).

The detailed design of the experimental system was described in Stone et al. (2013) and Bansemer et al. (2015a). Briefly, the culture system was supplied with 30 μm sand-filtered, UV treated seawater (Model 025120-2, 120w, Emperor Aquatics, Pottstown, PA, USA).

Forty eight 12.5 L blue plastic culture units (Nally IH305, ViscountPlastics Pty Ltd.; 39.2 × 28.8 × 11.0 cm) per system were each supplied with flow-through seawater (300 mL min^{-1}). The water depth in each culture unit was held at 2.5 cm using a standpipe with a mesh screen (0.8 mm) on the outlet to retain uneaten feed. The air temperature was adjusted based on the incoming water temperature and ranged from 16.0 to 19.3 °C while the water temperature was held at 14, 17 or 20 °C (±1 °C) throughout the experiment through the use of either immersion heaters (240 V, 3 kw, JQ20; Austin and Cridland, Carlton, NSW, Australia) or chillers (3 hp, 240 V, 50 Hz: Daeil Cooler Co., Ltd., Busan, Korea).

The photoperiod was 12 h low intensity fluorescent lighting at 3.4 lx and 12 h dark. The selected water temperatures represent the temperature range typically occurring from autumn, through winter, to early summer experienced by post-weaned juvenile greenlip abalone in South Australia (Bansemer et al., 2015a).

4.3.2 Experimental animals

Greenlip abalone were purchased from South Australian Mariculture (Boston Point, Port Lincoln, SA, Australia) and were then held at the South Australian Research and Development Institute (SARDI), Aquatic Sciences Centre in holding

tanks with a flow-through, UV-treated, seawater system for two weeks before stocking. During this period, animals were fed a 5-mm commercial chip (Abgrow premium abalone diet; Eyre Peninsula Aquafeed Pty Ltd, Lonsdale, SA, Australia) *ad libitum* daily.

4.3.3 Experimental stocking

Eighteen randomly selected animals (0.91 ± 0.00 g; shell length 19.46 ± 0.02 mm; n = 648) were stocked in each of the culture units with four replicates for each of the twelve diet-temperature treatments. Animals were acclimated to the system for 16 days and fed their respective diets. Water temperatures were maintained at levels of 14, 17 and 20 °C (± 1 °C) throughout the 91- day experiment. During the experiment, dead abalone were collected and replaced with similar size and weight abalone that had been cultured at the same water temperature and fed the commercial formulated diet.

4.3.4 Experimental diets, feeding and water management

Four diets containing four CP levels (27, 30, 33 and 36% CP) were used at each of the 14, 17 and 20 °C water temperature. These chosen CP levels were considered commercially applicable to land-based abalone production in southern Australia. The experimental diets were formulated on a digestible protein basis and contained highly palatable and digestible ingredients at realistic inclusion levels, using protein and energy digestibility results reported for greenlip abalone (Fleming et al., 1998; Vandepeer, 2005). Diets contained ~3.6% lipid, and ~17.4MJ kg^{-1} crude and ~12.5MJ kg^{-1} digestible energy levels. The diets were made using a TR110 pasta machine (La Prestigiosa medium; IPA. Vicenza. Italy). All diets were produced with a size dimension of 4 × 3 × 2 mm. Diets were then oven-dried at 45 °C for 48 h and stored at -20 °C. The experimental dietary ingredients and the proximate composition of the experimental diets are presented in Table 4.1.

Feeding occurred daily at 16:00 h and abalone were fed to excess (4% biomass day^{-1}). Tanks were cleaned between 08:30 h and 09:30 h the following day at which point uneaten feed was collected by pouring the entire tank contents through a fine mesh screen (500 μm). The uneaten feed then was weighed and oven-dried at 105 °C for 16 h. The difference between amount of uneaten feed and feed delivered was used to calculate daily feed consumption. For corrected feed consumption, leaching loss was taken into account. The dry matter leaching loss for each diet was determined in triplicate by submerging the diet (1 g) in seawater (25 mL) at 14, 17 and 20 °C for 16 h. After 16 h, the supernatant was removed using a syringe, and the remaining pellets were dried at 105 °C for 16 h. The dry matter leaching loss for all diets was highest at 20 °C, but was less than 8% dry weight in 16 h.

The water quality measurement was previously described in Chapter 2 (2.3.2).

Table 4.1 Ingredients and nutrient composition of experimental diets (reported by Bansemer et al., 2015a)

Ingredients (g 100 g^{-1} diet as fed)	**Crude protein level (%)**			
	27	**30**	**33**	**36**
Salmon fish meal	4.00	4.00	4.00	4.00
Solvent extracted soybean meal	18.90	21.40	23.90	26.47
Lupins (de-hulled)	20.80	23.60	26.40	29.14
Waxy maize starch	30.67	29.07	27.59	19.96
Pregelatinised waxy maize starch	10.00	5.62	1.15	0.00
Wheat gluten meal	5.00	5.00	5.00	5.00
Casein	5.48	6.53	7.59	8.63
Diatomaceous earth	1.76	1.79	1.77	4.60
Fish oil	1.22	0.84	0.46	0.10
EPA Vitamin/mineral premix	0.20	0.20	0.20	0.20
Sodium alginate	0.30	0.30	0.30	0.30
Vitamin E	0.01	0.01	0.01	0.01
Calcium sulphate	0.43	0.36	0.30	0.22
Monosodium phosphate	0.72	0.68	0.65	0.61
Arginine	0.31	0.37	0.41	0.46
Threonine	0.20	0.23	0.27	0.30
Ingredient composition (g 100 g^{-1} diet as feed), analysed and calculated				
Moisture	10.35	10.48	10.61	10.30
Crude protein	27.00	31.10	34.30	37.30
Digestible protein (calculated)	20.27	23.54	26.13	28.57
Lipid	3.60	3.60	3.70	3.50
Gross energy (MJ kg^{-1})	17.00	17.25	17.64	17.27
Digestible energy (MJ kg^{-1}) (calculated)	12.24	12.35	12.57	12.53
Ash	5.02	5.31	5.24	8.17
NFE (calculated)	64.38	59.99	56.76	51.03
Digestible CP:GE (g MJ^{-1})	16.57	19.06	20.79	22.80
Calculated amino acids (g 100 g^{-1})				
Arginine	2.22	2.50	2.77	3.04
Histidine	0.72	0.80	0.89	0.97
Isoleucine	1.28	1.44	1.59	1.74
Leucine	2.10	2.35	2.59	2.83
Lysine	1.52	1.71	1.90	2.09
Methionine	0.46	0.51	0.57	0.62
Phenylalanine	1.31	1.46	1.61	1.76
Threonine	1.22	1.37	1.53	1.67
Tryptophan	0.30	0.34	0.37	0.41
Valine	1.42	1.59	1.75	1.92

NFE = Nitrogen free extract = 100 % - (protein % + lipid % + ash % + moisture %); EPA: Eyre Peninsula Aquafeed.

4.3.5 Determination of the components of the energy budget

Energy budgets were calculated for greenlip abalone by measuring each component of the energy budget in the equation described in Chapter 2 (2.3.3).

4.3.5.1 Ingested food energy

The determination of ingested food energy was described in Chapter 2 (2.3.3.1).

4.3.5.2 Somatic growth energy

The determination of somatic growth energy was described in Chapter 2 (2.3.3.2). At 14 °C, the final weights of greenlip abalone (mean ± SE) were 1.59g (n=4) in 27% CP diet; 1.47g (n=4) in 30% CP diet; 1.53g (n=4) in 33% CP diet and 1.49g (n=4) in 36% CP diet. At 17 °C, the final weights of greenlip abalone (mean ± SE) were 1.92g (n=4) in 27% CP diet; 1.97g (n=4) in 30% CP diet; 1.95g (n=4) in 33% CP diet and 1.96g (n=4) in 36% CP diet. At 20 °C, the final weights of greenlip abalone (mean ± SE) were 2.58g (n=4) in 27% CP diet; 2.50g (n=4) in 30% CP diet; 2.57g (n=4) in 33% CP diet and 2.47g (n=4) in 36% CP diet (Bansemer et al. 2015a).

4.3.5.3 Respiration energy

The determination of respiration energy was described in Chapter 2 (2.3.3.3).

4.3.5.4 Ammonia excretion energy

The determination of ammonia excretion energy was described in Chapter 2 (2.3.3.4).

4.3.5.5 Egested faecal energy

The determination of egested faecal energy was described in Chapter 2 (2.3.3.5).

4.3.5.6 Pedal mucus production energy and shell growth energy

As only five animals were available for this bioenegetic study per replicate and abalone available were small (1.47 - 2.58 g) at the end of experiment, there was

insufficient mucus collected for analysis. Thus, collection of sufficient amount of mucus correspoding to dietary treatments for proper analysis on energy content and confidence in results was loow. In order to collect a sufficient amount of pedal mucus at 14, 17 or 20 °C, forty abalone with similar size were introduced in four containers (600 mL beaker) that had been previously weighed and immersed in a tank supplied with fresh seawater at each water temperature. Abalone were carefully removed after 6 h and the beakers were rinsed using distilled water to remove faeces and seawater. The beakers were then dried at 70 °C for 1 h, reweighed and pedal mucus production was calculated by substraction. The dried pedal mucus was carefully scraped from the plates and its energy content was finally analysed using combustion in a microbomb calorimeter (Lopez and Tyler, 2006). Because it was impossible to collect enough mucus from abalone per replicate, the pedal mucus production energy was assumed to be similar among abalone fed different diets at each temperature.

The calculation of pedal mucus production energy was described in Chapter 2 (2.3.3.6)

Shell growth energy was described in Chapter 2 (2.3.3.6).

2.5. Statistical analysis

All statistical analyses were carried out using IBM SPSS (Version 22 for Windows; IBM SPSS Inc., Chicago, IL, USA). To assess the effects of temperatures (14, 17 and 20 ºC) and CP level (27, 30, 33 and 36%) on each parameter of the energy budget for greenlip abalone, data were analysed using two-factor ANOVA. When a significant interaction between temperature and CP level was observed, differences of CP level are compared within 14, 17 or 20 ºC (one-factor ANOVA; Tukey's HSD test). When significant main effects were observed, a post-hoc test was

used to detect significant differences between treatment means (one-factor ANOVA; Tukey's HSD test).

4.4 Results

4.4.1 General observation and water quality

The overall mortality for the study was 4.05%. At 14 °C treatments, water temperature 14.0 ± 0.1 °C; dissolved oxygen 99.3 ± 1.4% saturation or 8.0 ± 0.3 mg L^{-1}; pH 8.14 ± 0.05; and salinity 35.7 ± 0.07 ppt. At 17 °C treatments, water temperature 17.0 ± 0.3 °C; dissolved oxygen 98.2 ± 1.2% saturation or 7.6 ± 0.2 mg L^{-1}; pH 8.15 ± 0.04; and salinity 35.7 ± 0.07 ppt. At 20 °C treatments, water temperature 19.9 ± 0.3 °C; dissolved oxygen 97.2 ± 1.6% saturation or 7.3 ± 0.2 mg L^{-1}; pH 8.15 ± 0.4; and salinity 35.7 ± 0.07 ppt. The water temperature was presented as mean ± SD, n = 75, while mean ± SD, n = 91 for other parameters. The water quality and mortality in this experiment was reported in Bansemer et al. (2015a).

4.4.2 Ingested food energy

The components of the energy budget of greenlip abalone fed different CP levels at seasonal temperatures are shown in Table 4.2. The ingested food energy rate was significantly affected by temperature ($P < 0.001$; two-factor ANOVA; Table 4.2) and the interaction between temperature and CP level ($P = 0.014$), but not by CP level ($P = 0.059$). Ingested food energy of abalone at 20 ºC (5.92 J g abalone^{-1} h^{-1}) was significantly higher than those at 17 (5.04 J g abalone^{-1} h^{-1}) and 14 ºC (5.18 J g abalone^{-1} h^{-1}) and was similar between 14 and 17 ºC. Ingested food energy of abalone fed 27, 30, 33 and 36% CP was 5.21, 5.17, 5.38 and 5.76 J g abalone^{-1} h^{-1}, respectively. The interactive effects of temperature and CP level on ingested food energy were due to a significant increase in ingested food energy for abalone fed 36% CP compared to 27 and 30% CP at 14 ºC, whereas ingested food energy for

abalone fed these CP levels was similar at 17 or 20 ºC. At 14 °C, abalone fed 36% CP expended significantly more ingested food energy than those fed 27% CP (P = 0.026) and 30% CP (P = 0.020), but not from those fed 33% (P > 0.05). There was no significant difference in ingested food energy among abalone fed graded levels of CP at 17 or 20 ºC (P > 0.05; one-factor ANOVA, Tukey's HSD test; Table 4.2).

4.4.3 Absorbed energy

Absorbed energy was significantly influenced by temperature (P < 0.001; two-factor ANOVA; Table 4.2), CP level (P < 0.001), and their interaction (P = 0.011). The absorbed energy was significantly improved in abalone cultured at 20 ºC (5.25 J g abalone^{-1} h^{-1}) compared to those culture at lower temperature and it was similar in abalone at 17 (4.42 J g abalone^{-1} h^{-1}) and 14 ºC (4.38 J g abalone^{-1} h^{-1}). Although abalone fed 36% CP diet (5.12 J g abalone^{-1} h^{-1}) had significantly higher absorbed energy than those fed 27 (4.49 J g abalone^{-1} h^{-1}) and 30% CP diet (4.42 J g abalone^{-1} h^{-1}), they had similar absorbed energy with those fed 33% CP diet (4.71 J g abalone^{-1} h^{-1}). The interaction between CP level and temperature was due to a significant increase in absorbed energy for abalone fed 36% CP diet compared to those fed 27, 30 and 33% CP diet at 14 ºC, while it was similar in abalone fed those CP levels at 17 or 20 ºC.

4.4.4 Somatic growth energy

There was significant effect from temperature on somatic growth energy (P < 0.001; two-factor ANOVA; Table 4.2). However, CP level and interaction between these two factors had no significant effect on the somatic growth energy (P > 0.05). Abalone significantly invested more energy for somatic growth as water temperature increased from 14 to 20 ºC (P < 0.001). Somatic growth energy of abalone at 14, 17 and 20 ºC was 0.92, 1.21 and 1.55 J g abalone^{-1} h^{-1}, respectively. Abalone fed 27, 30, 33 and 36% CP invested 1.28, 1.18, 1.22 and 1.23 J g abalone^{-1} h^{-1}, respectively.

4.4.5 Respiration energy

Respiration energy was significantly influenced by temperature ($P < 0.001$; two-factor ANOVA; Table 4.2), but was not by CP level ($P = 0.880$) or the interaction of these two factors ($P = 0.777$). Abalone cultured at 20 ºC (2.35 J g abalone^{-1} h^{-1}) had significantly higher respiration energy than those cultured at 14 or 17 ºC (1.92 J g abalone^{-1} h^{-1}) ($P < 0.001$). However, there was no difference in the respiration energy of abalone at 14 and 17 ºC ($P > 0.05$). Respiration of the abalone fed 27, 30, 33 and 36% CP was 2.10, 1.94, 2.03 and 2.19 J g abalone^{-1} h^{-1}, respectively.

4.4.6 Ammonia excretion energy

Ammonia excretion energy was significantly influenced by CP level ($P = 0.032$; two-factor ANOVA; Table 4.2), but not by temperature ($P = 0.503$) or the interaction between these two factors ($P = 0.259$). Ammonia excretion energy of abalone at 14, 17 and 20 ºC was 0.048, 0.048 and 0.059 J g abalone^{-1} h^{-1}, respectively. Abalone fed 36% CP diet (0.06 J g abalone^{-1} h^{-1}) lost significantly more energy due to ammonia excretion than those fed 27% CP diet (0.030 J g abalone^{-1} h^{-1}) ($P = 0.009$), but did not differ from those fed 30% CP (0.055 J g abalone^{-1} h^{-1}) ($P = 0.453$) and 33% CP diets (0.057 J g abalone^{-1} h^{-1}) ($P = 0.570$). Ammonia excretion energy was similar among abalone fed 27, 30 and 33% CP diets ($P > 0.05$).

4.4.7 Egestion faecal energy

Egested faecal energy was significantly influenced by temperature ($P < 0.001$; two-factor ANOVA; Table 4.2), CP level ($P < 0.006$), and their interaction ($P = 0.001$). The egested faecal energy was significantly higher in abalone cultured at 14 ºC (0.81 J g abalone^{-1} h^{-1}) compared to those cultured at higher temperatures and was similar for abalone at 17 (0.62 J g abalone^{-1} h^{-1}) and 20 ºC (0.67 J g abalone^{-1} h^{-1}). Abalone fed 36% CP diet (0.64 J g abalone^{-1} h^{-1}) had significantly lower egested

faecal energy than those fed 30% CP diet (0.76 J g abalone^{-1} h^{-1}) ($P < 0.05$), but had similar egested faecal energy to those fed 27 (0.72 J g abalone^{-1} h^{-1}) or 33% CP diet (0.68 J g abalone^{-1} h^{-1}) ($P > 0.05$). The interaction between CP level and temperature was due to a significant decrease in egested faecal energy for abalone fed 33% CP diet compared to those fed 36% CP diet at 14 or 20 ºC, while it increased in abalone at 17 ºC. At 14 ºC, abalone fed 36% CP had significantly lower egested faecal energy than those fed 30 and 33% CP ($P = 0.026$; $P = 0.003$, respectively), but it was similar compared to those fed 27% CP ($P > 0.05$; one-factor ANOVA, Tukey's HSD test; Table 4.2). At 17 ºC, egested faecal energy of abalone fed 36% CP was significantly higher than those fed 33% CP ($P = 0.007$), but it was similar to those fed 27 and 30% CP ($P > 0.05$). At 20 ºC, there were significant differences in egested faecal energy of abalone fed 36% CP and those fed lower CP level, abalone fed 33% CP and those fed lower CP level. However, the egested faecal energy was similar in abalone fed 27 and 30% CP ($P > 0.05$).

4.4.8 Pedal mucus production energy and shell growth energy

The pedal mucus production energy was 0.25, 0.28 and 0.29 J g abalone^{-1} h^{-1} in abalone cultured at 14 ºC, 17 and 20 ºC, respectively.

The shell growth energy was significantly affected by temperature ($P < 0.001$; two-factor ANOVA; Table 4.2), but not by CP level ($P = 0.654$) or interaction between these two factors ($P = 0.596$). Shell growth energy was 0.014, 0.019 and 0.026 J g abalone^{-1} h^{-1} as temperature increased from 14 to 20 ºC. Shell growth energy of abalone fed 27, 30, 33 and 36% CP was similar (0.02 J g abalone^{-1} h^{-1}).

Table 4.2 Energy components (J g abalone^{-1} h^{-1}) of greenlip abalone (*Haliotis laevigata*) at three water temperature fed four dietary protein levels[1].

Temp. (°C)	14				17				20				ANOVA							
CP (%)	27	30	33	36	27	30	33	36	27	30	33	36	Temp (°C) (A)			Protein level (%) (B)				A×B
													14	17	20	27	30	33	36	
I	4.79^{a}	4.74^{a}	5.21^{ab}	6.00^{b}	4.57	4.90	5.11	5.58	6.26	5.89	5.83	5.69	X	X	Y	NS				0.014
Ab	3.98^{a}	3.89^{a}	4.29^{a}	5.34^{b}	3.99	4.28	4.62	4.79	5.49	5.08	5.21	5.22	X	X	Y	A	A	AB	B	0.011
Pg	0.97	0.80	0.92	1.00	1.26	1.20	1.15	1.24	1.62	1.53	1.58	1.47	X	Y	Z	NS				0.578
R	2.09	1.73	1.77	2.10	1.75	1.77	1.97	2.20	2.47	2.31	2.36	2.26	X	X	Y	NS				0.777
U	0.013	0.039	0.080	0.057	0.037	0.052	0.047	0.056	0.040	0.073	0.043	0.078	NS			A	AB	AB	B	0.259
E	0.81^{ab}	0.85^{b}	0.91^{b}	0.66^{a}	0.59^{ab}	0.62^{ab}	0.49^{a}	0.79^{b}	0.77^{c}	0.80^{c}	0.63^{b}	0.47^{a}	Y	X	X	AB	B	AB	A	0.001
M	0.25	0.25	0.25	0.25	0.28	0.28	0.28	0.28	0.29	0.29	0.29	0.29	NA			NA				NA
S	0.015	0.013	0.013	0.013	0.019	0.020	0.020	0.018	0.026	0.025	0.027	0.025	X	Y	Z	NS				0.596

[1] Data is presented as mean +/- SE, n = 4. Where significant main effects were detected, post-hoc tests were used to determine differences between means (Tukey's HSD test; $P < 0.05$).

X, Y, Z: for variables with a significant effect of temperature (X indicates the lowest value; $P < 0.05$); A, B, C: for variables with a significant effect of protein level (A indicates the lowest value; $P < 0.05$); ^{a,b,c} For parameters with a significant interaction, differences in inclusion levels are compared within 14 ºC, 17 ºC and 20 ºC group (one-factor ANOVA, Tukey's HSD test). NS: denotes non significant differences ($P > 0.05$). NA: denotes not applicable.

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Ab: Absorbed energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy. Unavailable data of Pr as abalone showed not visible signs on gonad development.

4.4.9 Energy budgets of abalone fed formulated diets of different CP at different water temperatures

The distribution of energy, expressed as percentage of ingested food energy for greenlip abalone fed different CP level at seasonal temperature are shown in Table 4.3. Absorbed energy ranged from 82.1% to 91.8% of ingested food energy. The pattern of energy allocation to each component of energy budget was similar across treatments. The major portions of the ingested energy were respiration energy (34.1 - 43.9%) and somatic growth energy (17.0 - 28.1%), whereas, energy used for ammonia excretion, pedal mucus production and shell growth accounted for small portions of energy budget, ranging from 0.28 to 1.40%, 4.12 to 6.20% and 0.22 to 0.46%, respectively. The 6-month-old greenlip abalone expended 8.20 - 17.9% of ingested food energy via faecal egestion.

Table 4.3 Energy budgets (%) of greenlip abalone (*Haliotis laevigata*) at three water temperature fed CP levels [1].

Temp. (°C)	14				17				20			
CP (%)	27	30	33	36	27	30	33	36	27	30	33	36
I	100	100	100	100	100	100	100	100	100	100	100	100
Ab	83.2	82.1	82.5	89.0	87.3	87.3	90.3	85.9	87.8	86.3	89.3	91.8
Pg	20.7	17.0	18.0	16.8	28.1	24.7	22.8	22.1	26.0	26.1	27.2	25.8
R	43.9	36.5	34.1	35.0	38.6	36.6	38.2	39.7	39.4	39.3	40.6	40.1
U	0.28	0.82	1.40	0.98	0.87	1.08	0.95	1.00	0.64	1.25	0.74	1.38
E	16.9	17.9	17.5	11.0	12.8	12.7	9.70	14.2	12.2	13.7	10.8	8.20
M	5.17	5.19	4.76	4.12	6.20	5.69	5.45	4.96	4.65	4.95	5.02	5.12
S	0.31	0.28	0.25	0.22	0.42	0.42	0.40	0.33	0.41	0.43	0.46	0.44
Unexplained	12.7	22.3	23.9	31.9	13.0	18.8	22.5	17.7	16.7	14.3	15.2	18.9

[1] Data is presented as mean ± SE, n = 4.

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy. Absorbed energy (Ab), somatic growth energy, respiration energy, ammonia excretion energy, pedal mucus production energy and shell growth energy calculated as a percentage of ingested food energy. Egested faecal energy obtained from 100% ingested food energy minus absorbed energy. Unexplained was calculated as 100% - (Pg + Pr + R + U + M + S + E)

Unavailable data for Pr as abalone showed no visible signs on gonad development.

4.5 Discussion

In the present study, a significant increase in ingested food energy with increasing of temperature from 14 to 20 ºC was observed. Our results support Lopez and Tyler (2006) in that ingested food energy consumed by the green ormer fed seaweed or formulated diets increased with temperature from 15 to 22 ºC. Similarly, Stone et al. (2013) reported that the feed consumption rate of 1-y-old greenlip abalone fed graded protein levels at 27 - 36% was positively related to temperature from 14 to 22 ºC. However, ingested food energy did not appear to be protein dependent at 17 or 20 ºC in the current study. It has been reported that the abalone were consuming more food when fed lower protein and energy requirement in order to satisfy their energetic requirements. The higher consumption rate of abalone fed the higher protein was due to low energy content in the diets (Gómez-Montes., 2003; Green et al., 2011). Thus, in the present study, it is possible that no significance in ingested food energy of abalone fed graded protein levels was observed due to similar dietary digestible energy (~ 12.50 MJ kg^{-1}) and it is sufficient digestible energy for 6-month-old greenlip abalone at 17 or 22 ºC. Nevertheless, at 14 ºC, the increase in ingested food energy from 27 to 36% CP in the present study is unexpected as abalone fed low-protein diets increased their food consumption rate to enhance ingested food energy as an offset to increase protein intake (Stone et al., 2013). It is possible that the dietary digestible energy of ~ 12.50 MJ kg^{-1} was not sufficient for greenlip abalone at 14 ºC, but future research is warranted.

In the current study, greenlip abalone expended significantly more available energy for somatic growth as water temperature increased from 14 to 20 ºC. The results agree with findings of Stone et al. (2013) that the growth of 1-year-old greenlip abalone (1.8 g; shell length 23.3 mm) gradually increased with increasing water temperature from 14 ºC up to 22 ºC, where greenlip abalone had not reached their thermal tolerance level. In fact, above 23 °C, an increase in health problems and significant levels of mortality occur on greenlip abalone farms in Australia (Mozqueira, 1996; Stone et al., 2014a). These findings are consistent with Lopez and Tyler (2006) showing that despite significant decrease in somatic growth energy from 18 - 22 ºC, *H. tuberculata* fed formulated diet significantly invested more energy to somatic growth as water temperature increased from 15 to 18 ºC.

CP levels (27 - 36%) did not significantly affect the somatic growth energy of greenlip abalone at 17 and 20 °C in the present study. It has been reported that shell length and weight gain of South African abalone, *Haliotis midae,* fed diets containing graded levels of protein (18 - 26%) and energy (11.6-16.2 MJ kg^{-1}) at 18, 22 or 24 °C were independent of dietary protein when the digestible energy content of the diet was above 13.5 MJ kg^{-1} (Green et al., 2011). Thus, it is again possible that energy for somatic growth of greenlip abalone did not respond to dietary protein levels due to similar digestible energy content of ~12.5MJ kg^{-1} between diets.

The results of the current study showed that respiration energy increased considerably with increasing temperature from 14 to 20 °C. Lyon (1995) and Lopez and Tyler (2006) reported a similar trend with *H. midae* (from 16 to 23 °C) and *H. tuberculata* (from 15 to 22 °C), where respiration energy was found to increase proportionally to temperature elevation. Since there were no turning points observed in respiration energy over the range of temperature tested in this study, this gives

further support to the work showing that optimum temperatures for growth of this species are above 20 °C.

In the current study, the ratio between oxygen consumed and nitrogen excreted (O/N ratio) was additionally calculated to find out where the main sources of energy come from. This ratio represents the degree to which protein is utilised in metabolism by marine invertebrates (Bayne et al., 1985). If the O/N ratio is below 16, the energy source is from the exclusive use of proteins, whereas proteins and lipids are used equally as an energy source if a value fall between 50 and 60 (Mayzaud and Conover, 1988). The values obtained in the present work for the O/N ratio ranged from 22.1 - 61.8, except for treatment of 27% CP at 14 °C, which may be caused by underestimated ammonia production. This value indicates a higher use of dietary proteins as an energy source in abalone, which has been also reported in juvenile green abalone, *H. fulgens* (Farías et al., 2003).

In the current study, respiration energy accounted for the largest component of energy budgets in greenlip abalone across treatments (ranging 34.1 - 43.9%), which is higher than 4.0 - 200.0 g ormer (27.9 - 31.1%) but similar to 8.6 - 77.2g Thai abalone (33.2 - 42.5%) (Ganmanee et al., 2010; Peck et al., 1987). Respiration energy was also the largest component of both summer and winter budgets of 50.0 g the Northern abalone (59 and 77%, respectively) or in the ormer during summer (30%) (Donovan and Carefoot, 1998; McBride et al., 2001).

Faecal energy (from 8.2 - 17.9%) was the third major proportion of the energy budget across the treatments in the current study. However, egested faecal energy was not remarkably different from other *Haliotis* species. For example, egested faecal energy was 18.7 - 19.1% in *H. asinina* (Ganmanee et al., 2010), 11.1% in *H. tuberculata* (Lopez and Tyler, 2006) and 12.4% in *H. fulgens* (Farías et al., 2003). Although approximately 70% and 63% of the ingested food energy was lost due to

faecal egestion in pink abalone *Haliotis corrugata* and *H. midae*, those higher egested faecal energy values were attributed to overestimation of ingested food energy consumed by *H. corrugata* or low absorption efficiency by feeding tough *Ecklonia maxima* fronds in *H. midae* (Barkai and Griffths 1988; Montaño-Vargas et al., 2005). Since egested faecal energy accounted for a large proportion of the energy budget in this study, particularly at low temperature, it should be considered an important factor for the culture environment and economy.

Table 4.4 Energy budgets (% of ingested food energy) of some abalone species.

Species	Diets	CP (%)	Temp (ºC)	*Pg*	*Pr*	*R*	*U*	*F*	*M*	References
H. corrugata	Formulated diet	32-42	21	7.23 - 9.94	NA	4.95 - 7.68	0.52 - 1.03	72.8 - 79.2	0.73 -1.23	Montaño-Vargas et al. (2005)
H. tuberculata	Formulated diet	29.1	18	55.7	1.15	24.2	1.2	11.1	0.99	Lopez and Tyler (2006)
H. tuberculata	Macroalgae[1]	NA	24 - 28	2.0	NA	30.0	24.0	14.0	1.0	McBride et al. (2001)
	Macroalgae[1]	NA	20 - 22	39.0	NA	8.0	7.0	36.0	1.0	
H. tuberculata	Macroalgae[2]	NA	15	12.0 - 37.5	0 - 5.3	21.6 - 31.1	17.6 - 21.6[*]		23.3 - 29.1	Peck et al. (1987)
H. asinina	Formulated diet	28	28	37.8 - 45.8	NA	33.2 - 42.5	0.8 - 1.8	18.7 - 19.1	NA	Ganmanee et al. (2010)
H. fulgens	Formulated diet	30.8	16	38.8	NA	45.0	3.8%	12.4	NA	Farías et al. (2003)
H. fulgens	Macroalgae[1]	NA	24 - 28	19.0	NA	20.0	12.0	23.0	9.0	McBride et al. (2001)
	Macroalgae[1]	NA	20 - 22	46.0	NA	12.0	11.0	20.0	5.0	
H. kamtschatkana	Kelp[3]	NA	summer	6.0	3.0	59.0	<1	18.0	16.0	Donovan and Carefoot (1998)
	Kelp[3]	NA	winter	<1.0	0.0	77.0	<1.0	13.0	4.0	
H. midae	Kelp[4]	NA	14	1.4	3.3	32.2	0.1	63.0	< 1.0	Barkai and Griffiths (1988)
		NA	19	1.0	3.4	32.5	0.1	63.0	< 1.0	

[1] *Ulva lactuca* and *Gracilaria conferta* (3:1), [2]*Ulva lactuca*, [3]*Nereocystis luetkeana*, [4] *Ecklonia maxima*, [*] U + F

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy. Absorbed energy (Ab), somatic growth energy, respiration energy, ammonia excretion energy, pedal mucus production energy and shell growth energy calculated as a percentage of ingested food energy.

In the current study, pedal mucus production energy of greenlip abalone was similar at seasonal temperatures and ranged from 0.25 to 0.29 J g abalone^{-1} h^{-1}. Although greater mucus production was observed in summer than winter in the Northern abalone and the ormer (Donovan and Carefoot, 1998; Lopez and Tyler, 2006), our results support the finding in that the differences in seasonal water temperature did not affect mucus production energy of the green abalone and the ormer (McBride et al., 2001). Differences in pedal mucus production energy noticed here are likely due to differences between species and diets. Mucus production is used for locomotion and has been measured in most studies including the current study. However, mucus production is also used for other activities such as adhesion, feeding, keeping gills moist during aerial exposure, acting as an offensive agent and binding faecal pellets (Davies and Hawkins, 1998). It is possible that pedal mucus production in this energy budget may be underestimated and should be considered as part of the unexplained energy in greenlip abalone.

4.6 Conclusion

The data from this study enhances the knowledge of the physical responses of greenlip abalone to graded CP levels at seasonal water temperatures. Up to 20 °C, the two main components of the energy budget, respiration energy and somatic growth energy, proportionally increased with increasing water temperature, whereas dietary CP levels had little impact on energy budgets of greenlip abalone. An understanding of the energy budget of greenlip abalone would also assist in the formulation of practical diets at different water temperatures and help to predict waste outputs for water management.

Chapter 5

Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed probiotic and prebiotic supplementation in formulated diet at different temperatures

Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Krishna-Lee Currie, Thanh H. Hoang, Jian G. Qin and James O. Harris. Bioenergetics of greenlip abalone

(*Haliotis laevigata* Donovan) fed probiotic and prebiotic supplementation in formulated diet at different temperatures. In preparation.

5.1 Abstract

Supplements to greenlip abalone diets have been used to reduce mortality and optimise production efficiency at high summer temperatures in abalone culture, but the bioenergetic change associated with dietary supplementation is rarely tested. The bioenergetics of three-year-old greenlip abalone (weight: 81.04 g; 81.47mm SL) fed probiotic and prebiotic (pro/pre) diets were evaluated at both 22 (positive temperature control) and 25 °C (negative temperature control) for 38 days. These products were added into a formulated diet at levels of 0.1, 0.2 and 0.4% Pro 1 (probiotic *Bacillus nato*); 0.1 and 0.5% Pro 2 (probiotic *Bacillus subtilis* and *Bacillus licheniformis*); 0.1 and 0.2% Pre (prebiotic SafMannan yeast); 0.1% Pro 1 + 0.1% Pre; 0.5% Pro1 + 0.2% Pre. The supplementation of tested pro/prebiotic products did not affect any parameters of energy budgets at both 22 and 25 °C water temperatures. In contrast, with the exception of respiration energy, ingested food energy and absorbed energy were reduced, while ammonia excretion energy, egested faecal energy and pedal mucus production energy were increased in abalone under thermal stress (25 °C) compared to the preferred temperature (22 °C). Abalone had available energy for somatic growth and reproduction at 22 °C, while they had no energy for somatic growth and no visible signs of gonad development at 25 °C. The egested faecal energy was the main component of the energy budget at both 22 and 25 °C water temperatures. Somatic growth energy and reproduction energy was only available at 22 °C and accounted for 19.6 - 27.8% of energy ingestion and 2.03 - 2.90 %, respectively. Respiration energy was lower at 22 °C (14.2 - 22.9%) than at 25 °C (24.8 - 37.5%). Pedal mucus production energy was 3.32 - 3.63% at 22 °C and 7.97 - 10.0% at 25 °C. Ammonia excretion energy accounted for small portions of energy

ingestion, ranging from 0.40 - 0.83% at 22 °C and 0.75 - 2.30% at 25 °C. Shell growth energy accounted for the smallest proportion of ingested food energy, ranging from 0.14 to 0.29% at 22 °C or from 0.12 to 0.44% at 25 °C. Supplementation with pre/probiotic had no beneficial or adverse effects on greenlip abalone at 22 or 25 °C with respect to bioenergetics. As the main effects observed here were due to temperature, an increase of waste production and ammonia excretion in the summer months may contribute to bacterial proliferation and affect water quality in abalone farms.

Keywords: Greenlip abalone, bioenergetics, probiotic, prebiotic, thermal stress.

5.2 Introduction

Greenlip abalone aquaculture is an economically important industry in Australia (Hone and Fleming, 1998). Although commercial, land-based farmed, greenlip abalone, fed formulated diets grow faster than those fed other diets or wild abalone, they still require over 3 years to attain a market size of 60-90 mm in shell length or 35-90 g weight (Freeman, 2001; Hone and Fleming, 1998; Stone et al., 2013). Temperature is an important factor that affects survival, growth, feeding and metabolic rate of greenlip abalone. Generally, within optimum temperature ranges, feed intake, somatic growth and metabolic rates of greenlip abalone increase directly with temperature, whereas outside the optimum range, the growth rate rapidly decreases (Harris et al., 2005; Lange et al., 2014; Morash and Alter, 2015; Stone et al., 2013, 2014a, b). Land-based greenlip abalone farmed in southern Australia experience high mortality during summer months when water temperature exceeds 23 °C (Lange et al., 2014; Stone et al., 2014a, b). High water temperatures reduce dissolved oxygen in the water, and increase the proliferation of bacteria, reduce the abalone's antibacterial capacity, increase metabolism and respiration levels or cause oxidative stress by disrupting the oxidant-antioxidant equilibrium (Lange et al., 2014; Morash and Alter, 2015; Stone et al., 2014b).

In order to reduce mortality rates and optimise production efficiency at high water temperature during summer months, recent studies have focused on the effectiveness of adding various supplements to abalone diets such as macroalgae and grape seed extract (Lange et al., 2014; Stone et al., 2014b). The use of probiotics and prebiotics as feed additives in aquaculture has become more popular due to proving environmentally-friendly as well as improving nutrition, health and growth of animals (Irianto and Austin, 2002; Macey and Coyne 2005, Wang et al., 2008). Probiotics are defined as "live microbial feed supplements that improve the health of

human and terrestrial livestock" (Gatesoupe, 1999), while prebiotics are "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon" (Ringø et al., 2010). Probiotics and prebiotics have been examined in aquaculture for disease control (Balcázar et al., 2007; Macey and Coyne, 2005;) to increase growth and survival of some aquatic animals (Irianto and Austin, 2002; Krishnaprakash et al., 2009), to improve water quality (Balcázar et al., 2006; Dalmin et al., 2001), to enhance the immune response (Balcázar et al., 2006; Dalmin et al., 2001; Rengpipat et al., 2000), to decrease the presence of intestinal pathogens and change the production of health related bacterial metabolites (Manning and Gibson, 2004; Ringø et al., 2010). Probiotics have been evaluated in a wide range of invertebrates (Gomez-Gil et al., 2000; Irianto and Austin, 2002; Krishnaprakash et al., 2009). In one approach, bacteria isolated from the gut of *H. midae* were able to assist in the digestion of seaweed *Ecklonia maxima* or *Gracilaria verrucosa* (Erasmus et al., 1997). The growth and survival rate of *H. midae,* fed supplemented feed with two yeasts (strains SS1 and AY1) and one bacterial strain (SY9) were increased (Macey and Coyne, 2005), while supplementation of a combination of three probiotic species (the 3-probiotic conglomerate consisted of *Enterococcus* JHLDc in addition to *Exiguobacterium* JHEb1 and *Vibrio* JH1.) isolated from gut of abalone into the commercial feed of farmed New Zealand abalone, *Haliotis iris*, also improved growth and survival rate (Hadi et al., 2014). Similarly, Ten Doeschate and Coyne (2008) reported an increase in the growth rate of farmed *H. midae* fed a kelp diet supplemented with *Pseudoalteromonas* sp. strain C4 due to improvement in the nutritional status and digestibility of the feed.

Another approach would be to use probiotics that are readily available, rather than derived specifically from the host animal. Among probiotic products, the spores

of *Bacillus* spp. are easy to introduce in dry food, improve water quality, survival and growth rates and increased the health status of some species such as *Litopenaeus vannamei* and *Penaeus monodon* (Dalmin et al., 2001; Li et al., 2009; Rengpipat et al., 2000). In addition, the spores of *Bacillus* spp. Also have the capacity to resist extreme pH conditions, high temperatures, solvents and long-term storage without refrigeration (Olmos and Paniagua-Michel, 2014). The prebiotic mannanoligosaccharide (MOS), derived from yeast (*Saccharomyces cerevisiae*) cell walls, helps support the growth of beneficial gut microflora and has positive effects on immune responses, survival and growth rates in a range of aquatic animals (Ringø et al., 2010). Therefore, supplementation of those above probiotics or prebiotics may improve the activity of gastro-intestinal microbiota and enhance immune status, disease resistance, survival, feed utilisation and growth performance of greenlip abalone at high temperature, resulting in an improvement of the energy balance upon exposure to elevated temperatures.

Bioenergetics is a study of the transformation of energy in living organisms by understanding the balance between ingested food energy and energy use (Jobling, 1993). Since ingested feed energy, somatic growth energy and respiration energy are the main components of the energy budget, changing temperature can also directly affect directly the energy budgets of animals. Thus, measuring those parameters of the energy budget can provide an understanding of ingested food energy, absorbed energy, somatic growth energy and waste production energy during thermal stress. Since supplemented pre/probiotics have positive effects on abalone health, gastro-intestinal microbiota, feed utilisation and growth performance, it was hypothesised that pre/probiotics additive would help to improve aborption energy and energy for somatic growth.

As a part of a summer mortality project on abalone, aimed at improving abalone health, survival and growth during the summer period, this study was carried out to investigate the effects of pro/prebiotic supplementation in formulated abalone diets on each component of their energy budget at optimal and high water temperatures.

5.3 Materials and methods

5.3.1 Experimental animals

Three-year-old greenlip abalone (weight: 81.04 ± 0.11 g, n = 60; shell length: 81.47 ± 0.08 mm, n = 60) were obtained from South Australian Mariculture (Boston Point, Port Lincoln, South Australia). Upon arrival at the South Australian Research and Development Institute (SARDI), the abalone were transferred to 500 L flow through tanks supplied with aeration and seawater at ambient photoperiod and water temperatures and fed a 5-mm commercial Abgrow diet chips (Eyre Peninsula Aquafeed Pty Ltd [EPA], Lonsdale, South Australia, Australia) for one month prior to the experiment.

5.3.2 Experimental design and system

The study used the temperature challenge method developed by Stone et al. (2014b) to investigate the effects of the inclusion of pro/prebiotic products on each parameter of the energy budget of greenlip abalone at 22 or 25 °C water temperature. Abalone fed the basal diet (Abgrow diet mash) without dietary supplements at 22 or 25 °C served as the control.

The experiment used two identical water temperature controlled systems (22 and 25 °C) previously described in Stone et al. (2013) with 30-µm sand-filtered, and UV treated seawater. The systems consisted of 60 blue plastic experimental tanks (12.5 L, Nally IH305, Viscount Plastics Pty Ltd.; length, 39.2 cm; width, 28.8 cm; depth, 11.0 cm; and bottom surface area, 1129 cm^2), with a water depth of 5 cm

controlled by a standpipe resulting in a tank water volume of 5.4 L with a mesh screen (nominal mesh size, 0.8 mm) at the outlet to retain uneaten food. Since incoming water was 13.8 °C during experimental period, to achieve and maintain water temperature at 22 °C or 25 °C, 3-kW immersion heaters (240V, 3kw; JQ20, Austin & Cridland, Carlton, NSW, Australia) were used, while room temperature was adjusted to 24 °C. The experimental facility consisted of two identical temperature-controlled (22 °C and 25 °C) saltwater systems supplied with flow-through UV-treated seawater (model 025120-2, 120W; Emperor Aquatics, Pottstown, PA). Each system was comprised of a 780-L sump, 780-L intermediate tank, and 780-L header tank (Solid Nally MegaBins, MS7800; Viscount Plastics Pty Ltd., Hawthorn East, Vic., Australia).

5.3.3 Experimental stocking

Six hundred abalone were removed from the holding tank using a spatula. They were weighed (wet weight, mean ± SE, 81.04 ± 0.11 g, n = 600), measured (shell length, 81.47 ± 0.08 mm, n = 600) and randomly assigned among 60 tanks (ten animals per tank and three replicate tanks per treatment combination). The experiment ran for a total of 52 days, which excluded a 2-week acclimation period to slowly raise the water temperature from 13.8 °C (~1 °C d^{-1}) to the desired treatment temperatures of 22 and 25 °C, followed by a 38-day temperature challenge period. For each treatment, the size and weight of dead abalone were measured and in an attempt to keep stocking densities equal, replaced with tagged abalone of a similar weight and size that had been held at the same water temperature and fed their respective diets.

5.3.4 Diets, feeding and water management

Eyre Peninsula Aquafeeds provided the Abgrow diet mash. The Pro 1 (probiotic *Bacillus nato* 5.2 ± 0.5 × 10^{10} spores mL^{-1}, Agricure Pty. Ltd. Australia);

Pro 2 (probiotic *Bacillus subtilis* and *Bacillus licheniformis* $\times 10^9$ spores g^{-1}, Platypus In-Feed Concentrate, supplied by IAH Sales Pty. Ltd., Australia); and Pre [Prebiotic SafMannan yeast cell wall extract MOS (≥ 18 %), β Glucan (β 1,3 & 1,6) (≥ 20%), supplied by Leasaffre Feed Additive, France] were added into Abgrow diet mash at different levels. Therefore, there were ten diets used in this experiment: Diet 1) a basal diet (formulated from the Abgrow diet mash); Diet 2) 0.1% Pro 2; Diet 3) 0.2% Pro 2; Diet 4) 0.4% Pro 2; Diet 5) 0.1% Pro 1; Diet 6) 0.5% Pro 1; Diet 7) 0.1% Pre; Diet 8) 0.2% Pre; Diet 9) the mixed 0.1% Pro 1 + 0.1% Pre and Diet 10) the mixed 0.5% Pro 1 + 0.2% Pre.

Biochemical analyses on crude protein, crude lipid, gross energy, moisture and ash of the basal diet were analysed according to the methods of the AOAC (1995) and presented in Table 5.1.

To manufacture the diets, the proportions of pro/prebiotics were included as ingredients into dry Abgrow mash by removing a similar proportion of Abgrow mash. Both Abgrow mash and pro/prebiotic were in powder forms. The Ytterbium, Yttrium (supplied by IAH Sales Pty. Ltd., Australia) and fish oil inclusions were added in all diets at 0.02, 0.02 and 1.5%, respectively. All ingredients were mixed in a Hobart mixer (Hobart Corp., Troy, OH, USA) for 5 min. Water (~ 30%) and the sodium alginate binder (0.36%) was then added to the diet mash and mixed for further 3 min. The diets were cold pelleted using a TR110 pasta machine (MacchinePer Pasta SRL, Molina Di Malo, VI, Italy) to produce a 5 mm flat sinking pellet. Diets were then dried at 50 ºC for approximately 48 h until the diets were less than 10% moisture and stored at room temperature. Although there is little information about the commercial dietary mash (basal diet) which was supplied by the feed company due to confidentiality, the actual dietary nutrient and energy data

was still calculated so significant differences between other experimental diets were absolute.

Abalone were fed to excess (0.6% body weight (bw d^{-1}) at 16:00 daily. Cleaning and collection of uneaten food was undertaken at 08:30 daily by straining the entire tank contents through a fine mesh (500 µm). The uneaten feed was weighed, stored frozen at -20 °C and then was dried in an oven at 105 °C for 16 h. The proportion of uneaten feed that was lost through leaching was estimated in a tank without animals and the correction factor was used to calculate the apparent feed intake. Feed intake was determined and used to calculate ingested food energy.

The water quality measurement was previously described in Chapter 2 (2.3.2).

Table 5.1. Analysed nutrient composition of Abgrow diet mash.

Nutrition composition	The Abgrow diet
Moisture (%)	11.5
Ash (%)	6.90
Crude protein (%)	32.07
Crude lipid (%)	4.50
Crude carbohydrate (%)*	45.03
Gross energy (MJ kg^{-1})	14.77

* Carbohydrate was calculated by difference [carbohydrate % = 100% - (protein % + lipid % + ash % + moisture %]

5.3.5 Determination of the components of the energy budget

Energy budgets were calculated for greenlip abalone by measuring each component of the energy budget in the equation as described in Chapter 2 (2.3.3). Three animals were collected for bioenegetics study in this experiment due to the sufficiently large size of the animals.

5.3.5.1 Ingested food energy

The determination of ingested food energy was described in Chapter 2 (2.3.3.1).

5.3.5.2 Somatic growth energy and reproduction energy

The determination of somatic growth energy was described in Chapter 2 (2.3.3.2). The final weights of greenlip abalone (mean ± SE) at in 22 °C were 82.7 ± 1.12g (n = 3) in Diet 1; 82.0 ± 0.35g (n = 3) in Diet 2; 84.6 ± 0.17g (n = 3) in Diet 3; 83.9± 0.88g (n = 3) in Diet 4; 83.6 ± 1.65g (n = 3) in Diet 5; 82.7 ± 1.27g (n = 3) in Diet 6; 83.5 ± 1.64g (n = 3) in Diet 7; 83.0 ± 0.44g (n = 3) in Diet 8; 85.3 ± 1.11g (n = 3) in Diet 9 and 84.5 ± 0.34g (n = 3) in Diet 10.

At in 25°C, the final weights of greenlip abalone (mean ± SE) were 76.2 ± 3.52g (n = 3) in Diet 1; 75.8 ± 2.32g (n = 3) in Diet 2; 80.8 ± 0.96g (n = 3) in Diet 3; 78.3 ± 0.76g (n = 3) in Diet 4; 76.3 ± 1.68g (n = 3) in Diet 5; 77.2 ± 1.65g (n = 3) in Diet 6; 76.7 ± 1.45g (n = 3) in Diet 7; 79.3 ± 1.13g (n = 3) in Diet 8; 75.7 ± 0.36g (n = 3) in Diet 9 and 78.4 ± 1.40g (n = 3) in Diet 10.

Abalone with gonad development were collected and disected to extract the gonad. The gonad gland from each treatment was freeze dried for 48 h to constant mass and then combusted in a microbomb calorimeter to determine the energy content (Lopez and Tyler, 2006).

Reproduction energy (J g abalone^{-1} h^{-1}) = [(energy content of gonad × gonad weight) / (initial weight + final weight)/ 2]/ time.

5.3.5.3 Respiration energy

Oxygen consumption was measured both in the morning (at 9:00 h) and night (at 22:00 h) because greenlip abalone show a circadian rhythm (Buss et al., 2015). The respiratory rate was determined by the difference between the initial and final oxygen levels measured over a 0.5 h period of incubation of three animals in a sealed respiratory chamber beginning at full oxygen saturation. This was completed at the end of the growth experiment. Three abalone of each treatment were introduced into a 5 L chamber (n = 3, 5 L chambers per treatment) and supplied with controlled seawater at 22 °C or 25 °C for three days to reduce the effects of handling on oxygen consumption. For the first two days, abalone were fed (1% body weight) then deprived of food on the third day. At 9:00 h or 22:00 h on the fourth day, initial disolved oxygen concentrations in water in each chamber were measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). Subsequently, the water supply was cut off for 0.5 h of incubation. Oxygen levels in the chambers were always above 70% oxygen saturation during the incubation, to avoid hypoxia depressing their metabolic rates (Harris et al., 1999b). At the end of the 0.5 h incubation period, the chambers were carefully opened and the final dissolved oxygen concentration in water was measured in each chamber. Three abalone in each chamber were weighed and measured. Oxygen levels in four control chambers with no abalone were also determined to account for background biological oxygen consumption.

Oxygen uptake rate (mg g abalone^{-1} h^{-1}) = [(initial levels of oxygen in the chamber - final levels of oxygen in the chamber) × volume of the chamber] / (biomass × time)

The oxygen used for respiration was then transformed to energy equivalents by multiplying by 14.77 J mg O_2^{-1} for carbohydrate respiration, 13.72 J mg O_2^{-1} for

lipid respiration and 13.39 J mg O_2^{-1} for protein respiration (Elliott and Davison, 1975).

5.3.5.4 Ammonia excretion energy

The determination of ammonia excretion energy was described in Chapter 2 (2.3.3.4).

5.3.5.5 Egested faecal energy

Throughout the experiment, faecal material from each tank was collected daily at 12:00 h using a pasteur pipette and then dispensing the faeces onto fine mesh to remove water. The faeces was then scraped off the mesh and transferred to a 70 mL container and stored at - 20 °C. At the end of experiment, samples were freeze dried for 48 h to constant mass. Samples were then combusted in the microbomb calorimeter to determine energy content. The apparent digestibility coefficient (ADC) was also analysed in the freeze dried faecal material by using the soil chemical methods to analyse concentration of Ytterbium in feed and faeces (Rayment and Lyons, 2010).

The apparent digestible energy (%) = $100 \times [1 - (F/D \times D_{marker}/F_{marker})]$, where F is the percent of nutrient or energy in faeces; D is the percent of nutrient or energy in diet; D_{marker} is the percent of Ytterbium in diet; and F_{marker} is the percent of Ytterbium in faeces (Cho and Kaushik, 1990).

Absorbed energy (J g abalone^{-1} h^{-1}) = Ingested food energy × Apparent digestible energy

Egestion faecal energy (J g abalone^{-1} h^{-1}) = Ingested food energy - Absorbed energy

5.3.5.6 Mucus production energy and shell growth energy

This experiment was a part of the summer mortality project which had been carried out to improve health and reduce mortality in abalone cultured at high water temperatures. At the end of experiment, we were not able to collect enough mucus for each dietary treatment to analyse the energy content due to two reasons: 1) high mortality (up to 50%) at 25 °C; 2) the surviving experimental animals per tank were also shared between another immune study (Currie et al., 2015a) and the current bioenergetic study. Thus, in order to obtain necessary quantity of mucus, ten abalone of a similar size were introduced into each of four replicate 5-L containers for each temperature, that had been previously weighed and immersed in a tank supplied with fresh seawater. Abalone were carefully removed after 6 h and the containers were rinsed using distilled water to remove faeces and seawater. The containers were then dried at 70 °C for 1 h, reweighed and mucus production was calculated by subtraction. The dried mucus was carefully scraped from the plates and its energy content was finally analysed using combustion in a microbomb calorimeter (Lopez and Tyler, 2006).

The calculation of mucus production energy was described in Chapter 2 (2.3.3.5). The mucus value was assumed to be similar among of abalone fed different diets at 25 °C and its proportion in energy budget was calculated as percentage of ingested food energy.

Shell growth energy was described in Chapter 2 (2.3.3.6).

5.3.6 Statistical analysis

To assess the effects of diet type (prebiotic and probiotic) and water temperature (22 and 25 °C) on each parameter of the energy budget for greenlip abalone, the data was analysed using the statistical program IBM SPSS (Version 22

for Windows; IBM SPSS Inc., Chicago, IL, USA). In order to ensure normal distribution, the data was transformed where appropriate. Levene's test for equality of variance was used to assess the homogeneity of the variance among means prior to running the ANOVA and the post-hoc comparisons. When significant main effects were observed, Tukey's HSD post-hoc test was used to detect significant differences between treatment means. When significant interactions between diet type and water temperature were observed, differences in diet type are compared within 22 °C or 25 °C (one-factor ANOVA; Tukey's HSD test). The Independent-Samples T Test was used to compare mucus production energy of the abalone cultured at 22 and 25 °C. A significance level of $P < 0.05$ was used for all statistical tests.

5.4 Results

5.4.1 General observation and water quality

The survival rate was 98% at 22 °C or from 50 to 90% at 25 °C (Currie et al., 2015a). Over the course of the experiment, greenlip abalone were more active and exhibited normal feeding behaviour at 22 °C compared to those at 25 °C, which exhibited less movement, released mucus from the foot, displayed limited attachment to the tank substrate, and often detached and inverted before mortality occurred.

In the 22 °C treatment, the water temperature (mean ± SE, n = 39) was 21.8 ± 0.04 °C; dissolved oxygen 82.6 ± 0.32% saturation or 5.9 ± 0.03 mg L^{-1}, pH 8.08 ± 0.01 and salinity 36.0 ± 0.01 ppt. In the 25 °C treatment, water temperature was 24.8 ± 0.05 °C, dissolved oxygen 84.2 ± 0.53% saturation or 5.8 ± 0.03 mg L^{-1}, pH 8.09 ± 0.01 and salinity 36.0 ± 0.01 ppt.

5.4.2 Ingested food energy

The components of the energy budget in greenlip abalone fed different diets at 22 °C and 25 °C are shown in Table 5.2. The ingested food energy was significantly affected by water temperature ($P < 0.001$; two-factor ANOVA; Table 2), but not by

feed (P = 0.694) or the interaction between these two factors (P = 0.715). Abalone cultured at 25 °C had significantly lower ingested food enrgy (1.12 J g abalone^{-1} h^{-1}) than those at 22 °C (1.96 J g abalone^{-1} h^{-1}).

5.4.3 Absorbed energy

Absorbed energy was significantly influenced by water temperature (P < 0.001; two-factor ANOVA; Table 5.2), but not by diet type (P = 0.580) or the interaction between these two factors (P = 0.700). Absorbed energy of abalone cultured at 25 °C (0.55 J g abalone^{-1} h^{-1}) was significantly lower than those at 22 °C (1.13 J g abalone^{-1} h^{-1}).

5.4.4 Somatic growth energy and reproduction energy

Somatic growth energy of the abalone was significantly influenced by temperature (P < 0.001; two-factor ANOVA; Table 5.2), but not by diet type (P = 0.583) or the interaction between these two factors (P = 0.809). Abalone had available energy for somatic growth and gonad development at 22 °C, while they had no somatic growth energy at 25 °C and showed no sign of gonad development.

5.4.5 Respiration energy

Respiration energy was not significantly influenced by diet type (P = 0.877; two-factor ANOVA; Table 5.2), water temperature (P = 0.872) or the interaction between these two factors (P = 0.442). Respiration energy of greenlip abalone was 0.37 J g abalone^{-1} h^{-1} at 25 °C and 0.38 °C J g abalone^{-1} h^{-1} at 22 °C.

5.4.6 Ammonia excretion energy

Ammonia excretion energy was significantly affected by water temperature (P = 0.013; two-factor ANOVA; Table 5.2), but not by diet type (P = 0.765) or the interaction between these two factors (P = 0.387). Ammonia excretion energy of the abalone at 25 °C (0.019 J g abalone^{-1} h^{-1}) was significantly higher than those at 22 °C (0.013 J g abalone^{-1} h^{-1}).

5.4.7 Egested faecal energy

Egested faecal energy was significantly influenced by water temperature ($P < 0.001$; two-factor ANOVA; Table 5.2), but not by diet type ($P = 0.469$) or the interaction of these two factors ($P = 0.265$). Abalone cultured at 25 °C expended significantly less energy (0.57 J g abalone^{-1} h^{-1}) through faecal egestion than those at 22 °C (0.83 J g abalone^{-1} h^{-1}).

5.4.8 Pedal mucus production energy and shell growth energy

Pedal mucus production energy was significantly influenced by temperature ($P = 0.037$; Independent-Samples T Test). Abalone lost significantly more energy through pedal mucus production at 25 °C (0.10 J g abalone^{-1} h^{-1}) than those at 22 °C (0.07 J g abalone^{-1} h^{-1}).

Shell growth energy of abalone was not affected by temperature, diet or their interaction ($P > 0.05$; one-factor ANOVA; Table 5.2). Shell growth energy ranged from 0.001 - 0.007 (J g abalone^{-1} h^{-1}) across the treaments.

Table 5.2 Two-factor ANOVA results for energy components of abalone fed experimental diets [1]

Temp	Diet [2]	Energy components (J g abalone^{-1} h^{-1})								
		I	Ab	Pg	Pr	R	U	F	M	S
22 ℃	1	1.89 ± 0.05	1.15 ± 0.04	0.37 ± 0.08	0.043 ± 0.009	0.43 ± 0.08	0.012 ± 0.004	0.74 ± 0.01	0.07 ± 0.01	0.004 ± 0.001
	2	1.87 ± 0.09	0.94 ± 0.06	0.40 ± 0.09	0.039 ± 0.009	0.26 ± 0.06	0.015 ± 0.003	0.92 ± 0.04	0.07 ± 0.01	0.007± 0.001
	3	1.92 ± 0.15	1.14 ± 0.09	0.54 ± 0.11	0.049 ± 0.009	0.39 ± 0.02	0.012 ± 0.004	0.78 ± 0.06	0.07 ± 0.01	0.005 ± 0.002
	4	2.02 ± 0.11	1.16 ± 0.14	0.50 ± 0.07	0.048 ± 0.007	0.36 ± 0.04	0.013 ± 0.002	0.85 ± 0.06	0.07 ± 0.01	0.005 ± 0.000
	5	2.10 ± 0.08	1.17 ± 0.02	0.58 ± 0.04	0.061 ± 0.004	0.38 ± 0.02	0.009 ± 0.001	0.93 ± 0.07	0.07 ± 0.01	0.006 ± 0.002
	6	2.02 ± 0.09	1.12 ± 0.05	0.43 ± 0.09	0.044 ± 0.009	0.42 ± 0.02	0.010 ± 0.001	0.89 ± 0.04	0.07 ± 0.01	0.003 ± 0.001
	7	1.92 ± 0.05	1.12 ± 0.08	0.50 ± 0.04	0.051 ± 0.005	0.43 ± 0.01	0.016 ± 0.004	0.81 ± 0.04	0.07 ± 0.01	0.004 ± 0.001
	8	1.95 ± 0.05	1.12 ± 0.07	0.49 ± 0.11	0.047 ± 0.010	0.29 ± 0.07	0.014 ± 0.003	0.83 ± 0.06	0.07 ± 0.01	0.003 ± 0.001
	9	2.00 ± 0.12	1.23 ± 0.09	0.46 ± 0.09	0.048 ± 0.009	0.39 ± 0.04	0.015 ± 0.005	0.77 ± 0.03	0.07 ± 0.01	0.005 ± 0.002
	10	1.93 ± 0.03	1.12 ± 0.07	0.53 ± 0.04	0.053 ± 0.004	0.35 ± 0.07	0.012 ± 0.004	0.81 ± 0.04	0.07 ± 0.01	0.003 ± 0.000
25 ℃	1	1.14 ± 0.04	0.49 ± 0.17 [(2)]	- 0.12 ± 0.01	-	0.28 ± 0.00 [(1)]	0.026 ± 0.000 [(1)]	0.68 ± 0.13 [(2)]	0.10 ± 0.01	0.004 ± 0.001
	2	1.05 ± 0.13	0.50 ± 0.13 [(2)]	- 0.11 ± 0.04	-	0.35 ± 0.00 [(1)]	0.015 ± 0.000 [(1)]	0.50 ± 0.08 [(2)]	0.10 ± 0.01	0.002 ± 0.002
	3	1.12 ± 0.04	0.61 ± 0.05	- 0.08 ± 0.03	-	0.31 ± 0.05 [(2)]	0.012 ± 0.003 [(2)]	0.50 ± 0.08	0.10 ± 0.01	0.007 ± 0.002
	4	1.05 ± 0.02	0.47 ± 0.07	- 0.10 ± 0.01	-	0.30 ± 0.00 [(1)]	0.021 ± 0.000 [(1)]	0.58 ± 0.09	0.10 ± 0.01	0.003 ± 0.001
	5	1.04 ± 0.08	0.40 ± 0.01	- 0.10 ± 0.05	-	0.28 ± 0.01 [(2)]	0.020 ± 0.009 [(2)]	0.64 ± 0.06	0.10 ± 0.01	0.002 ± 0.001
	6	1.17 ± 0.10	0.61 ± 0.11	- 0.16 ± 0.04	-	0.46 ± 0.00 [(1)]	0.020 ± 0.000 [(1)]	0.55 ± 0.03	0.10 ± 0.01	0.003 ± 0.001
	7	1.09 ± 0.06	0.58 ± 0.12	- 0.14 ± 0.02	-	0.38 ± 0.05	0.015 ± 0.002	0.51 ± 0.13	0.10 ± 0.01	0.005 ± 0.002
	8	1.11 ± 0.08	0.55 ± 0.09	- 0.07 ± 0.02	-	0.32 ± 0.10 [(2)]	0.024 ± 0.009 [(2)]	0.56 ± 0.05	0.10 ± 0.01	0.005 ± 0.002
	9	1.29 ± 0.10	0.61 ± 0.06	- 0.11 ± 0.04	-	0.39 ± 0.03 [(2)]	0.010 ± 0.001 [(2)]	0.68 ± 0.05	0.10 ± 0.01	0.001 ± 0.001
	10	1.14 ± 0.09	0.65 ± 0.06	- 0.16 ± 0.04	-	0.44 ± 0.03 [(2)]	0.023 ± 0.010 [(2)]	0.49 ± 0.05	0.10 ± 0.01	0.006 ± 0.001
ANOVA	Diet (A)	0.694	0.580	0.583	0.791	0.558	0.765	0.469	-	0.112
(*P* value)[3]	Temp (B)	< 0.001	< 0.001	< 0.001	-	0.478	0.013	< 0.001	0.037	0.999
	A x B	0.715	0.700	0.809	-	0.643	0.387	0.265	-	0.318

[1] Data is presented as mean +/- SE, n = 3 with the exception of variances [(2)] as n= 2 and [(1)] as n= 1 due to high mortality rates prior to sampling.

[2] Diet 1) a basal diet (the Abgrow diet mash); Diet 2) 0.1% Pro 2; Diet 3) 0.2% Pro 2; Diet 4) 0.4% Pro 2; Diet 5) 0.1% Pro 1; Diet 6) 0.5% Pro 1; Diet 7) 0.1% Pre; Diet 8) 0.2% Pre; Diet 9) the mixed 0.1% Pro 1 + 0.1% Pre and Diet 10) the mixed 0.5% Pro 1 + 0.2% Pre.

[3] Where significant main effects were detected, post-hoc tests were used to determine differences between means (Tukey's HSD test; $P < 0.05$). - : denotes unavailable data as abalone showed not visible signs on gonad development.

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy.

5.4.9 Energy budgets of abalone

Energy budgets for the greenlip abalone fed different dietary prebiotic and probiotic supplement at different temperatures are shown in Table 5.3. Absorbed energy was lower at high temperature (37.3 - 57.6% of energy ingestion at 25 °C compared to 50.5 - 61.4% at 22 °C). A major portion of the ingested food energy was used for egested faecal particles at both water temperatures (38.6 - 49.5% at 22 °C or 42.4 - 62.4% at 25 °C). Somatic growth energy accounted for 19.6 - 27.8% of the ingested food energy at 22 °C, but abalone did not have available energy for somatic growth at 25 °C. Reproduction energy was only found at 22 °C, ranging 2.03 - 2.90 % energy ingestion. Respiration energy was lower at 22 °C (14.2 - 22.9%) than at 25 °C (24.8 - 37.5%). Pedal mucus production energy was 3.32 - 3.63% at 22 °C, and increased to 7.97 - 10.0% at 25 °C. Ammonia excretion energy accounted for only a small proportion of ingested food energy, ranging 0.40 - 0.83% at 22 °C and 0.75 - 2.30% at 25 °C. Shell growth energy accounted for the smallest proportion of the ingested food energy, ranging from 0.14 to 0.29% at 22 °C or 0.12 - 0.44% at 25 °C.

Table 5.3 Energy budgets of abalone fed experimental diets [1]

Temp	Diet	Energy budgets (%)									
		I	Ab	Pg	Pr	R	U	E	M	S	Unexplained
22 °C	1	100	60.7 ± 0.60	19.6 ± 3.46	2.27 ± 0.38	22.9 ± 4.48	0.63 ± 0.23	39.3 ± 0.58	3.57 ± 0.09	0.23 ± 0.05	11.5
	2	100	50.5 ± 1.26	21.4± 3.81	2.03± 0.35	14.2 ± 2.92	0.83 ± 0.18	49.5 ± 1.26	3.63 ± 0.18	0.14 ± 0.02	8.3
	3	100	59.4 ± 0.52	27.8 ± 3.79	2.50 ± 0.35	20.5 ± 2.44	0.63 ± 0.20	40.6 ± 0.52	3.57 ± 0.26	0.25 ± 0.08	4.2
	4	100	57.4 ± 4.13	24.7 ± 2.38	2.37 ± 0.24	18.1 ± 2.40	0.67 ± 0.15	42.6 ± 4.13	3.33 ± 0.18	0.26 ± 0.01	8.0
	5	100	55.5 ± 1.63	27.8 ± 1.06	2.90 ± 0.12	18.1 ± 0.75	0.40 ± 0.06	44.4 ± 1.61	3.23 ± 0.13	0.29 ± 0.09	2.9
	6	100	55.7 ± 0.57	21.5 ± 4.25	2.17 ± 0.43	20.8 ± 0.32	0.53 ± 0.07	44.2 ± 0.57	3.37 ± 0.15	0.14 ± 0.04	7.3
	7	100	58.1 ± 2.64	25.9 ± 1.67	2.63 ± 0.19	22.6 ± 0.10	0.83 ± 0.18	41.9 ± 2.64	3.53 ± 0.09	0.21 ± 0.02	2.4
	8	100	57.3 ± 3.10	24.8 ± 5.00	2.40 ± 0.47	14.9 ± 3.35	0.73 ± 0.12	42.7 ± 3.10	3.47 ± 0.12	0.15 ± 0.05	10.9
	9	100	61.4 ± 0.85	23.3 ± 5.18	2.43 ± 0.52	19.4 ± 1.82	0.70 ± 0.21	38.6 ± 0.85	3.40 ± 0.21	0.25 ± 0.06	11.9
	10	100	58.2 ± 2.49	27.5 ± 2.38	2.77 ± 0.23	18.1 ± 3.64	0.60 ± 0.21	41.8 ± 2.49	3.50 ± 0.06	0.14 ± 0.02	5.6
25 °C	1	100	41.5 ± 12.5**	- 10.6 ± 2.60	-	24.8 ± 0.00 (1)	2.30 ± 0.00 (1)	58.4 ± 12.8 (2)	8.93 ± 0.29	0.23 ± 0.17	15.9
	2	100	49.0 ± 2.00**	- 11.0 ± 1.60	-	29.5 ± 0.00 (1)	1.20 ± 0.00 (1)	50.9 ± 2.29 (2)	10.0 ± 1.45	0.12 ± 0.07	19.3
	3	100	55.3 ± 5.93	- 6.80 ± 3.00	-	28.8 ± 1.35 (2)	1.10 ± 0.02 (2)	44.9 ± 6.09	9.10 ± 0.32	0.44 ± 0.13	22.5
	4	100	45.3 ± 7.62	- 9.40 ± 0.06	-	28.9 ± 0.00 (1)	2.10 ± 0.00 (1)	54.8 ± 7.49	9.67 ± 0.19	0.30 ± 0.04	13.6
	5	100	37.3 ± 7.13	- 9.70 ± 4.20	-	25.1 ± 0.19 (2)	1.80 ± 0.08 (2)	62.4 ± 7.09	9.87 ± 0.82	0.25 ± 0.06	10.3
	6	100	52.0 ± 5.29	- 13.4 ± 2.30	-	36.2 ± 0.00 (1)	1.50 ± 0.00 (1)	48.2 ± 5.42	8.80 ± 0.80	0.22 ± 0.07	18.5
	7	100	53.6 ± 11.2	- 12.6 ± 1.30	-	34.9 ± 2.43	1.33 ± 0.13	46.4 ± 11.2	9.33 ± 0.52	0.32 ± 0.09	20.3
	8	100	49.0 ± 4.93	- 6.10 ± 1.60	-	29.8 ± 4.40 (2)	2.30 ± 0.11 (2)	51.1 ± 4.89	9.20 ± 0.64	0.30 ± 0.09	13.4
	9	100	47.4 ± 0.69	- 8.40 ± 2.80	-	31.9 ± 7.05 (2)	0.75 ± 0.25 (2)	52.6 ± 0.69	7.97 ± 0.72	0.18 ± 0.05	15.0
	10	100	57.6 ± 3.01	- 13.6 ± 2.50	-	37.5 ± 1.15 (2)	1.95 ± 0.06 (2)	42.4 ± 3.01	8.97 ± 0.67	0.34 ± 0.09	22.4

[1] Data is presented as mean +/- SE, n = 3 with the exception of variances (2) as n = 2 and (1) as n = 1 due to high mortality rates prior to sampling.

[2] Diet 1) a basal diet (the Abgrow diet mash); Diet 2) 0.1% Pro 2; Diet 3) 0.2% Pro 2; Diet 4) 0.4% Pro 2; Diet 5) 0.1% Pro 1; Diet 6) 0.5% Pro 1; Diet 7) 0.1% Pre; Diet 8) 0.2% Pre; Diet 9) the mixed 0.1% Pro 1 + 0.1% Pre and Diet 10) the mixed 0.5% Pro 1 + 0.2% Pre. - : denotes unavailable data as abalone showed not visible signs on gonad development.

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy. Absorbed energy, somatic growth energy, respiration energy, ammonia excretion energy, pedal mucus production energy and shell growth energy calculated as a percentage of ingested food energy. Egested faecal energy obtained from 100% ingested food energy minus absorbed energy. Unexplained was calculated as 100% - (Pg + Pr + R + U + M + S + E)

5.5 Discussion

Land-based farmed greenlip abalone are significantly impacted by seasonally fluctuating water temperatures (12 - 26 °C) (Stone et al., 2014a). High mortality of 3-year-old abalone (> 60 mm SL) has been reported during summer months when water temperatures exceed 22 °C (Lange et al., 2014; Stone et al., 2013). The purpose of this study was to evaluate the effects of the supplementation of pro/prebiotic products on the energy budget of greenlip abalone at high water temperatures. The results of study show that pro/prebiotic additives tested did not influence any components of the energy budget of greenlip abalone. However, water temperature had a significant impact on the energy budget.

Food ingestion and the digestion rate of abalone normally increase with increasing temperature up to certain limits and show a rapid reduction during thermal stress (Barkai and Griffiths, 1987; Currie et al. 2015b; Lange et al., 2014; Lopez and Tyler, 2006; Stone et al., 2014a, b). Our results support previous studies on greenlip abalone by Lange et al. (2014) and Stone et al. (2014b) in that ingested food energy of greenlip abalone decreases as water temperature increased from 22 to 25 °C. At the digestive level, the rate of transit of feed through the digestive compartments, digestibility of major nutrients and the activities of digestive enzymes are all affected by water temperature (Bansemer et al., 2016a; Currie et al. 2015b; Kaushik, 1986). In general, the longer transit time through the digestive tract, should result in a higher absorption of feed nutrients (Taghon et al., 1978). However, a reduction in enzyme activity with declining temperatures may conversely slow the absorption process. As water temperature increases, digestive efficiency decreases due to reduction of food retention time in the gut, decrease in duration of contact between feed nutrients and the digestive enzymes and less time for absorption, thus impinging on nutrient digestibility (Brett and Higgs, 1970; Kaushik, 1986; McGaw and Whiteley, 2012). It

was initially expected that supplementation of pro/prebiotics in the formulated diet of greenlip abalone could aid digestive processes due to improvement in the intestinal microbial balance, higher activity of digestive enzymes and enhancement of food absorption in the gut at elevated water temperature. However, additive diets did not improve absorbed energy at 22 or 25 °C.

Bacillus bacteria were chosen in this study due to a number of advantages including having positive effects on food digestion and assimilation, water quality, survival, growth rates and increasing the health status of some aquacultured species (Dalmin et al., 2001; Li et al., 2009; Rengpipat et al., 2000). The supplementation of the commercial probiotic contained spores of five species of *Bacillus* in a formulated diet. This improved specific activities of amylase, total protease, and lipase in the Indian white shrimp, *Fenneropenaeus indicus*. Consequently, both survival and growth of the shrimp were significantly higher than those fed the control diet (Ziaei-Nejad et al., 2006). In abalone, microorganisms (one bacterial SY9 and two yeast strains SS1 and AY1) isolated from the gastrointestinal tract of *H. midae* increased the growth rate of abalone due to increases in protein digestion and absorption within the intestine (Macey and Coyne, 2005). Incorporating the probiotic bacteria with specific enzymatic abilities adds to the pool of digestive enzymes leading to improvements in digestion and absorption of nutrients in New Zealand abalone (Hadi et al., 2014). Alginate lyase activity in the *H. midae* digestive tract was significantly higher in abalone fed kelp supplemented with *Pseudoalteromonas* sp. (Ten Doeschate and Coyne, 2008). However, the addition of two commercial probiotic products (*Bacillus subtilis* and *Bacillus licheniformis* or *Bacillus nato*) did not influence survival (Currie et al., 2015a) or somatic growth energy of greenlip abalone at 25 °C or even at 22 °C which have been reported as appropriate water temperatures for growth of greenlip abalone in the current study. It is possible that

the tested commercial pro/prebiotic products were not suitable candidates for greenlip abalone.

Most studies on probiotic supplementation in the diet investigated the identification of potential probiotic bacteria in the gut of abalone to produce probiotic products (Erasmus et al., 1997; Hadi et al., 2014; Macey and Coyne, 2005). For example, some *Vibrio* sp. or *Pseudoalteromonas* sp. in abalone gut can provide vital contributions to abalone digestive process (Macey and Coyne, 2005, 2006; Sawabe et al., 2003; Ten Doeschaten and Coyne, 2008). Thus, these isolated pro/prebiotics from abalone may have more effectiveness than commercial products. However, there may be beneficial effects to the addition of those available commercial pro and prebiotics on other parameters such as immune function and gut microbiota not reported in the present work. Future research is recommended to identify and isolate potential probiotic bacterial in greenlip abalone gut for probiotic treatment.

Positive energy balance occurs when metabolised energy intake exceeds expenditure, resulting in available energy for somatic growth. Conversely, negative energy balance occurs when the metabolic demand for energy frequently exceeds supply, causing growth to cease (Jobling, 1993). In the present study, it is possible that due to the high-energy expenditure, but less feed ingestion due to stress, greenlip abalone did not have available energy for somatic growth at 25 °C due to spending available body reserve energy. Our results are in accordance with Stone et al. (2013) in that during the period of prolonged exposure to high summer water temperatures, dietary energy will be partitioned preferentially to support essential metabolic functions. Similarly, negative somatic growth energy was found in the energy budget during the summer months and a high use of body reserve energy for maintenance

metabolism was indicated when *H. tuberculata* were cultured under heat stress conditions (McBride et al., 2001).

The ability of abalone to cope with temperature stress by altering or maintaining their metabolism is species-dependent. The stress caused by elevated seawater temperatures in summer increased respiration energy of *H. tuberculata* (McBride et al., 2001; Morash and Alter, 2015). Vosloo et al. (2013) also reported that an acclimation to 19 °C and high oxygen decreased oxygen consumption rates of juvenile *H. midae*. According to Vosloo and Vosloo (2010), during acute (24 h) exposure to 16, 19 and 22 °C, the rate of oxygen consumption of adult South African abalone gradually increased with increasing temperature, but significantly increased with the increase in temperature after chronic exposure for a month. Interestingly, the greenlip abalone at 25 °C were able to maintain oxygen consumption energy to a level comparable with those at 22 °C in the present study. Our results are in accordance with Carefoot et al. (1993) in that oxygen consumption of the Northern abalone, *Haliotis kamtschatkana* appeared to be maintained at normal levels during a starvation period. In addition, Kaushik (1986) and Lopez and Tyler (2006) have reported that the routine metabolic rate increases with temperature, but only within a limited range of temperatures. It is possible that greenlip abalone may be able to use energy from dietary energy and energy stored in the muscle to maintain respiration energy.

Pedal mucus production energy of greenlip abalone was greater at 25 °C (7.97 - 10.0%) than at 22 °C (3.32 - 3.63%), again reflecting thermal stress in this species. Similarly, the large energy loss (23.3- 29.1%) via mucus production in *H. tuberculata* was attributed to stress, although the causes of stress and production of larger quantities of mucus at thermal stress are not clear (Peck et al., 1987).

In response to prolonged exposure at 25 °C, greenlip abalone channelled a major proportion of ingested energy to egested faecal energy (42.4 - 62.4%) and to support respiration (24.8 - 37.5%). When compared to other abalone species, the quantity of egested faecal energy presented in the present study is higher than that reported in *H. tuberculata* (3.0 - 11.2%), *H. asinina* (18.7 - 19.1%) and the green abalone, *Haliotis fulgens* (12.4%) (Farías et al., 2003; Ganmanee et al., 2010; Lopez and Tyler, 2006). This large energy loss via egested faeces can probably be attributed to low absorbed energy caused by thermal stress. Our result agrees with a previous study by Barkai and Griffiths (1988) in that 63% of the ingested food energy is lost in the faeces in *H. midae* due to low absorption efficiency.

5.6 Conclusion

The energy budgets of greenlip abalone provides a good indication of this animal's energy partitioning response to high water temperature. Apart from energy expended through respiration, thermal stress impacts negatively on the energy budget of greenlip abalone. Pro/prebiotic supplementation had no effect on energy budget at 22 or 25 °C reflecting that these tested products were inappropriate for greenlip abalone for somatic growth energy improvement. The negative value of somatic growth energy indicates that abalone used ingested food energy and body energy reserves for maintaining metabolism and produced large quantities of mucus to cope with stress at 25 °C. Further study should identify potential microorganisms in the gut of greenlip abalone prior to producing pro/prebiotics. Abalone farms should be aware of increases in waste and ammonia production, while concurrent reductions in ingested feed energy and somatic growth energy when periods of prolonged exposure to high summer water temperatures occur.

Chapter 6

Bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) fed antioxidant additives at high water temperature

Duong N. Duong, David A.J. Stone, Matthew S. Bansemer, Thanh H. Hoang, Jian G. Qin and James O. Harris. Effects of dietary feed additives on bioenergetics of greenlip abalone (*Haliotis laevigata* Donovan) prolonged exposure elevated water temperature. In preparation.

6.1 Abstract

A major problem confronting the abalone farming industry in Australia is elevated mortality during summer months. Recent research suggests that nutritional supplementation can have positive effects on health and survival. In order to understand this further, we used bioenergetics to study the physical responses of abalone, examining the growth pattern and waste output under chronic thermal stress. The aim of this study was to investigate the bioenergetics of 3-year-old greenlip abalone (49.21 g; 70.26 mm) fed a range of antioxidant additives at 25 °C for 38 days. The graded levels of peanut skin extract (PE; 0.5, 1.0, 2.5 and 5.0%), green tea extract (GTE; 0.5, 1.0, 2.5 and 5.0%), vitamin C (1.0% vitamin C; 1.0% vitamin C + 1.0% GTE; and 1.0% vitamin C + 1.0% PE) and 5.0% GSE were added to the commercial diet. Abalone fed all diets at 25 °C were compared against those fed the commercial diet at 22 °C (positive temperature control). Supplements did not significantly affect ingested food energy, respiration energy, pedal mucus production energy and shell growth energy, but absorbed energy, somatic growth energy, ammonia excretion energy and egested faecal energy were dietary dependent. Abalone fed 5.0% GSE had significantly higher absorbed energy than those fed 0.5, 2.5 and 5.0% GTE, 0.5% PE, 1.0 % Vit C or Vit C 1.0%/GTE 1.0%. At 25 °C, with the exception of the GSE treatment, somatic growth energy was negative, indicating that abalone utilised their energy reserves. The ammonia excretion energy was the lowest in abalone fed 0.5% GTE and significantly lower than those fed 1.0% Vit C/1.0% PE, while abalone fed 0.5% PE had less egested faecal energy than those fed 1.0% and 5.0% PE. Abalone fed the commercial control diet at 22 °C had significantly higher ingested food energy, absorbed energy and somatic growth energy than those fed all diets at 25 °C, whereas the respiration energy, ammonia excretion energy, pedal mucus production energy and shell growth energy of abalone

were similar between 22 and 25 °C. Egested faecal energy of abalone fed the commercial diet at 22 °C was significantly higher than those fed GTE inclusion (excepted for 1.0% GTE), 0.5% PE and Vit C supplement. A major portion of the ingested food energy was lost through egested faecal energy (23.7 - 47.2%) and respiration energy (11.8 - 39.9%). Somatic growth energy accounted for 18.0% of ingested food energy in abalone fed 5.0% GSE at 25 °C or 26.0% in abalone fed the commercial diet at 22 °C, but abalone cultured at 25 °C fed the rest of the diets had negative energy for somatic growth. Energy allocated to mucus production, ammonia excretion and shell growth was 3.96 - 15.6%, 0.34 - 3.21% and 0.11 - 0.75%, respectively. Supplementation with PE, GTE and vitamin C had little impact on energy budgets, while the inclusion of 5.0% GSE in the commercial diet had some positive effects on the energy budget of greenlip abalone cultured at high summer water temperatures. These results are useful to manage feed intake, production, water quality and waste production during chronic thermal stress.

Keywords: Greenlip abalone, grape seed extract, peanut skin extract, green tea extract, water temperature, energy budgets.

6.2 Introduction

The greenlip abalone, *Haliotis laevigata*, is an important species in Australian aquaculture and is grown mainly in land-based systems (Stone et al., 2013, 2014a, b). Abalone cannot maintain constant body temperature when the ambient temperature changes and a fluctuation of one or two degrees will have significant repercussions on metabolism (Van Barneveld, 2008). Therefore, they are classified as thermoconformers as their body temperature varies depending on their surrounding environment (Prosser, 1991). Feeding, respiration and growth rates generally increase as water temperature increases, until the temperature reaches a level that causes stress, and ultimately death of the animal (Mozqueira, 1996).

Higher mortalities occur over the summer months, when temperatures approach or exceed the thermal limits of abalone. According to Stone et al. (2014b), summer mortality refers to a disease caused by an interaction between biotic and abiotic environmental factors at high water temperatures (> 22 °C) and significantly impacting health, growth and mortality of all age classes abalone, especially, up to 50% of larger (≥ 60 mm) cultured abalone in southern Australia. In order to reduce mortality rates and optimise production efficiency, recent studies have focused on the effectiveness of adding various supplements to abalone diets, such as macro and microalgae (Dang et al., 2011a; Lange et al., 2014; Stone et al., 2014b) and grape seed extract (GSE) (Lange et al., 2014). The results from Lange et al. (2014) demonstrated that those dietary supplements had positive effects on survival and health of abalone.

Green tea extract (GTE), GSE and peanut skin extract (PE) have been recognized to have diverse health benefits including antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic properties (Perumalla and Hettiarachchy, 2011; Saito et al., 1998; Yu et al., 2005). In terrestrial animal models, GTE has been tested

in rat, mice and rabbits (Bose et al., 2008; Bruno et al., 2008; Bursill et al., 2007; Inagake et al., 1995; Löest et al., 2002; Yokozawa et al., 2002), while GSE has been examined in mice, rat, rabbit, horse and hamsters (Bagchi et al., 1998; Davies et al., 2009; Décordé et al., 2009; Koga et al., 1999; Yamakoshi et al., 1999; Yousef et al., 2009). In aquaculture, GTE has been evaluated in fish where it significantly improved growth and feed utilisation and was effective in lowering serum lipoprotein cholesterol and glutamic oxaloacetic transaminase in olive flounder, *Paralichthys olivaceus* (Cho et al., 2007). Abdel-Tawwab et al. (2010) reported that green tea supplement as a promising immunostimulant could improve fish performance, health, and prevent tilapia *aeromoniasis*. According to Hwang et al. (2013), GTE diet increases the survival of juvenile black rockfish, *Sebastes schlegeli* due to improving immunity reinforcement from lysozyme activity and improvements in stress recovery from 2-phenoxyethanol and air exposure.

Similarly, positive health benefits from dietary GSE supplementation have also been identified in several fish species and greenlip abalone. Dietary inclusion of 0.2g GSE kg^{-1} feed had positive effects on growth and body composition, and ameliorated serum biochemistry parameters of tilapia, *Oreochromis niloticus* (Zhai et al., 2014). Kao et al. (2010) reported significant reductions in inflammatory responses and mortality in zebrafish, *Danio rerio*, infected with *Staphylococcus aureus* pre-incubated with GSE. Specifically relevant to abalone, Lange et al. (2014) reported improved survival and feed intake at 26 °C in greenlip abalone fed a commercial diet containing 5.0% GSE.

Peanut skin is a by-product of the production of peanut oil, peanut butter, snack peanuts and confectionary, and is mainly used as a low-value stock feed ingredient (Yu et al., 2006). It has a low economic value despite its relatively high concentration of antioxidants including catechins, procyanidins (mainly A1), and

other phenolic compounds (Nepote et al., 2005; Yu et al., 2005). It has been reported that one gram of dry peanut skin contained 90 - 125 mg total phenolics (Yu et al., 2005) and 16, 111, 221 and 296 mg 100 g^{-1}, respectively of total catechins, procyanidin dimers, trimers and tetramers in directly peeled peanut skin (Yu et al., 2006). Therefore, extracts from peanut skin are suggested as an inexpensive source of antioxidants for use as functional ingredients in foods or as dietary supplements (Yu et al., 2006). Limited data are available regarding the use of PE to improve animal health and survival.

High water temperature and low oxygen levels can cause oxidative stress in aquatic animals by disrupting the oxidant-antioxidant equilibrium (Lushchak, 2011). Vitamin C is a recognised exogenous dietary antioxidant and is capable of preventing oxidation of molecules in humans and animals (Cederberg et al., 2001; Frei et al., 1988). It was reported that supplementation of 2 g vitamin C kg^{-1} diet enhanced the resistance to stress conditions and bacterial infections in shrimp post larvae (Merchie et al., 1997). Similarly, positive effects on stress resistance in salinity stress tests have been observed in larvae European sea bass, *Dicentrarchus labrax*, fed high concentrations of vitamin C (20%) (Merchie et al., 1995). Also for turbot, *Scophthalmus maximus*, feeding vitamin C-enriched diets reduced mortality by 10% after a challenge with *Vibrio anguillarum* (Merchie et al., 1996). Recently, vitamin C has also been shown to influence the immune response and disease resistance of large yellow croaker, *Pseudosciaena crocea* (Ai et al., 2006) and Indian major carp, *Labeo rohita* (Misra et al., 2007). However, little research has been carried out on the effects of vitamin C in molluscs, particularly in abalone (Mai, 1998).

Bioenergetics study quantifies the allocation of ingested food energy, absorbed energy, somatic growth energy, respiration energy, ammonia excretion energy, shell growth energy and mucus production energy and is one way to understand the basic

biology of an animal and its energy partitioning for maintenance and growth under environmental variation. It has been reported that significant reductions in feed intake and growth of abalone (Lange et al., 2014; Stone et al., 2014b) and changed respiration rate (McBride et al., 2001; Morash and Alter, 2015; Vosloo et al., 2013) occur during periods of prolonged exposure to high summer water temperatures. Since somatic growth energy and respiration energy are the main components of the energy budget, those parameters along with others are expected to change in response to thermal stress. Thus, the aims of this study were to established an energy model for each dietary treatment and to investigate the effects of graded levels of GTE, PE, vitamin C and 5.0% GSE supplementation in a commercial abalone diet on each component of energy budget of greenlip abalone at 25 °C. This information will assist in inproving feeding regimes and maintaining optimum physiological conditions, predicting production and managing water quality through waste output in abalone farming.

6.3 Materials and methods

6.3.1 Experimental animals

Three-year-old greenlip abalone were obtained from South Australian Mariculture (Boston Point, Port Lincoln, South Australia). Upon arrival at the South Australian Research and Development Institute (SARDI), the abalone were transferred to 500 L flow through tanks supplied with aeration and seawater at ambient temperature (21 °C) and photoperiod and fed 5 mm commercial Abgrow diet chips (Eyre Peninsula Aquafeed Pty Ltd [EPA], Lonsdale, South Australia, Australia) for one month prior to the experiment.

6.3.2 Experimental design and system

The study used the temperature challenge method developed by Stone et al. (2014b) to investigate the effects of the inclusion of graded levels of GTE or PE (0.5,

1.0, 2.5 and 5.0%), vitamin C supplementation (1.0% vitamin C; 1.0% vitamin C + 1.0% GTE; and 1.0% vitamin C + 1.0% PE) and 5.0% GSE in a commercial diet (5 mm commercial Abgrow diet chips (Eyre Peninsula Aquafeed Pty Ltd [EPA], Lonsdale, South Australia, Australia) on bioenergetics of greenlip abalone at 25 °C water temperature. Abalone fed a commercial diet without dietary supplements and maintained at 22 °C served as the positive control, whereas abalone fed the same diet and maintained at 25 °C served as a negative control. Abalone in the remaining treatment groups were all maintained at 25 °C. The commercial diet containing 5.0% Australian grapeseed extract (GSE) fed abalone at 25 °C was also included due to improved survival and health of abalone at high temperatures in our previous study (Lange et al., 2014). The reason for using this commercial diet was that it is the diet currently used by Australian greenlip abalone growers. The commercial diet formulations are relatively constant and have been used for several years prior to this study. Unfortunately, the actual ingredient composition of these diets was limited due to confidentiality issues. However, the comparison could be performed between treatments since the dietary analysis of the nutrients and energy were done for all diets including the commercial diets.

Experimental system was previously described in Chapter 5 (5.3.2).

6.3.3 Experimental stocking

Five hundred and sixty abalone were removed from the holding tank using a spatula. They were weighed (weight, 49.21 ± 0.05 g), measured (shell length, 70.26 ± 0.09 mm) (mean ± SE, n = 56) and systematically interspersed among four replicate tanks per treatment combination. The experiment ran for a total of 38 days, including a one-week acclimation period to slowly raise the water temperature from 21 °C (~1 °C d^{-1}) to the desired treatment temperatures of 22 and 25 °C, followed by a 33-day temperature challenge period. For each treatment, the size and weight of

dead abalone were measured in an attempt to keep stocking densities equal by replacing with tagged abalone of a similar weight and size that had been treated identically and held at the same water temperature and fed their respective diets.

6.3.4 Experimental diets, feeding and water management.

Eyre Peninsula Aquafeeds provided the Abgrow diet mash. PE and GTE (Shenzhen Naturactive Inc., Shenzhen, Guangdong, People's Republic of China) were formulated into the Abgrow mash at graded levels of 0.5, 1.0, 2.5 and 5.0%, while vitamin C (ROVIMIX® STAY-C® 35; DSM, Heerlen, the Netherlands) supplementations were 1.0% vitamin C; 1.0% vitamin C + 1.0% GTE; and 1.0% vitamin C + 1.0% PE. A positive control diet (positive control diet B) containing 5% Australian GSE (GSeedEX grape seed tannin, Tarac Technologies Pty Ltd., Nuriootpa, SA, Australia) was also formulated into the Abgrow mash based on previous research (Lange et al., 2014).

To manufacture the diets, the required amounts of dry Abgrow mash, vitamin/mineral premix and fish oil were weighed out and mixed in a Hobart mixer (Hobart Corp., Troy, OH, USA) for 5 min. The vitamin/mineral premix and fish oil inclusions were kept constant in all diets at 2 and 15 g kg^{-1}, respectively. Water (~ 30%) and the sodium alginate binder (0.36%) were then added to the diet mash and mixed for a further 3 minutes. The diets were cold pelleted using a TR110 pasta machine (MacchinePer Pasta SRL, Molina Di Malo, VI, Italy) to produce a 5 mm flat sinking pellet. Diets were then dried at 50 ^{o}C for approximately 48 h until the diets were less than 10% moisture.

Abalone were fed to excess (1% body weight (bw) d^{-1}) at 16:00 h daily. Cleaning and collection of uneaten food was performed at 08:30 h daily by straining the entire tank contents through a fine mesh. The uneaten feed was weighed, first stored frozen at - 20 °C and then was dried in an oven at 105 °C for 16 h. The

proportion of uneaten feed lost through leaching was estimated in a tank without animals and the correction factor was used to calculate the apparent feed intake. Feed intake was determined and used to calculate ingested food energy.

The water quality measurement was previously described in Chapter 2 (2.3.2).

Table 6.1 The nutrient composition of additive ingredients and diets used in this study (reported by Duong et al., 2016)

Biochemical composition	Diets												
	Comm. 0.0	GSE 5.0	GTE 0.5	GTE 1.0	GTE 2.5	GTE 5.0	PE 0.5	PE 1.0	PE 2.5	PE 5.0	Vit C 1.0	Vit C 1.0 GTE 1.0	Vit C 1.0 PE 1.0
Commercial diet mash (g kg $^{-1}$)	979.4	929.4	974.4	969.4	954.4	929.4	974.4	969.4	954.4	929.4	969.4	959.4	959.4
GSE (g kg $^{-1}$)	0.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PE (g kg $^{-1}$)	0.0	0.0	0.0	0.0	0.0	0.0	5.0	10.0	25.0	50.0	0.0	0.0	10.0
GTE (g kg $^{-1}$)	0.0	0.0	5.0	10.0	25.0	50.0	0.0	0.0	0.0	0.0	0.0	10.0	0.0
Sodium alginate (g kg $^{-1}$)	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6
Vitamin C (g kg $^{-1}$)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0	10.0	10.0
Vitamin–mineral premix (g kg $^{-1}$)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Salmon oil (mL kg $^{-1}$)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Sum	1000.0	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Moisture (g kg $^{-1}$)	63.0	62.4	63.1	63.1	63.4	66.5	63.1	63.2	63.6	64.2	63.1	63.1	63.1
Crude protein (g kg $^{-1}$)	309.0	295.9	307.5	306.0	301.5	307.9	294.1	306.0	301.6	294.1	306.0	306.0	306.0
Crude lipid (g kg $^{-1}$)	49.0	46.9	48.8	48.6	48.1	49.4	46.7	48.5	47.9	46.7	48.6	48.6	48.6
Ash (g kg $^{-1}$)	62.0	62.5	61.8	61.6	61.1	63.0	60.9	61.8	61.5	60.9	61.6	61.6	61.6
Carbohydrate (g kg $^{-1}$)	517.0	532.4	518.8	520.6	526.0	558.2	536.0	520.8	526.5	536.0	520.6	520.6	520.6
NFE (g kg $^{-1}$) [1]	517.0	532.3	518.8	520.7	525.9	513.2	535.2	520.5	525.4	534.1	520.7	520.7	520.7
Gross energy (MJ kg $^{-1}$)	15.9	15.9	15.9	15.9	15.9	16.6	15.9	15.9	15.9	15.9	15.9	15.9	15.9

[1] NFE = nitrogen-free extract was calculated by difference = 1000 g kg^{-1} - (moisture g kg^{-1} + crude protein g kg^{-1} + crude lipid g kg^{-1} + ash g kg^{-1}).

Table 6.2. The composition of grape seed extract (GSE), green tea extract (GTE) and peanut skin extract (PE) (reported by Duong et al., 2016).

Nutrient composition	GSE	GTE	PE
Moisture (g kg^{-1})	51.0	77.0	86.0
Crude protein (g kg^{-1})	46.0	9.0	10.0
Crude lipid (g kg^{-1})	7.0	12.0	3.0
Ash (g kg^{-1} as fed)	71.0	26.0	40.0
Carbohydrate (g kg^{-1})	852.0	876.0	896.0
NFE (g kg^{-1}) [a]	876.0	953.0	946.0
Gross energy (MJ kg^{-1})	15.1	15.1	15.5
FRAP (mmol Fe^{2+} equivalent g^{-1}) [b]	6.02	9.78	5.55
HPLC-MS qualitative profile (mAU)			
Gallic acid	1700	NA	NA
Procyanidin	450	NA	NA
Flavan-3-ol (catechin)	1850	NA	250
Procyanidin (dimer Type-B1)	1650	NA	NA
Flavan-3-ol (epicatechin)	2000	NA	350
Multiple procyanidin polymers (>dimeric)	1000	NA	NA
Catechin gallate	NA	250	NA
Epgallocatechin	NA	1800	NA
Catechin	NA	700	NA
Caffeine	NA	300	NA
Epicatechin, epigallo-catechin gallate (EGCG)-mixed peak	NA	2100	NA
Epicatechin gallate (ECG)	NA	2050	NA
Procyanidin (flavan-3-ol dimer)	NA	NA	650
procyanidin (Type-A, two bonds between flavan-3-ol units)	NA	NA	1800
procyanidin (Type-A1)	NA	NA	1350

NA: not available

[a] NFE = nitrogen-free extract was calculated by difference = 100% − (crude protein% + total fat% + ash%).

[b] FRAP = ferric-reducing antioxidant potential was measured by homogenising the ingredient and feed sample in DMSO and assaying the supernatant. Test ingredient FRAP values (mmol Fe^{2+} equivalent g^{-1}).

6.3.5. Determination of the components of the energy budget

Energy budgets were calculated for greenlip abalone by measuring each component of the energy budget in the equation as described in Chapter 2 (2.3.3).

6.3.5.1 Ingested food energy

The determination of ingested food energy was described in Chapter 2 (2.3.3.1).

6.3.5.2 Somatic growth and reproduction energy

The determination of energy content for somatic growth and reproduction was described in Chapter 5 (5.3.5.2). The final weights of greenlip abalone (mean ± SE) were 56.4 ± 1.41g (n = 4) in 22°C Commercial; 48.7 ± 0.41 g (n = 4) in 25°C Commercial; 49.7 ± 0.32 g (n = 4) in 25°C GSE 5.0%; 49.1 ± 0.24 g (n = 4) in 25°C GTE 0.5%; 48.4 ± 0.13 g (n = 4) in 25°C GTE 1.0%; 47.9 ± 0.85 g (n = 4) in 25°C GTE 2.5%; 46.8 ± 1.39 g (n = 4) in 25°C GTE 5.0%; 48.6 ± 0.41 g (n = 4) in 25°C PE 0.5%; 48.5 ± 0.09 g (n = 4) in 25°C PE 1.0%; 47.3 ± 1.12 g (n = 4) in 25°C PE 2.5%; 47.4 ± 0.46 g (n = 4) in 25°C PE 5.0%; 48.5 ± 0.31 g (n = 4) in 25°C Vit C 1.0%; 48.9 ± 1.60 g (n = 4) in 25°C Vit C 1.0%/GTE 1.0% and 47.6 ± 0.68 g (n = 4) 25°C Vit C 1.0%/PE 1.0%.

There was no sign of gonad development in this study at 25 °C. Therefore, the gonad of the abalone was not separated.

6.3.5.3 Respiration energy

The determination of respiration energy was described in Chapter 5 (5.3.5.3).

6.3.5.4 Energy rate for ammonia excretion

The determination of ammonia excretion energy was described in Chapter 2 (2.3.3.4).

6.3.5.5 Energy rate for faecal egestion

The determination of egested faecal energy was described in Chapter 2 (2.3.3.5).

6.3.5.6 Energy rate for mucus production and shell

The determination of pedal mucus production energy was described in Chapter 5 (5.3.5.6).

The calculation of energy of mucus production rate was described in Chapter 2 (2.3.3.5)

Energy for shell was described in Chapter 2 (2.3.3.6).

6.3.6. Biochemical analyses

The biochemical compositions of the diets and test ingredients were analysed according to the methods of the AOAC (1995) and are displayed in Table 6.1. Crude protein (N × 6.25) was determined by the Kjeldahl method. Crude lipids were analysed with a Soxtherm rapid extraction system (Gerhardt GmbH and Co. KG, Konigswinter, Germany) with petroleum liquid (BP 100 °C) as the extracting solvent. Ash was determined using a muffle furnace at 550 °C for 16 h. Carbohydrate was calculated by difference. GSE, GTE and PE were assayed for ferric reducing antioxidant power (FRAP) using the methods of Cheah (2011), slightly modified by Xu et al. (2010). The polyphenol composition of GSE, GTE and PE were also assessed qualitatively at the Analytical Research Laboratory, Plant Science, Southern Cross University, Lismore, New South Wales, Australia using an Agilent 1100 HPLC coupled in series to an Agilent PDA (Photo-Diode Array) and an Agilent 1100 MS (Mass Selective) detectors. Test specimens were solubilised in 70:30 ethanol: water and injected onto a reverse phase (Phenomenex Luna C18 100mm x 4.6mm ID, 3um) over a broad gradient eluting from 10% Acetonitrile (0.005% % TFA): 0.005% TFA (Triflouroacetic acid) to 95% Acetonitrile (0.005% % TFA): 5%,

0.005% TFA over 20 mins. The composition of each specimen extract was examined for composition using characteristic UV-Vis profiles and MS fragment ions as markers for respective ID of catechins, oligomeric procyanidins and condensed tannins (Table 6.2)

6.3.7 Statistical analysis

Statistics were computed using Statistical Package for the Social Sciences (SPSS) for Windows (version 22, IBM Corp., Armonk, NY, USA). In order to ensure normal distribution, data were transformed where appropriate, while Levene's test for equality of variance was used to assess the homogeneity of variance among means prior to running the ANOVA and the post-hoc comparisons. One factor ANOVA was used to compare the effects of additive diets on each parameter of the energy budget at 25 °C. The Dunnett's 2-tailed test was used to compare ingested feed energy, absorbed energy, respiration energy, ammonia excretion energy, egested faecal energy, shell growth energy and pedal mucus production energy of abalone fed all diets at 25 °C against the commercial diet at 22 °C as the positive control. The Independent-Samples T Test was used to compare pedal mucus production energy of abalone cultured at 22 and 25 °C.

6.4 Results

6.4.1 General observation and water quality

The survival rate was 85% at 22 °C or from 17.5 to 77.5% at 25 °C (Duong et al., 2016). The details of experimental water parameters were reported in Duong et al. (2016). At the 22 °C treatment, water temperature averaged (mean ± SE, n = 38) 22.2 ± 0.04 °C; dissolved oxygen 84.8 ± 0.5% saturation or 5.9 ± 0.04 mg L^{-1}, pH 8.17 ± 0.01 and salinity 36.0 ± 0.01 ppt. At 25 °C treatment, water temperature 24.7 ± 0.14 °C, dissolved oxygen 88.3 ± 0.23% saturation or 6.0 ± 0.02 mg L^{-1}, pH 8.20 ± 0.01 and salinity 36.0 ± 0.01 ppt.

6.4.2 Ingested food energy

The ingested food energy of abalone at 25 °C was not significantly affected by diets containing GTE, PE and vitamin C or GSE ($P = 0.069$; one factor ANOVA; Table 6.3). The ingested food energy ranged from 0.48 to 0.86 (J g abalone^{-1} h^{-1}) across treatments at 25 °C. Abalone fed the commercial diet at 22 °C had significantly higher ingested food energy than those fed the rest at 25 °C ($P < 0.001$; Dunnett's 2-tailed test; Table 6.3).

6.4.3 Absorbed energy

Absorbed energy of abalone cultured at 25 °C was significantly affected by diets ($P = 0.010$; one factor ANOVA; Table 6.3). Abalone fed 5.0% GSE had significantly higher absorbed energy than those fed 0.5, 2.5 and 5.0% GTE, 0.5% PE, 1.0 % vitamin C or vitamin C 1.0%/GTE 1.0%. The absorbed energy of abalone fed the commercial diet at 22 °C was significantly higher than those fed all diets at 25 °C ($P < 0.001$; Dunnett's 2-tailed test; Table 6.3).

6.4.4 Somatic growth energy and reproduction energy

At 25 °C, only abalone fed 5.0% GSE had available energy for somatic growth and its treatment was significantly different to those fed other diets ($P < 0.001$; one factor ANOVA; Table 6.3). However, there was no sign of gonad development. At 22 °C, greenlip abalone had available energy for somatic growth as well as reproduction. The somatic growth energy was significantly higher in abalone fed the commercial diet at 22 °C than those fed all diets at 25 °C ($P < 0.001$; Dunnett's 2-tailed test; Table 6.3)

6.4.5 Respiration energy

There was no significant effect of diet supplementation on respiration energy of abalone at 25 °C ($P > 0.05$; one factor ANOVA; Table 6.3). The respiration energy of greenlip abalone ranged from 0.14 to 0.20 (J g abalone^{-1} h^{-1}) for all

treatments at 25 °C. Respiration energy of abalone fed the control diet at 22 °C was similar to those fed all additive diets at 25 °C (P = 0.131; Dunnett's 2-tailed test; Table 6.3).

6.4.6 Ammonia excretion energy

There was significant effect of diet on ammonia excretion energy of abalone at 25 °C (P = 0.010; one factor ANOVA; Table 6.3). Ammonia excretion energy was the lowest in abalone fed 0.5% GTE and significant from those fed 1.0% vitamin C/1.0% PE, but it was similar to the rest (P > 0.05). Abalone fed the control diet at 22 °C had similar ammonia excretion energy with those fed all diets at 25 °C (P = 0.270; Dunnett's 2-tailed test; Table 6.3).

6.4.7 Egested faecal energy

Egested faecal energy of abalone cultured at 25 °C was significantly affected by diet (P = 0.009; one factor ANOVA; Table 6.3). Abalone fed 0.5% PE had lower egested faecal energy than those fed 1.0% (P = 0.041) and 5.0% (P = 0.047) PE, but it was similar to the rest of the diets (P > 0.05). There was significantly higher egested faecal energy for abalone fed the commercial control diet at 22 °C compared to those fed 0.5, 2,5 and 5.0% GTE, 0.5% PE and vitamin C series diets, but it was similar to the rest at 25 °C (P > 0.05; Dunnett's 2-tailed test; Table 6.3).

6.4.8 Pedal mucus production energy and shell growth energy

Pedal mucus production energy was significantly influenced by water temperature (P = 0.025; Independent-Samples T Test). Abalone lost significantly more energy through mucus production at 25 °C (0.07 J g abalone^{-1} h^{-1}) than those at 22 °C (0.05 J g abalone^{-1} h^{-1}).

There was no significant difference in shell growth energy of abalone fed additive diets at 25 °C (P > 0.05; one-factor ANOVA; Table 4) or the commercial diet at 22 °C (P > 0.05; Dunnett’s test; Table 6.3).

Table 6.3 Energy component (J g abalone^{-1} h^{-1}) of greenlip abalone (*Haliotis laevigata*) fed commercial diet at 22 °C and the commercial diet and diets supplemented with GSE, GTE, PE and vitamin C at 25 °C [1].

	Energy components (J g abalone^{-1} h^{-1})								
Temperature and diet	I	Ab	Pg	Pr	R	U	E	M	S
Control series									
22°C Commercial (Positive temperature control)	1.41 ± 0.10	0.96 ± 0.07	0.37 ± 0.04	0.04 ± 0.01	0.16 ± 0.01	0.011 ± 0.002	0.45 ± 0.03	0.05 ± 0.01	0.007 ± 0.004
25°C Commercial (Negative temperature control)	0.71 ± 0.09*	0.44 ± 0.04^{ab} *	- 0.08 ± 0.03 ^{b} *	-	0.20 ± 0.02	0.009 ± 0.004^{ab}	0.27 ± 0.05^{ab}	0.07 ± 0.01	0.001 ± 0.001
25°C GSE 5.0%	0.86 ± 0.05*	0.58 ± 0.02^{b} *	0.15 ± 0.03 ^{a} *	-	0.16 ± 0.01	0.004 ± 0.001^{a}	0.28 ± 0.04^{ab}	0.07 ± 0.01	0.003 ± 0.001
GTE series									
25°C GTE 0.5%	0.59 ± 0.08*	0.35 ± 0.03^{a} *	- 0.05 ± 0.02 ^{b} *	-	0.14 ± 0.02	0.003 ± 0.002^{a}	0.23 ± 0.05^{ab}*	0.07 ± 0.01	0.002 ± 0.001
25°C GTE 1.0%	0.74 ± 0.10*	0.43 ± 0.03^{ab} *	- 0.03 ± 0.01 ^{b} *	-	0.14 ± 0.01	0.004 ± 0.001^{a}	0.31 ± 0.07^{ab}	0.07 ± 0.01	0.002 ± 0.001
25°C GTE 2.5%	0.54 ± 0.02*	0.36 ± 0.01^{a} *	- 0.06 ± 0.01^{b} *	-	0.15 ± 0.01	0.011 ± 0.005^{ab}	0.19 ± 0.01^{ab}*	0.07 ± 0.01	0.004 ± 0.002
25°C GTE 5.0%	0.52 ± 0.05*	0.32 ± 0.03^{a} *	- 0.08 ± 0.01^{b} *	-	0.14 ± 0.01	0.006 ± 0.001^{ab}	0.20 ± 0.03^{ab}*	0.07 ± 0.01	0.002 ± 0.001
PE series									
25°C PE 0.5%	0.48 ± 0.04*	0.36 ± 0.02^{a} *	- 0.03 ± 0.01 ^{b} *	-	0.18 ± 0.01	0.013 ± 0.002^{ab}	0.12 ± 0.03^{a}*	0.07 ± 0.01	0.003 ± 0.002
25°C PE 1.0%	0.87 ± 0.08*	0.46 ± 0.05^{ab} *	- 0.08 ± 0.01 ^{b} *	-	0.16 ± 0.03	0.004 ± 0.002^{a}	0.41 ± 0.03^{b}	0.07 ± 0.01	0.002 ± 0.001
25°C PE 2.5%	0.69 ± 0.08*	0.40 ± 0.06^{ab} *	- 0.06 ± 0.02 ^{b} *	-	0.16 ± 0.04	0.009 ± 0.002^{ab}	0.29 ± 0.02^{ab}	0.07 ± 0.01	0.005 ± 0.001
25°C PE 5.0%	0.82 ± 0.16*	0.46 ± 0.07^{ab} *	- 0.07 ± 0.02 ^{b} *	-	0.14 ± 0.02	0.004 ± 0.001^{a}	0.36 ± 0.09^{b}	0.07 ± 0.01	0.003 ± 0.001
Vitamin C series									
25°C Vit C 1.0%	0.52 ± 0.04*	0.31 ± 0.02^{a} *	- 0.05 ± 0.02 ^{b} *	-	0.15 ± 0.01	0.005 ± 0.002^{a}	0.21 ± 0.03^{ab}*	0.07 ± 0.01	0.001 ± 0.001
25°C Vit C 1.0%/GTE 1.0%	0.51 ± 0.03*	0.32 ± 0.01^{a} *	- 0.03 ± 0.01 ^{b} *	-	0.16 ± 0.01	0.007 ± 0.001^{ab}	0.19 ± 0.02^{ab}*	0.07 ± 0.01	0.002 ± 0.001
25°C Vit C 1.0%/PE 1.0%	0.65 ± 0.09*	0.44 ± 0.06^{ab} *	- 0.09 ± 0.01 ^{b} *	-	0.16 ± 0.01	0.018 ± 0.004^{b}	0.22 ± 0.04^{ab}*	0.07 ± 0.01	0.002 ± 0.001
One factor - ANOVA (*P* value)	0.069	0.010	< 0.001	NA	0.134	0.010	0.009	NA	0.208
Dunnett's test (*P* value)	< 0.001	< 0.001	< 0.001	NA	0.131	0.270	< 0.001	NA	0.228

[1] Data is presented as mean +/- SE, n = 4

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Ab: is the absorbed energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy.

- denotes unavailable data as abalone showed no visible signs of gonad development.

* denote significantly different compared to the control treatment at 22 °C.

[a,b] superscripts for variables with a significant effect of diets at 25 °C.

6.4.9 Energy budgets of abalone

The components of energy budgets for greenlip abalone fed the commercial diet at 22 °C or 25 °C and diets supplemented with GSE, GTE, PE and vitamin C at 25 °C are shown in Table 6.4. Absorbed energy ranged from 52.8 to 76.3%. The major proportions of the ingested food energy were egested faecal energy (23.7 - 47.2%) and respiration energy (11.8 - 39.9%) across the treatments. Somatic growth energy accounted for 18.0% of ingested food energy in abalone fed 5.0% GSE at 25 °C and 26.0% in abalone fed the commercial diet at 22 °C. However, abalone cultured at 25 °C fed the rest of the diets had negative somatic growth energy, ranging from -14.2 to -5.09% of ingested food energy. Pedal mucus production was 3.96 - 15.6%, while ammonia excretion energy was accounted for small proportions of ingested food energy, ranging from 0.34 to 3.21%. Shell growth energy accounted for the smallest proportion of ingested food energy, ranging from 0.11 to 0.75%.

Table 6.4 Energy budgets (%) of greenlip abalone (*Haliotis laevigata*) fed commercial diet at 22 °C and the commercial diet and diets supplemented with GSE, GTE, PE and vitamin C at 25 °C [1].

	Energy budgets (%)									
Temperature and diet	I	Ab	Pg	Pr	R	U	E	M	S	Unexplained
Control series										
22°C Commercial (Positive temperature control)	100	68.0 ± 0.41	26.0 ± 1.15	3.10 ± 0.51	11.8 ± 0.96	0.77 ± 0.15	32.0 ± 0.41	3.96 ± 0.24	0.52 ± 0.31	21.9
25°C Commercial (Negative temperature control)	100	62.3 ± 2.17	- 10.5 ± 3.36	-	29.0 ± 4.80	1.10 ± 0.39	37.8 ± 2.17	10.9 ± 1.38	0.13 ± 0.07	21.2
25°C GSE 5.0%	100	68.3 ± 2.50	18.0 ± 1.86	-	18.6 ± 2.26	0.45 ± 0.15	31.8 ± 2.50	8.70 ± 0.57	0.35 ± 0.11	22.1
GTE series										
25°C GTE 0.5%	100	62.0 ± 3.00	- 10.2 ± 3.57	-	25.7 ± 5.02	0.66 ± 0.35	38.0 ± 3.00	13.2 ± 1.55	0.31 ± 0.12	22.4
25°C GTE 1.0%	100	59.5 ± 3.57	- 5.09 ± 1.12	-	19.9 ± 3.25	0.58 ± 0.16	40.5 ± 3.57	10.5 ± 1.24	0.28 ± 0.19	28.5
25°C GTE 2.5%	100	66.0 ± 0.91	- 11.3 ± 1.12	-	28.0 ± 2.40	1.88 ± 0.82	34.0 ± 0.91	13.6 ± 0.52	0.75 ± 0.42	22.5
25°C GTE 5.0%	100	62.3 ± 1.70	- 14.2 ± 1.88	-	28.0 ± 3.00	1.30 ± 0.34	37.8 ± 1.70	14.7 ± 1.68	0.27 ± 0.07	18.2
PE series										
25°C PE 0.5%	100	76.3 ± 4.06	- 6.38 ± 1.92	-	39.2 ± 5.20	2.46 ± 0.66	23.7 ± 4.06	15.6 ± 1.42	0.65 ± 0.29	19.0
25°C PE 1.0%	100	52.8 ± 1.03	- 9.15 ± 1.60	-	18.0 ± 1.49	0.34 ± 0.16	47.2 ± 1.03	8.65 ± 0.71	0.18 ± 0.06	25.8
25°C PE 2.5%	100	57.5 ± 2.72	- 9.37 ± 2.44	-	22.2 ± 3.34	1.31 ± 0.34	42.5 ± 2.72	11.1 ± 1.20	0.67 ± 0.21	22.9
25°C PE 5.0%	100	57.8 ± 4.19	- 8.94 ± 2.40	-	19.7 ± 5.55	0.47 ± 0.14	42.2 ± 4.19	9.88 ± 1.51	0.33 ± 0.05	27.8
Vitamin C series										
25°C Vit C 1.0%	100	60.3 ± 2.06	- 9.78 ± 2.67	-	28.3 ± 2.93	0.98± 0.53	39.7 ± 2.06	14.5 ± 1.26	0.11 ± 0.02	16.5
25°C Vit C 1.0%/GTE 1.0%	100	63.8 ± 1.80	- 6.11 ± 2.27	-	30.8 ± 1.80	1.22 ± 0.19	36.2 ± 1.80	14.5 ± 0.85	0.29 ± 0.11	17.3
25°C Vit C 1.0%/PE 1.0%	100	67.5 ± 1.66	- 14.0 ± 1.20	-	39.9 ± 1.73	3.21 ± 0.77	32.5 ± 1.66	11.9 ± 1.37	0.46 ± 0.33	12.5

[1] Data is presented as mean +/- SE, n = 4.

Energy budget equation: I - E = Pg + Pr + R + U + M + S where I is ingested food energy; E is egested faecal energy; Pg is the somatic growth energy; Pr is the reproduction energy; R is the respiration energy; U is the ammonia excretion energy; M is the pedal mucus production energy; and S is the shell growth energy. Absorbed energy (Ab), somatic growth energy, respiration energy, ammonia excretion energy, pedal mucus production energy and shell growth energy calculated as a percentage of ingested food energy. Egested faecal energy obtained from 100% ingested food energy minus absorbed energy. Unexplained was calculated as 100% - (Pg + Pr + R + U + M + S + E)

- : denotes unavailable data as abalone showed no visible signs of gonad development.

6.5 Discussion

The results of the present study show that supplementing GTE, PE or Vit. C had minimal effects on energy budget of greenlip abalone, but addition of 5.0% GSE in the commercial diet had significant impacts on absorbed energy and somatic growth energy.

When abalone are chronically exposed to high summer water temperatures, they will re-allocate available energy such as dietary energy or body reserve energy to enhance survival functions such as immune functions and heat tolerance (Lange et al., 2014; Lushchak, 2011; Stone et al., 2014b; Van Barneveld, 2008). A number of studies with abalone show a low growth during long exposure to thermal stress. For example, the growth rate of backlip abalone, *Haliotis rubra* decreased at 19 °C compared to those at 17 °C (Harris et al., 2005). Similarly, juvenile *H. midae* showed a reduction in tissue mass between 12 and 20 °C (Britz et al., 1997b). Stone et al. (2014b) also reported that growth rate and feed intake were suppressed in greenlip abalone grown at 26 °C as they may be exposed to the extremes of their temperature and oxygen tolerance limits. Our results support previous reports that the growth of abalone declines at 25 °C and mortality rates rise (Lange et al., 2014; Stone et al., 2014b). Furthermore, it is possible that greenlip abalone had also used body reserve energy to cope with thermal stress since abalone lost weight in all treatments at 25 °C, except for the treatment with 5.0% GSE.

5.0% GSE supplement was chosen in this study because it has been reported to improve survival in previous research (Lange et al., 2014), which was carried out under the same research facilities using animals of a similar size. A part from health benefits which have been reported in previous studies (Kao et al., 2010; Lange et al., 2014; Zhai et al., 2014), the improvement of somatic growth energy in abalone fed GSE may have been partly due to significant higher absorbed energy and

numerically higher ingested food energy in the current study. Lange et al. (2014) reported an increase in feed intake and meal acceptance in abalone fed 5.0% GSE addition at 26 °C, which would likely have caused the abalone to be in a more nutritionally nourished state, and in turn, have supported the ability to tolerate the temperature stress and improve survival.

A number of studies have previously reported on changing metabolic rate in response to thermal stress (McBride et al., 2001; Morash and Alter, 2015; Vosloo et al., 2013). For example, *H. tuberculata* increased respiration rate (McBride et al., 2001), while *H. midae* reduced oxygen uptake when exposed to elevated seawater temperatures in summer (Vosloo and Vosloo, 2010; Vosloo et al., 2013). In the current study, the respiration energy of greenlip abalone after chronic exposure to 25 °C was similar to those at 22 °C. It is possible that, apart from supporting essential immune functions and heat tolerance (McBride et al., 2001; Stone et al., 2014), greenlip abalone were able to use ingested food energy or body reserves energy to maintain respiration as normal during periods of prolonged exposure to high summer water temperatures.

Based on our observation, when the abalone were held at 25 °C, they exhibited similar physical behaviour as described for the 3-year-old abalone held at 26 °C in Stone et al. (2014b). For example, abalone intermittently raising their shells, rapidly twisted rotation, occasionally detaching from the tank walls or losing their attachment to the tank and landing upside down on the base of the tank, appearing unhealthy symptoms and mortality a few days later. Additionally, a higher amount of mucus secretion was observed in the water of culture units at 25 °C compared to 22 °C in the present study, even though it was not mentioned in previously (Stone et al., 2014). Similarly, Hooper et al. (2014) reported an increase of mucus after increased water temperature in abalone farms. The higher mucus production than normal is

likely to be a general stress response in gastropods, including heat stress (Hooper et al., 2014; Peck et al., 1987).

Up until now, there has been little available information on shell growth energy in *Haliotis* spp. And this is not taken into account in most studies. Thus, we are unable to make any direct comparison between shell growth energy or the proportion of shell growth energy of greenlip abalone and other *Haliotis* species. However, the energy content of greenlip abalone shell in the current study (181.5 J g abalone shell^{-1}) was comparable to the Japanese cockle, *Ruditapes philippinarum* (190.35 J g^{-1}), the European cockle, *Cardium edule* (157.12 J g^{-1}) or the lagoon cockle, *Cardium glaucum* (88.92 - 106.8 J g^{-1}) (Goulletquer and Wolowicz, 1989).

Among the energy budget parameters, pedal mucus production energy accounted for 3.96 - 15.6% of the energy budgets. This is likely to be an underestimation (especially at 25 °C) in the current study due to numbers of factors. As mentioned above, some mucus was present in the water and was lost due to water replacement. Furthermore, although mucus also is used for other function, such as egestion of faeces and coating all epithelial surfaces, only pedal mucus production energy was measured in most abalone energy budget studies, including the present study (Barkai and Griffiths, 1988; Farías et al., 2003; Ganmanee et al., 2010; Lopez and Tyler 2006; McBride et al., 2001; Peck et al., 1987). It is most likely that the unexplained component of the energy budgets is also associated with mucus.

6.6 Conclusion

5.0% GSE supplement had positive effects on ingested food energy, absorbed energy and somatic growth energy, but other supplements had little impact on the energy budgets at 25 °C. Across the treatments, the greenlip abalone were able to maintain energy for respiration at normal levels over an extended period at elevated water temperature. Greenlip abalone allocated the most energy to faecal egestion and

respiration. Overall, this study supports the use of GSE as a dietary additive during periods of higher summer water temperatures.

Chapter 7

General discussion and conclusion

7.1 Introduction

An understanding of the bioenergetics of greenlip abalone is necessary as a basis for providing an adequate dietary regime for any particular physical environment and also a prediction on waste output for culture management. Several studies have focused on the energy budgets of abalone, but limited information was available for greenlip abalone, an important commercial species of land-based systems in Australia. The main purposes of this thesis were: 1) to establish energy budgets for greenlips abalone, 2) to use energy budgets to understand the effects of some factors on abalone culture, specifically water temperature and diet. Addittionally, to add value to the current program of nutritional research conducted on greenlip abalone, I manipulated fish diets through adding dried macroalgae, changing protein levels, and selecting prebiotics, probiotics and antioxidants. This research has provided insights into new diet development and feeding management for abalone farming.

7.2 Summary of major findings and knowledge advancement

The results have shown that ingested food energy, absorbed energy, somatic growth energy, respiration energy, ingested faecal energy, pedal mucus production energy and shell growth energy changed in response to different diets or water temperatures.

1) Live macroalgae (*G. cliftonii* and *Ulva* sp. with or without enrichment) was firstly chosen in this study as they are the natural feed of abalone. Abalone fed live non-enriched *G. cliftonii* or the commercial diet showed an increase in ingested food energy, absorbed energy and somatic growth energy. Those improvements were due to a preference for red macroalgae over others in greenlip abalone and better digestibility and utilisation of nutrients in red macroalgae (Bansemer et al., 2016c; Shepherd, 1973; Stuart and Brown, 1994).

However, macroalgae feeding trials to date have often been nutritionally inferior which may be improved by feeding abalone with protein enhancement algae or mixed species of macroalgae.

2) Enriched macroalgae had significant impacts on the energy bugets of greenlip abalone. However, the advantages of nutrient enrichment on components of the energy budget in the current study depended on the type of macroalgae species. The benefits of nutrient enrichment were observed in greenlip abalone fed enriched live *Ulva* sp., but no effects of nutrient enrichment were found in enriched *G. cliftonii* treatments since dietary energy may be more important than dietary protein for greenlip abalone fed *G. cliftonii*. Thus, it is strongly recommended to enrich *Ulva* sp. before feeding it to greenlip abalone, while *G. cliftonii* can be given to abalone without enrichment. However, we should be aware of the potential for elevated levels of ammonia in the water when protein-enhanced macroalgae are used as feed.

3) Feeding equal amount of mixed of red macrolgae *G. cliftonii* and green macroalgae *Ulva* sp. had little benefits to the bioenergetics of greenlip abalone over a single species. It has been reported that the advantages of feeding mixed macroalgae diets over a single species diet were due to a superior balance of essential nutrients, such as amino acids, compared to mono-specific macroalgal diets (Viera et al., 2011). However, based on our observations, when the mixed diet (equal mixed of *G. cliftonii* and *Ulva* sp.) was provided, greenlip abalone firstly consumed *G. cliftonii* and switched to *Ulva* sp. after *G. cliftonii* was removed. Thus, those feeding habits may contribute to no differences between feeding the sole *G. cliftonii* and the mixed diet. However, a combination of some red macrolgae may be of more benefit since greenlip abalone prefer red macroalgae over green macroalgae.

4) In the current study, the improvement in ingested food energy, absorbed energy and somatic growth energy was observed in abalone fed inclusion of ≥ 10% *G. cliftonii* in the diet. As mentioned above, greenlip abalone in land-based systems are fed commercial formulated diets in Australia. It is possible that although the protein content and energy ratios may be suitable in most formulated feeds, the nutrients or other factors in macroalgae may be missing in formulated feeds, which may limit optimal development of abalone (Kirkendale et al., 2010). The inclusion of dried macroalgae meal to a formulated diet is a way to gain benefits of feeding live macroalgae to improve the feeding activity, health and marketability of abalone (Bansemer et al., 2014). Previous studies have investigated the growth performance of abalone fed the inclusion of dried macroalgae meal in formulated diets (O'Mahoney et al., 2014; Viera et al., 2005). However, those studies evaluated the inclusion of mixed species macroalgae meal did not investigate graded levels of inclusion. Nevertheless, inclusion of *G. cliftonii* in the formulated resulted in low diet water stability in our recent study (Bansemer et al., 2016b), which also was reported by Viera et al. (2005). A binder should be added to improve water stability when dietary macroalgae is included

5) The results in the current study show that the dietary energy level for 6-month-old greenlip abalone fed 27, 30, 33 and 36% CP at 14, 17 and 22 °C, is more important than the protein level, if energy requirements are met. Additionally, the dietary level of ~ 27% CP is recommended for 6-month-old greenlip abalone grwon at 17 and 22 °C as long as the digestible energy is close to 12.50 MJ kg^{-1}. The results of study are in agreement with Green et al. (2011) in that shell length and weight gain of South African abalone fed diets containing graded levels of protein (18 - 26%) and energy (11.6 - 16.2 MJ kg^{-1}) were independent of dietary

protein when digestible energy content of the diet was not lower than 13.5 MJ kg^{-1}. However, the existing commercial diets which are used to feed different sized of abalone throughout the year contain 30 - 35% CP and ~ 12.50 MJ kg^{-1} as recommened by Stone et al. (2013). Stone et al. (2013) also reported that dietary CP of greenlip abalone was size and water temperature dependent. For example, at the similar digestible energy ~ 12.50 MJ kg^{-1}, the optimum crude dietary protein levels increased from ~29% to ~ 35% as temperature increased from 14 to 22 °C for one-year-old abalone, whereas it appeared to be less in two-year-old, increased from 24% at 14 °C to 34% at 22 °C. Thus, there is a chance to introduce multi-diet feeding strategies to provide the optimum dietary CP level to abalone in response to age and seasonal fluctuations in water temeprature. Based on the bioenergetics, dietary CP for 6-month-old greenlip abalone can be ~ 27% when water temperature at 17 or 20 °C. Nevertheless, at 14 °C, it is not clear whether dietary protein or dietary energy play an important role in the energy budgets of greenlip abalone in the current study. Therefore, it is worth investigating dietary energy in response to water temperatures in further study.

6) The abalone must have been using energy previously stored in tissues to support maintenance costs such as metabolism, since they lost weight during prolonged exposure to elevated temperature (25 °C). In addition, greenlip abalone also lost more energy through pedal mucus production and ammonia excretion at high water temperature. Thus, it is predicted a decline of feed intake, absorption and production, while there is an increase in ammonia excretion and mucus production during themal stress. High water temperature caused a reduction in feed intake, it should be adjusted and water quality should be carefully monitored during summer months. Furthermore, dietary intervention or feed additives such as antioxidants are also promissing ways to reduce mortality and optimise

production efficiency at high summer temperatures (Lange et al., 2014; Stone et al., 2014b).

7) The addition of pro/prebiotics were initially expected to improve absorbed energy and somatic growth energy in 3-year-old greenlip at 22 or especially at 25 °C as near thermal maximum of 27.5 °C (Gilroy and Edwards, 1998). However, the energy budgets of greenlip abalone were not affected by dietary pro/prebiotic products, reflecting that those commercial pro/prebiotics are not appropriate for greenlip abalone in the present study. The use of probiotics and prebiotic (pro/prebiotic) has been reported to improve digestibility, health and growth of some abalone species (Erasmus et al., 1997; Hadi et al., 2014; Macey and Coyne, 2005; Ten Doeschate and Coyne, 2008). The less effective pro/prebiotic products in our study may be due to the diference in the source of pro/prebiotic. The pro/prebiotics used in previous studies were isolated from the gut of abalone prior to experiments (Erasmus et al., 1997; Hadi et al., 2014; Macey and Coyne, 2005; Ten Doeschate and Coyne, 2008). However, those pro/prebiotics from those above studies have limited application to the commercial scale due to the use of live probiotics, thus the feed in those studies may need to be freshly made weekly or monthly. In contrast, the commercial pro/prebiotics were selected in the current study due to numbers of benefit such as spore forms, easy introduction in dry food, improved water quality, survival and growth rates and increases in the health status of some aquatic species (Dalmin et al., 2001; Li et al., 2009; Rengpipat et al., 2000). However, as mentioned above, no alterations of bioenergetics were observed in the current study.

8) Supplementation of the 5.0% GSE diet had positive effects on ingested food energy and absorbed energy at 25 °C, while GTE, PE and Vit. C did not. It has been reported that inclusion of 5.0% GSE in the formulated diet improved the

survival of greenlip abalone chronically exposed to high water temperature (> 25 °C) compared to 22 °C, due to containing antioxidant and antibacterial activities and high levels of bioactive polyphenol compounds (Duong et al., 2016; Lange et al., 2014). However, previous studies have mainly focused on survival, growth and health (Lange et al., 2014; Stone et al., 2014b). Other parameters such as absorbed energy, respiration energy, ingested faecal energy and pedal mucus production energy were not measured, even though they might be important to assist in minimising the negative effects of high water temperature and water management.

9) For the energy budgets, in general, the main components are altered in response to a change in dietary type or water temperature. The largest component of the energy budget in 1-year-old abalone fed most diets was somatic growth energy, except for abalone fed live *Ulva* sp. where it was respiration energy. However, the largest proportion of the energy budget was respiration energy in 6-month-old abalone fed a range of CP levels (27, 30, 33 and 36%) at seasonal water temperatures (14, 17 and 20 °C). For 3-year-old abalone, a major proportion of the ingested food energy was egested faecal energy at both 22 and 25 °C water temperatures. This information will assist in predicting production and managing water quality through waste output in abalone farming regarding diets and water temperature.
10) In the current study, all components of the energy budget were measured, whereas, in several other studies, some components of the energy budget have not been measured and only obtained by subtraction or interpolating from the other terms (Barkai and Griffiths, 1988; Ganmanee et al., 2010; Peck et al., 1987). For example, Peck et al. (1987) did not measure ingested food energy instead used a of sum all parameters of the energy budget of *H. tuberculata* and

those authors also did not have separated values for egested faecal energy and ammonia excretion energy in the energy budget. Pedal mucus production was not assessed in the energy budgets of *H. asinina* and *H. midae* (Barkai and Griffiths, 1988; Ganmanee et al., 2010). Energy losses in the form of ammonia excretion of *H. midae* was negligible, accounting for less than 1% of consumption in the study of Barkai and Griffiths (1988).

11) Among the components of the energy budget, shell growth energy is rare and has not been considered in previous studies. However, I was able to measure shell growth energy even though it accounted for a small proportions of energy budgets (< 1%).

12) Mucus production energy was underestimated in the current studies as well as in other published works. Mollusc mucus is used in several processes such as locomotion, epithelial protection, egestion of faeces and cleaning the gills (Peck et al., 1987). However, only pedal mucus production energy is measured in most studies of energy budget due to difficulty in mucus collection from other parts. There is also another possible issue that may contribute to underestimated mucus production energy, which is the amount of mucus which was washed away due to water replacement in the current studies. Particularly, an increase in mucus production was observed in the water of culture tank when greenlip abalone were chronically exposured to thermal stress (> 25 °C) throughout the last two experiments. However, we were unable to collect mucus dissolved in the water. Thus, the amount of unmeasurable mucus production energy was attributed to some of unexplained energy in the current study.

13) It is likely that respiration energy was varied according to size of animals in this study. The 6-month-old animals (in Chapter 4) had higher respiration energy compared to 1-year-old abalone (in Chapter 2 and 3). This result is supported by

Jobling (1993) where large animals generally consume more oxygen than small ones, but on a unit weight basis, small animals will consume more oxygen than larger con-specifics.

14) Absorbed energy was varied according to age. It is likely that absorbed energy was higher in smaller animals (≤ 1-year-old) which was shown in the first three trials 75 - 91.8%, while it ranged from 37.3 to 76.3% in bigger abalone (3-year-old). The values in big abalone are similar to those reported for algae consumed by other gastropod molluscs including the exploited South African abalone *H. midae* feeding on *E. maxima* 37%, (Barkai and Griffiths, 1987), but for smaller abalone, it was higher than that of *H. tuberculata* (from 22.1 to 65.9 in) feeding on a diet consist of two macroalgae (*Ulva lactuca* and *Palmaria palmata*) or formulated diets. The reasons for the energy difference due to abalone size are not known and require further investigation.

7.3 Recommendation for management

1) Live non-enriched *G. cliftonii* or an inclusion of ≥ 10% dried *G. cliftonii* meal are recommended to feed greenlip abalone due to their positive effects on growth energy. However, stability of the dietary macroalgae inclusion should be also taken into account.
2) Australian feed companies have produced commercial diets containing CP level of ~ 35% for the production of greenlip abalone (Stone et al., 2013). However, 6-month-old abalone cultured at water temperatures up to 20 °C should be fed the dietary CP level of ~ 27 % due to no differenes in somatic growth energy when abalone were fed graded levels of 27, 30, 33 and 36% CP.
3) Dietary supplementation of 5.0% GSE is recommended for feeding greenlip abalone during the summer months when water temperature exceed 23 °C to improve survival rate and feed intake of abalone. However, the supplementation

of the commercial pro/prebiotics and other antioxidants (GTE, PE and VTM C) are unlileky to be useful.

4) Water quality should be carefully managed at low temperature (14 °C) due to an increase in egested faecal energy or at high water temperatures (> 25 °C) due to an increase in ammonia excretion and mucus production energy.

7.4 Conclusions and recommendations for future research

This thesis has laid the foundation for improving the knowledge of energy budgets in greenlip abalone. In respect to bioenergetics, an inclusion of dried or live red macroalgae meal is the better choice for feeding greenlip abalone compared to green macroalgae, while a fomulated diet of ~ 27% CP is recommended for 6-month-old greenlip abalone at seasonal water temperatures. High water temperature negatively affected the bioenergetics of greenlip abalone. An inclusion of 5% GSE has some positive effects on energy budgets. It is recommended that the approach of Macy and Coyne (2005) be adopted for pro/prebiotic application. The results of these studies will assist in predicting ingested food energy, respiration energy, ammonia excretion energy, nutrient effluent levels and somatic growth energy at optimal water temperature (22 ºC) or high temperature (25 ºC) for these tested diets.

To further our understanding on the bioenergetics of greenlip abalone and also to address some questions which are still remainin due to various limitations, further research is recommended as below:

1) Bioenergetics of greenlip abalone fed a combination of live red macroalgae or graded levels of their inclusion in the formulated diet needs further investigation since single red macroalage species had positive effects on bioenergetics of greenlip abalone in the current study. Therefore, mixed red macroalgae is expected to provide better nutrition over single species diets.

2) The bioenergetics of 2-year-old greenlip abalone have not been investigated in the current study due to low availability in anaimals of this size. Nutritional requirements for greenlip abalone may change over time with respect to protein. Therefore, bioenergetics of 2-year-old greenlip abalone is expected to be different from the 1 and 3 year-old greenlip abalone.

3) The results of Chapter 4 show that digestible energy (~ 12.50 MJ kg^{-1}) is sufficient for 6-month-old greenlip abalone at 17 or 22 ºC, but not clear at 14 ºC. Additionally, as this dietary digestible energy was calculated in the recent greenlip abalone studies (Stone et al., 2013; Bansemer et al., 2015a), it is necessary to investigate the optimal dietary digestible energy in response to water temperature and sizes.

4) Mucus production is underestimated in previous reports as well as in the current study due to difficulty in collecting sufficient amounts of mucus which is used in other biological processes, dissolved in the water or washed away by water replacement, developing better techniques to improve the collection of mucus is strongly recommended.

References

Abdel-Tawwab, M., Ahmad, M.H., Seden, M.E., Sakr, S.F., 2010. Use of green tea, *Camellia sinensis* L., in practical diet for growth and protection of Nile tilapia, *Oreochromis niloticus* (L.), against *Aeromonas hydrophila* infection. J. World Aquacult. Soc. 41, 203-213.

Abare., 2011. Australian Bureau of Agricultural and Resource Economics, Canberra, Australia.

Ai, Q., Mai, K., Tan, B., Xu, W., Zhang, W., Ma, H., Liufu, Z., 2006. Effects of dietary vitamin C on survival, growth, and immunity of large yellow croaker, *Pseudosciaena crocea*. Aquaculture 261, 327-336.

Allen V.J., Marsden I.D., Ragg N.L.C., Gieseg S., 2006. The effects of tactile stimulants on feeding, growth, behaviour, and meat quality of cultured Blackfoot abalone, *Haliotis iris*. Aquaculture 257, 294-308.

Angell, A.R., Pirozzi, I., De Nys, R., Paul, N.A., 2012. Feeding preferences and the nutritional value of tropical algae for the abalone *Haliotis asinina*. PloS One 7, p.e. 38857.

AOAC., 1995. Official methods of analysis (16th ed.) Association Official Analytical Chemists, Washington, DC, USA.

Azaza, M.S., Mensi, F., Ksouri, J., Dhraief, M.N., Brini, B., Abdelmouleh, A., Kraiem, M.M., 2008. Growth of Nile tilapia (*Oreochromis niloticus* L.) fed with diets containing graded levels of green algae ulva meal (*Ulva rigida*) reared in geothermal waters of southern Tunisia. J. Appl. Ichthyol. 24, 202-207.

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Bagchi, D.J., Balmoori, J., Stohs, S.J., 1998. Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and

DNA fragmentation, and peritoneal macrophage activation in mice. Gen. Pharmacol- Vasc. S. 30, 771-776.

Balcázar, J.L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Múzquiz, J.L., 2006. The role of probiotics in aquaculture. Vet. Microbiol. 114, 173-186.

Balcázar, J.L., Rojas-Luna, T., Cunningham, D.P., 2007. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. J. Invertebr. Pathol. 96, 147-150.

Bansemer, M.S., Qin, J.G., Harris, J.O., Howarth, G.S., Stone, D.A., 2014. Nutritional requirements and use of macroalgae as ingredients in abalone feed. Rev. Aquaculture 5, 1-15.

Bansemer, M.S., Harris, J.O., Qin, J.G., Adams, L.R., Duong, D.N., Stone, D.A., 2015a. Growth and feed utilisation of juvenile greenlip abalone (*Haliotis laevigata*) in response to water temperatures and increasing dietary protein levels. Aquaculture 436, 13-20.

Bansemer, M.S., Qin, J.G., Currie, K.L., Stone, D.A.J., 2015b. Temperature-dependent feed consumption patterns of greenlip (*Haliotis laevigata*) and hybrid (*H. laevigata* × *Haliotis rubra*) abalone fed fresh macroalgae or a formulated diet. J. Shellfish Res. 34, 885-892.

Bansemer, M.S., Qin, J.G., Harris, J.O., Schaefer, E.N., Wang, H., Mercer, G.J., Howarth, G.S., Stone, D.A., 2016a. Age-dependent response of digestive enzyme activities to dietary protein level and water temperature in greenlip abalone (*Haliotis laevigata*). Aquaculture 451, 451-456.

Bansemer, M.S., Qin, J.G., Harris, J.O., Duong, D.N., Howarth, G.S., Currie, K.L., Stone, D.A., 2016b. Dietary inclusions of dried macroalgae meal in formulated

diets improve the growth of greenlip abalone (*Haliotis laevigata*). J. Appl. Phycol. 1-14

Bansemer, M.S., Qin, J.G., Harris, J.O., Duong, D.N., Hoang, T.H., Howarth, G.S., Stone, D.A., 2016c. Growth and feed utilisation of greenlip abalone (*Haliotis laevigata*) fed nutrient enriched macroalgae. Aquaculture 452, 62-68.

Barkai, R., Griffiths, C.L., 1987. Consumption, absorption efficiency, respiration and excretion in the South African abalone *Haliotis midae*. S. Afr. J. Mar. Sci. 5, 523-529.

Barkai, R., Griffiths, C.L., 1988. An energy budget for the South African abalone, *Haliotis midae* Linnaeus. J. Molluscan Stud. 54, 43-51.

Bautista-Teruel, M.N., Millamena, O.M., 1999. Diet development and evaluation for juvenile abalone, *Haliotis asinina*: protein/energy levels. Aquaculture 178, 117-126.

Bautista-Teruel, M.N., Koshio, S.S., Ishikawa, M., 2011. Diet development and evaluation for juvenile abalone, *Haliotis asinina* Linne: lipid and essential fatty acid levels. Aquaculture 312, 172-179.

Bayne, B.L., Newell, R.C., 1983. Physiological energetics of marine molluscs. In: K.M. Wilbur, editor. The Mollusca 4, 109-139.

Boarder, S.J., Shpigel, M., 2001. Comparative performances of juvenile *Haliotis roei* fed on enriched *Ulva rigida* and various artificial diets. J. Shellfish Res. 20, 653-657.

Bose, M., Lambert, J.D., Ju, J., Reuhl, K.R., Shapses, S.A., Yang, C.S., 2008. The major green tea polyphenol,(-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. J. Nutr. 138, 1677-1683.

Bower, C.E., Holm-Hansen, T., 1980. A salicylate–hypochlorite method for determining ammonia in seawater. Can. J. Fish. Aquat. Sci. 37, 794-798.

Brett, J.R., Higgs, D.A., 1970. Effect of temperature on the rate of gastric digestion in fingerling sockeye salmon, *Oncorhynchus nerka*. J. Fish. Res. Board Can. 27, 1767-79.

Britz, P.J., Hecht, T., Knauer, J., Dixon, M.G., 1994. The development of an artificial feed for abalone farming. S. Afr. J. Sci. 90, 7-8.

Britz, P. J., 1996a. Effect of dietary protein level on growth performance of South African abalone, *Haliotis midae*, fed fishmeal-based semipurified diets. Aquaculture 140, 55-61

Britz, P.J., 1996b. The suitability of selected protein sources for inclusion in formulated diets for the South African abalone, *Haliotis midae*. Aquaculture 140, 63-73.

Britz, P.J., Hecht, T., 1997a. Effect of dietary protein and energy level on growth and body composition of South African abalone, *Haliotis midae*. Aquaculture 156, 195-210.

Britz, P.J., Hecht, T., Mangold, S., 1997b. Effect of temperature on growth, feed consumption and nutritional indices of *Haliotis midae* fed a formulated diet. Aquaculture 152, 191-203.

Bruno, R S., Dugan, C.E., Smyth, J.A., DiNatale, D.A., Koo, S.I., 2008. Green tea extract protects leptin-deficient, spontaneously obese mice from hepatic steatosis and injury. J. Nutr. 138, 323-331.

Bureau, D.P., Hua, K., 2010. Towards effective nutritional management of waste outputs in aquaculture, with particular reference to salmonid aquaculture operations. Aquac. Res. 41, 777-792.

Bursill, C.A., Abbey, M., Roach, P.D., 2007. A green tea extract lowers plasma cholesterol by inhibiting cholesterol synthesis and upregulating the LDL receptor in the cholesterol-fed rabbit. Atherosclerosis 193, 86-93.

Buss, J.J., Jones, D.A., Lumsden, A., Harris, J.O., Bansemer, M.S., Stone, D.A.J., 2015. Restricting feed ration has more effect than diet type on the feeding behaviour of greenlip abalone *Haliotis laevigata*. Mar. Freshw. Behav. Physiol. 48, 51-70.

Carefoot, T.H., Qian, P.Y., Taylor, B.E., West, T., Osborne, J., 1993. Effect of starvation on energy reserves and metabolism in the Northern abalone, *Haliotis kamtschatkana*. Aquaculture 118, 315-325.

Cederberg, J., Simán, C.M., Eriksson, U.J., 2001. Combined treatment with vitamin E and vitamin C decreases oxidative stress and improves fetal outcome in experimental diabetic pregnancy. Pediatr. Res. 49, 755-762.

Cheah, K., 2011. Grape sourced bioactives: a potential new treatment strategy for intestinal mucositis and colon cancer (Ph.D thesis). The University of Adelaide, 131 pp.

Cho, C.Y., Kaushik, S.J., 1990. Nutritional energetics in fish: energy and protein utilization in rainbow trout (*Salmo gairdneri*). World Rev. Nutr. Diet. 61, 132-172.

Cho, S.H., Lee, S.M., Park, B.H., Ji, S.C., Lee, J., Bae, J., Oh, S.Y., 2007. Effect of dietary inclusion of various sources of green tea on growth, body composition and blood chemistry of the juvenile olive flounder, *Paralichthys olivaceus*. Fish Physiol. Biochem. 33, 49-57.

Clarke, S.M., 1988. Abalone mariculture: the significance of algae. Austasia Aquac 3, 9-11.

Cook, P.A., Gordon, R.H., 2010. World abalone supply, markets, and pricing. J. Shellfish Res. 29, 569-571.

Cook, P.A., 2014. The worldwide abalone industry. Mod. Econ. 05, 1181–1186.

Coote, T.A., Hone, P.W., Van Barneveld, R.J., Maguire, G.B., 2000. Optimal protein level in a semipurified diet for juvenile greenlip abalone, *Haliotis laevigata*. Aquacult. Nutr. 6, 213-220.

Cui, Y., Liu, J., 1990. Comparison of energy budget among six teleosts-II. Metabolic rates. Comp. Biochem. Physiol. Part A. Physiology 97, 169-174.

Currie, K.L., Debasis, D., Duong, N.D., Stone, D.A., 2015a. The effect of dietary probiotics and prebiotics on feed intake, health and survival of greenlip abalone, *Haliotis laevigata* Donovan at high water temperature. Abstract presented (oral presentation). 9th International Abalone Symposium 2015. Yeosu, Korea.

Currie, K.L., Lange, B., Herbert, E.W., Harris, J.O., Stone, D.A., 2015b. Gastrointestinal evacuation time, but not nutrient digestibility, of greenlip abalone, *Haliotis laevigata* Donovan, is affected by water temperature and age. Aquaculture 448, 219-228.

Dalmin, G., Kathiresan, K., Purushothaman, A., 2001. Effect of probiotics on bacterial population and health status of shrimp in culture pond ecosystem. Indian J. Exp. Biol. 39, 939-942.

Dang V.T., Li Y., Speck P., Benkendorff K., 2011a. Effects of micro and macroalgal diet supplementations on growth and immunity of greenlip abalone, *Haliotis laevigata*. Aquaculture 320, 91-98.

Dang V.T., Speck P., Doroudi M., Smith B., Benkendorff K., 2011b. Variation in the antiviral and antibacterial activity of abalone *Haliotis laevigata*, *H. rubra* and their hybrid in South Australia. Aquaculture 315, 242-249.

Dang, V.T., Speck, P., Benkendorff, K., 2012. Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*. Fish Shellfish Immunol 32,732-740.

Davies, J.A., Krebs, G.L., Barnes, A., Pant, I., McGrath, P.J., 2009. Feeding grape seed extract to horses: effects on health, intake and digestion. Animal 3, 380-384.

Davies, M.S., 1993. Energetic implications of variation in pedal mucus production by *Patella vulgata Linnaeus*, 1758. Veliger 36, 203-208.

Davies, M.S., Hawkins, S.J., 1998. Mucus from marine molluscs. Adv. Mar. Biol. 34, 1-71.

Décordé, K., Teissèdre, P. L., Sutra, T., Ventura, E., Cristol, J. P., Rouanet, J. M., 2009. Chardonnay grape seed procyanidin extract supplementation prevents high-fat diet-induced obesity in hamsters by improving adipokine imbalance and oxidative stress markers. Mol. Nutr. Food Res. 53, 659-666.

Donovan, D.A., Carefoot, T.H., 1998. Effect of activity on energy allocation in the northern abalone *Haliotis kamtschatkana* (Jonas). J. Shell Res. 17, 729-736.

Duong, D.N., Qin, J.G., Harris, J.O., Hoang, T.H., Bansemer, M.S., Currie, K.L., Phan-Thien, K.Y., Dowell, A., Stone, D.A., 2016. Effects of dietary grape seed extract, green tea extract, peanut extract and vitamin C supplementation on metabolism and survival of greenlip abalone (*Haliotis laevigata* Donovan) cultured at high temperature. Aquaculture 464, 364-373.

Elliott, J.M., Davison, W., 1975. Energy equivalents of oxygen consumption in animal energetics. Oecologia 19, 195-201.

Erasmus, J.H., Cook, P.A., Coyne, V.E., 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. Aquaculture 155, 377-386.

Farías, A., García-Esquivel, Z., Viana, M.T., 2003. Physiological energetics of the green abalone, *Haliotis fulgens*, fed on a balanced diet. J. Exp. Mar. Biol. Ecol. 289, 263-276.

Fleming, A.E., 1995. Digestive efficiency of the Australian abalone *Haliotis rubra* in relation to growth and feed preference. Aquaculture 134, 279-293

Fleming, A. E., Van Barneveld, R.J., Hone P. W. 1996. The development of artificial diets for abalone: A review and future directions. Aquaculture 140, 5-53.

Fleming, A.E., Barneveld, R.J.V., Hone, P.W., Vandepeer, M.E., Kruk, J.A., 1998. Complementary additivity of the digestibility coefficients of feed ingredients fed to juvenile greenlip abalone (*Haliotis laevigata*). J. Shellfish Res. 17, 641-648.

Freeman, K.A., 2001. Aquaculture and related biological attributes of abalone species in Australia-a review. Fisheries Research Report, No. 128, Fisheries Western Australia, 48 pp.

Freeman, K., Daume, S., Rowe, M., Parsons, S., Lambert, R., Maguire, G.B., 2006. Effects of season, temperature control, broodstock conditioning period and handling on incidence of controlled and uncontrolled spawning of greenlip abalone (*Haliotis laevigata* Donovan) in Western Australia. J Shellfish Res. 25, 187-194.

Frei, B., Stocker, R., Ames, B.N., 1988. Antioxidant defenses and lipid peroxidation in human blood plasma. P. Natl. Acad. Sci. USA 85, 9748-9752.

Ganmanee, M., Sirirustananun, N., Jarayabhand, P., 2010. Energy budget of the Thai abalone, *Haliotis asinina*, reared in a semiclosed recirculating land-based system. J. Shellfish Res. 29, 637-642.

García-Esquivel, Z., Montes-Magallón, S., González-Gómez, M.A., 2007. Effect of temperature and photoperiod on the growth, feed consumption, and

biochemical content of juvenile green abalone, *Haliotis fulgens*, fed on a balanced diet. Aquaculture 262, 129-141.

Gatesoupe, F. J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147-165.

Gilroy, A., Edwards, S.J., 1998. Optimum temperature for growth of Australian abalone: preferred temperature and critical thermal maximum for blacklip abalone, *Haliotis rubra* (Leach), and greenlip abalone, *Haliotis laevigata* (Leach). Aquacult. Res. 29, 481-485.

Gomez-Gil, B., Roque, A., Turnbull, J.F., 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191, 259-270.

Gómez-Montes, L., García-Esquivel, Z., D'Abramo, L.R., Shimada, A., Vásquez-Peláez, C., Viana, M.T., 2003. Effect of dietary protein: energy ratio on intake, growth and metabolism of juvenile green abalone *Haliotis fulgens*. Aquaculture 220, 769-780.

Goulletquer, P., Wolowicz, M., 1989. The shell of *Cardium edule*, *Cardium glaucum* and *Ruditapes philippinarum*: organic content, composition and energy value, as determined by different methods. J. Mar. Biol. Assoc. U.K. 69, 563-572.

Green, A.J., Jones, C.L., Britz, P.J., 2011. The protein and energy requirements of farmed South African abalone, *Haliotis midae* L. cultured at optimal and elevated water temperatures. Aquacult. Res. 42, 1653-1663.

Guillard, R.R., Ryther, J.H., 1962. Studies of marine planktonic diatoms: I. *Cyclotella nana Hustedt*, and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol. 8, 229–239.

Hadi, J.A., Gutierrez, N., Alfaro, A.C., Roberts, R.D., 2014. Use of probiotic bacteria to improve growth and survivability of farmed New Zealand abalone (*Haliotis iris*). New Zeal. J. Mar. Fresh. Res. 48, 405-415.

Hahn, K.O., 1989. Handbook of culture of abalone and other marine gastropods. CRC Press, Boca Raton, Florida, 348 pp.

Harris, J.O., Maguire, G.B., Edwards, S., Hindrum, S.M., 1997. Effect of nitrite on growth and oxygen consumption for juvenile greenlip abalone *Haliotis laevigata* Donovan. J. Shellfish Res. 16, 395-401.

Harris, J.O., Maguire, G.B., Edwards, S., Hindrum, S.M., 1998a. Effect of ammonia on the growth rate and oxygen consumption of juvenile greenlip abalone *Haliotis laevigata* Donovan. Aquaculture 160, 259-272.

Harris, J.O., Maguire, G.B., Handlinger, J.H., 1998b. Effects of chronic exposure of greenlip abalone, *Haliotis laevigata* Donovan, to high ammonia, nitrite, and low dissolved oxygen concentrations on gill and kidney structure. J. Shellfish Res. 17, 683-687.

Harris, J.O., Maguire, G.B., Edwards, S.J., Hindrum, S.M., 1999a. Effect of pH on growth rate, oxygen consumption rate, and histopathology of gill and kidney tissue for juvenile greenlip abalone, *Haliotis laevigata* Donovan and blacklip abalone, *Haliotis rubra* Leach. J. Shellfish Res. 18, 611-619.

Harris, J.O., Maguire, G.B., Edwards, S.J., Johns, D.R., 1999b. Low dissolved oxygen reduces growth rate and oxygen consumption rate of juvenile greenlip abalone, *Haliotis laevigata* Donovan. Aquaculture 174, 265-278.

Harris, J.O., Burke, C.M., Edwards, S.J., Johns, D.R., 2005. Effects of oxygen supersaturation and temperature on juvenile greenlip, *Haliotis laevigata* Donovan, and blacklip, *Haliotis rubra* Leach, abalone. Aquacult. Res. 36, 1400-1407.

Hart, A. M., Fabris, F., Brown, J., Caputi, N., 2013. Biology, history, and assessment of Western Australian abalone fisheries. Fisheries Research Report, pp 241.

Hindrum, S., Burke, C., Edwards, S.J., Johns, D., 2001. Effects of combined exposure to elevated ammonia and low dissolved oxygen levels in greenlip (*Haliotis laevigata* Donovan) and blacklip (*H. rubra* Leach) abalone. 1. Growth and mortality data from simulated systems failure. J. Shellfish Res. 20, 679-684.

Hoang, T.H., Qin, J.G., Stone, D.A., Harris, J.O., Duong, D.N., Bansemer, M.S., 2016. Colour changes of greenlip abalone (*Haliotis laevigata* Donovan) fed fresh macroalgae and dried algal supplement. Aquaculture 456, 16-23.

Hone, P.W., Fleming, A.E., 1998. Abalone, in: Hyde, K. (Ed). The new rural industries - A handbook for farmers and investors. Rural Industries and Development Corporation, Canberra, pp. 85-92.

Hone, P.W., Van Barneveld, R.J., Maguire, G.B., 2000. Optimal protein level in a semipurified diet for juvenile greenlip abalone *Haliotis laevigata*. Aquacult. Nutr. 6, 213-220.

Hooper, C., Day, R., Slocombe, R., Benkendorff, K., Handlinger, J., Goulias, J., 2014. Effects of severe heat stress on immune function, biochemistry and histopathology in farmed Australian abalone (hybrid *Haliotis laevigata* × *Haliotis rubra*). Aquaculture 432, 26-37.

Huchette, S.M., Koh, C.S., Day, R.W., 2003. Growth of juvenile blacklip abalone (*Haliotis rubra*) in aquaculture tanks: effects of density and ammonia. Aquaculture 219, 457-470.

Hwang, J.H., Lee, S.W., Rha, S.-J., Yoon, H.S., Park, E.S., Han, K.H., Kim, S.J., 2013. Dietary green tea extract improves growth performance, body

composition, and stress recovery in the juvenile black rockfish, *Sebastes schlegeli*. Aquacult. Int. 21, 525-538.

Inagake, M., Yamane, T., Kitao, Y., Oya, K., Matsuraoto, H., Kikuoka, N., Nakatani, H., Takahashi, T., Nishimura, H., Iwashiraa, A., 1995. Inhibition of 1,2-dimethylhydrazine-induced oxidative DNA damage by green tea extract in rat. Cancer Sci. 86, 1106-1111.

Irianto, A., Austin B., 2002. Probiotics in aquaculture. J. Fish. Dis. 25, 633-642.

Jobling, M., 1993. Bioenergetics: feed intake and energy partitioning. In: J.C. Rankin and F.B. Jensen (Editors), Fish Ecophysiology. Chapman and Hall, London, pp. 1-44.

Kao, T.T., Tu, H.C., Chang, W.N., Chen, B.H., Shi, Y.Y., Chang, T.C., Fu, T.F., 2010. Grape seed extract inhibits the growth and pathogenicity of *Staphylococcus aureus* by interfering with dihydrofolate reductase activity and folate-mediated one-carbon metabolism. Int. J. Food Microbiol. 141, 17-27.

Kaushik, S.J., 1986. Environmental effects on feed utilization. Fish Physiol. Biochem. 2, 131-140.

Kirkendale, L., Robertson-Andersson, D.V., Winberg, P.C., 2010. Review on the use and production of algae and manufactured diets as feed for sea-based abalone aquaculture in Victoria. University of Wollongong, Shoalhaven Marine & Freshwater Centre, Nowra, 198 pp.

Koga, T., Moro, K., Nakamori, K., Yamakoshi, J., Hosoyama, H., Kataoka, S., Ariga, T., 1999. Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds. J. Agr. Food Chem. 47, 1892-1897.

Krishnaprakash, R., Saravanan, R., Murugesan, P., Rajagopal, S., 2009. Usefulness of probiotics in the production of high quality shrimp (*Penaeus monodon*) seeds in hatcheries. J. World. Zool. 4, 144-147.

Lange, B., Currie, K.-L., Howarth, G.S., Stone, D.A.J., 2014. Grape seed extract and dried macroalgae, *Ulva lactuca* Linnaeus, improve survival of greenlip abalone, *Haliotis laevigata* Donovan, at high water temperature. Aquaculture 433, 348-360.

Lawrence, J.M., Lane, J.M., 1982. The utilization of nutrients by postmetamorphic echinoderms. In: Jangoux, M., Lawrence, J.M. (Eds.), Echinoderm Nutrition. A.A. Balkema Publishers, Rotterdam, Netherlands, 368–371.

Leighton, D.L., 1974. The influence of temperature on larval and juvenile growth in three species of southern California abalones. Fish. Bull 72, 1137-1145.

Li, J., Tan, B., Mai, K., 2009. Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). Aquaculture 291, 35-40.

Lim, T.J., Lee, S.M., 2003. Effect of dietary pigment sources on the growth and shell color of abalone (*Haliotis discus hannai*). Korean J. Fisher. Aqua. Sci. 36, 601-605.

Löest, H.B., Noh, S.K., Koo, S.I., 2002. Green tea extract inhibits the lymphatic absorption of cholesterol and α-tocopherol in ovariectomized rats. J. Nutr. 132, 1282-1288.

Lopez, L.M., Tyler, P., 2006. Energy budget of cultured female abalone *Haliotis tuberculata* (L). J. Shellfish Res. 25, 385-389.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13-30.

Lyon, G., 1995. Aspects of the physiology of the South African abalone *Haliotis midae* L., and implications for intensive abalone culture. M.Sc. thesis, Rhodes University, Grahamstown, 85 pp.

Macey, B.M., Coyne, V.E., 2005. Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. Aquaculture 245, 249-261.

Macey, B.M., Coyne, V.E., 2006. Colonization of the gastrointestinal tract of the farmed South African abalone *Haliotis midae* by the probionts *Vibrio midae* SY9, *Cryptococcus* sp. SS1, and *Debaryomyces hansenii* AY1. Mar. Biotechnol. 8, 246-259.

Mai, K., Mercer, J.P., Donlon, J., 1994. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino: II. Amino acid composition of abalone and six species of macroalgae with an assessment of their nutritional value. Aquaculture 128, 115-130.

Mai, K., Mercer, J.P., Donlon, J., 1995a. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. III. Response of abalone to various levels of dietary lipid. Aquaculture 134, 65-80.

Mai, K., Mercer, J.P., Donlon, J., 1995b. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. IV. Optimum dietary protein level for growth. Aquaculture 136, 165-180.

Mai, K., Mercer, J.P., Donlon, J., 1996. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. V. The role of polyunsaturated fatty acids of macroalgae in abalone nutrition. Aquaculture 139, 77-89.

Mai, K., 1998. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino.: VII. Effects of dietary

vitamin C on survival, growth and tissue concentration of ascorbic acid. Aquaculture 161, 383-392.

Manning, T. S., G. R. Gibson., 2004. Prebiotics. Best Pract. Res. Clin. Gastroenterol. 18, 287-298.

McBride, S.C., Rotem, E., Ben-Ezra, D., Shpigel, M., Cook, P.A., 2001. Seasonal energetics of *Haliotis fulgens* (Philippi) and *Haliotis tuberculata* (L). J. Shellfish Res. 20, 659-665.

McGaw, I.J., Whiteley, N.M., 2012. Effects of acclimation and acute temperature change on specific dynamic action and gastric processing in the green shore crab, *Carcinus maenas*. J. Therm. Biol. 37, 570-578.

Mercer, J.P., Mai, K.S., Donlon, J., 1993. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* Linnaeus and *Haliotis discus hannai* Ino. I. Effects of algal diets on growth and biochemical composition. Invertebr. Repr. Dev. 23, 75-88.

Merchie, G., Lavens, P., Dhert, P., Pector, R., Soni, A.F., Nelis, H., Sorgeloos, P., 1995. Live food mediated vitamin C transfer to *Dicentrarchus labrax* and *Clarias gariepinus*. J. Appl. Ichthyol. 11, 336-341.

Merchie, G., Lavens, P., Dhert, P., Góamez, M., Nelis, H., De Leenheer, A., Sorgeloos, P., 1996. Dietary ascorbic acid requirements during the hatchery production of turbot larvae. J. Fish Biol. 49, 573-583.

Merchie, G., Lavens, P., Sorgeloos, P., 1997. Optimization of dietary vitamin C in fish and crustacean larvae: a review. Aquaculture, 155, 165-181.

Misra, C. K., Das, B. K., Mukherjee, S. C., Pradhan, J., 2007. Effects of dietary vitamin C on immunity, growth and survival of Indian major carp *Labeo rohita*, fingerlings. Aquacult. Nutr. 13, 35-44.

Montaño-Vargas, J., Shimada, A., Vásquez, C., Viana, M.T., 2002. Methods of measuring feed digestibility in the green abalone (*Haliotis fulgens*). Aquaculture 213, 339-346.

Montaño-Vargas, J., Viana, M.T., D'abramo, L.R., Shimada, A., Vasquez-Pelaez, C., 2005. Growth and energy utilization of juvenile pink abalone, *Haliotis corrugata* fed diets containing different levels of protein and two starch: lipid ratios. J. Shellfish Res. 24, 1179-1185.

Morash, A.J., Alter, K., 2015. Effects of environmental and farm stress on abalone physiology: perspectives for abalone aquaculture in the face of global climate change. Rev. Aquacult. 1-27.

Mottet, M.G., 1978. A review of the fishery biology of abalones. Washington Department of Fisheries. Technical Report. No. 37, 81 pp.

Mozqueira, A., 1996. Site selection for land based abalone farming. In: Forster, A. (Ed). Proceedings of the Abalone Aquaculture Workshop, December, 1995, Albany, Western Australia. Aquaculture Development Council and Fisheries Department of Western Australia. 17-21.

Naidoo, K., Maneveldt, G., Ruck, K., Bolton, J.J., 2006. A comparison of various seaweed-based diets and formulated feed on growth rate of abalone in a land-based aquaculture system. J. Appl. Phycol. 18, 437-443.

Neori, A., Shpigel, M., Ben-Ezra, D., 2000. A sustainable integrated system for culture of fish, seaweed and abalone. Aquaculture 186, 279-291.

Nepote, V., Grosso, N.R., Guzmán, C.A., 2005. Optimization of extraction of phenolic antioxidants from peanut skins. J. Sci. Food Agri. 85, 33-38.

Ogino, C., Ohta, E., 1963. Studies on the nutrition of abalone. I. Feeding trials of abalone, *Haliotis discus* Reeve, with artificial diets. Bull. Jpn. Soc. Sci. Fish., 29, 691-694.

Ogino, C., Kato, N., 1964. Studies on the nutrition of abalone. II. Protein requirements for growth of abalone, *Haliotis discus*. Bull. Jpn. Soc. Sci. Fish. 30, 523-526.

Olmos, J., Paniagua-Michel, J., 2014. *Bacillus subtilis* a potential probiotic bacterium to formulate functional feeds for aquaculture. J. Microbial. Biochem. Technol. 6, 361-365.

O'Mahoney, M., Rice, O., Mouzakitis, G., Burnell, G., 2014. Towards sustainable feeds for abalone culture: evaluating the use of mixed species seaweed meal in formulated feeds for the Japanese abalone, *Haliotis discus hannai*. Aquaculture 430, 9-16.

Park, C.J., Kim, S.Y., 2013. Abalone aquaculture in Korea. J. Shellfish Res. 32, 17–19.

Peck, L.S., Culley, M.B., Helm, M.M., 1987. A laboratory energy budget for the ormer *Haliotis tuberculata* L. J. Exp. Mar. Biol. Ecol. 23, 104-123.

Perumalla, A.V.S., Hettiarachchy, N.S., 2011. Green tea and grape seed extracts - Potential applications in food safety and quality. Food Res. Int. 44, 827-839.

Prosser, C.L., 1991a. Comparative animal physiology, environmental and metabolic animal physiology. New York: Wiley-Liss. 1-13

Prosser, C.L., 1991b. Temperature, in: Prosser, C.L. (Ed.), Comparative Animal Physiology, Environmental and Metabolic Animal Physiology. Wiley-Liss, New York, 109-165.

Randall, D.J., Wright, P.A., 1987. Ammonia distribution and excretion in fish. Fish Physiol. Biochem. 3, 107-120.

Rayment, G., Lyons, D., 2011. Soil Chemical Methods - Australasia. CSIRO Publishing, Collingwood. 495 pp.

Reddy-Lopata, K., Auerswald, L., Cook, P., 2006. Ammonia toxicity and its effect on the growth of the South African abalone *Haliotis midae* Linnaeus. Aquaculture 261, 678-687.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasaveta, P., 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture 191, 271-288.

Ringø, E., Olsen, R.E., Gifstad, T.Ø., Dalmo, R.A., Amlund, H., Hemre, G.I., Bakke, A.M., 2010. Prebiotics in aquaculture: a review. Aqua. Nutr. 16, 117-136.

Rychly, J., 1980. Nitrogen balance in trout: II. Nitrogen excretion and retention after feeding diets with varying protein and carbohydrate levels. Aquaculture 20, 343-350.

Saito, M., Hosoyama, H., Ariga, T., Kataoka, S., Yamaji, N., 1998. Antiulcer activity of grape seed extract and procyanidins. J. Agric. Food Chem. 46, 1460-1464.

Sales, J., Truter, P.J., Britz, P.J., 2003. Optimum dietary crude protein level for growth in South African abalone (*Haliotis midae* L.). Aquacult. Nutr. 9, 85-89.

Sawabe, T., Setoguchi, N., Inoue, S., Tanaka, R., Ootsubo, M., Yoshimizu, M., Ezura, Y., 2003. Acetic acid production of *Vibrio halioticoli* from alginate: a possible role for establishment of abalone *V. halioticoli* association. Aquaculture 219, 671-679.

Shepherd, S.A., 1973. Studies on southern Australian abalone (genus *Haliotis*). I. Ecology of five sympatric species. Mar. Freshwater Res. 24, 217-258.

Shepherd, S.A., Turner, J.A., 1985. Studies on southern Australian abalone (genus *Haliotis*). VI. Habitat preference, abundance and predators of juveniles. J. Exp. Mar. Biol. Ecol. 93, 285-298.

Shpigel, M., Ragg, N.L., Lupatsch, I., Neori, A., 1999. Protein content determines the nutritional value of the seaweed *Ulva lactuca* L. for the abalone *Haliotis tuberculata* L. and *H. discus hannai* Ino. J. Shellfish Res. 18, 227-234.

Shumway S.E., 1982. Oxygen consumption in oysters: an overview. Mar. Biol. Lett. 3, l-23

Stone, D.A., Harris, J.O., Wang, H., Mercer, G.J., Schaefer, E.N., Bansemer, M.S., 2013. Dietary protein level and water temperature interactions for greenlip abalone *Haliotis laevigata*. J. Shellfish Res. 32, 119-130.

Stone, D.A.J., Bansemer, M.S., Harris, J.O., 2014a. Development of formulated diets for cultured abalone Final report. Australian Seafood Cooperative Research Centre Bedford Park SA. 131 pp. http://pir.sa.gov.au/data/assets/pdf_file/0007/262987/CRC_Cultured_Abalone_Report_-_FINAL.pdf.

Stone, D.A., Bansemer, M.S., Lange, B., Schaefer, E.N., Howarth, G.S., Harris, J.O., 2014b. Dietary intervention improves the survival of cultured greenlip abalone (*Haliotis laevigata* Donovan) at high water temperature. Aquaculture 430, 230-240.

Stuart, M.D., Brown, M.T., 1994. Growth and diet of cultivated black-footed abalone, *Haliotis iris* (Martyn). Aquaculture 127, 329-337.

Taghon, G.L., Self, R.F., Jumars, P.A., 1978. Predicting particle selection by deposit feeders: a model and its implications. Limnol. Oceanogr. 23, 752-759.

Taylor, B., 1992. Abalone nutrition: optimum protein levels in artificial diets for *Haliotis kamtschatkana*. J. Shellfish Res. 11, pp 556.

Ten Doeschate, K.I., Coyne, V.E., 2008. Improved growth rate in farmed *Haliotis midae* through probiotic treatment. Aquaculture 284, 174-179.

Tung, C.H., Alfaro, A.C., 2011. Effect of dietary protein and temperature on the growth and health of juvenile New Zealand black-footed abalone (*Haliotis iris*). Aquacult. Res. 42, 366-385.

Uki, N., Kemuyama, A., Watanabe, T., 1986. Optimum protein level in diets for abalone *Haliotis discus hannai*. Bull. J. Soc. Sci. Fish. 52, 1005-1012.

Van Barneveld, R., 2008. Aquaculture nutrition subprogram: technical, project management and review services. Fisheries Research and Development Corporation and Barneveld Nutrition Pty Ltd, 2008.

Van Keulen, J., Young, B.A., 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. J. Anim. Sci. 44, 282-287.

Vandepeer M., 2005. Abalone aquaculture subprogram: manufactured diet development. FRDC project No. 96/385. Fisheries Research and Development Corporation and the Abalone Aquaculture Subprogram 2005. Alice Springs, N. T. pp 193.

Vandepeer, M., 2006. Abalone aquaculture subprogram: preventing summer mortality of abalone in aquaculture systems by understanding interactions between nutrition and water temperature. Fisheries Research and Development Corporation and the South Australian Research and Development Institute Aquatic Sciences Centre, West Beach, South Australia. Publication No. RD02/0035-2. SARDI Aquatic Sciences, 54pp.

Viana, M.T., López, L.M., Salas, A., 1993. Diet development for juvenile abalone *Haliotis fulgens* evaluation of two artificial diets and macroalgae. Aquaculture 117, 149-156.

Viana, M.T., Cervantes-Trujano, M., Solana-Sansores, R., 1994. Attraction and palatability activities in juvenile abalone (*Haliotis fulgens*): nine ingredients used in artificial diets. Aquaculture 127, 19-28.

Viera, M.P., Pinchetti, J.G., de Vicose, G.C., Bilbao, A., Suárez, S., Haroun, R.J., Izquierdo, M.S., 2005. Suitability of three red macroalgae as a feed for the abalone *Haliotis tuberculata coccinea* Reeve. Aquaculture 248, 75-82.

Viera, M.P., de Vicose, G.C., Gómez-Pinchetti, J.L., Bilbao, A., Fernandez-Palacios, H., Izquierdo, M.S., 2011. Comparative performances of juvenile abalone (*Haliotis tuberculata coccinea* Reeve) fed enriched vs non-enriched macroalgae: effect on growth and body composition. Aquaculture 319, 423-429.

Vosloo, D., Vosloo, A., 2010. Response of cold-acclimated, farmed South African abalone (*Haliotis midae*) to short-term and long-term changes in temperature. J. Therm. Biol 35,317-323.

Vosloo, D., Vosloo, A., Morillion, E.J., Samuels, J.N., Sommer, P., 2013. Metabolic readjustment in juvenile South African abalone (*Haliotis midae*) acclimated to combinations of temperature and dissolved oxygen levels. J. Therm. Biol. 38, 458-466.

Wahbeh, M.I., 1997. Amino acid and fatty acid profiles of four species of macroalgae from Aqaba and their suitability for use in fish diets. Aquaculture 159, 101-109.

Wang, Y.B., Li, J.R., Lin, J., 2008. Probiotics in aquaculture: challenges and outlook. Aquaculture 281, 1-4.

Weber, J.M., Ruzindana-Umunyana, A., Imbeault, L., Sircar, S., 2003. Inhibition of adenovirus infection and adenain by green tea catechins. Antiviral Res. 58,167-173.

Wells, F.E., Mulvay, P., 1995. Good and bad fishing areas for *Haliotis laevigata*: a comparison of population parameters. Mar. Freshwater Res. 46, 591-598.

Xu, X., Xie, H., Wang, Y., Wei, X., 2010. A-type proanthocyanidins from lychee seeds and their antioxidant and antiviral activities. J. Agric. Food Chem. 58, 11667-11672.

Yamakoshi, J., Kataoka, S., Koga, T., Ariga, T., 1999. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. Atherosclerosis 142, 139-149.

Yang, S.D., Liou, C.H., Liu, F.G., 2002. Effects of dietary protein level on growth performance, carcass composition and ammonia excretion in juvenile silver perch (*Bidyanus bidyanus*). Aquaculture 213, 363-372.

Yokozawa, T., Nakagawa, T., Kitani, K., 2002. Antioxidative activity of green tea polyphenol in cholesterol-fed rats. J. Agri. Food Chem. 50, 3549-3552.

Yousef, M.I., Saad, A.A., El-Shennawy, L.K., 2009. Protective effect of grape seed proanthocyanidin extract against oxidative stress induced by cisplatin in rats. Food Chem. Toxicol. 47, 1176-1183.

Yu, J., Ahmedna, M., Goktepe, I., 2005. Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. Food Chem. 90, 199-206.

Yu, J., Ahmedna, M., Goktepe, I., Dai, J., 2006. Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. J. Food Compos. Anal. 19, 364-371.

Yukihira, H., Lucas, J.S., Klumpp, D.W., 2000. Comparative effects of temperature on suspension feeding and energy budgets of the pearl oysters, *Pinctada margaritifera*, and *P. maxima*. Mar. Ecol. Prog. Ser. 195, 179-188.

Zhai, S.W., Lu, J.J., Chen, X.H., 2014. Effects of dietary grape seed proanthocyanidins on growth performance, some serum biochemical

parameters and body composition of tilapia (*Oreochromis niloticus*) fingerlings. Ital. J. Anim. Sci. 13, 536-540.

Ziaei-Nejad, S., Rezaei, M.H., Takami, G.A., Lovett, D.L., Mirvaghefi, A.R. and Shakouri, M., 2006. The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus.* Aquaculture 252, 516-524.