# Digestive physiology and utilisation of macroalgae as feed for Australian abalone


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## Summary

Greenlip (*Haliotis laevigata*) and hybrid (*H. laevigata* × *H. rubra*) abalone are extensively farmed marine gastropods, and primarily grown in land-based systems throughout southern Australia. Limited understanding of nutritional requirement impedes Australian abalone aquaculture, which forms the main thrust of this thesis. The main objectives of this thesis were to: (i) understand the interaction between age, water temperature and dietary protein on abalone growth and digestive physiology; and (ii) provide a fundamental understanding on the effective use of macroalgae as feed for cultured Australian abalone.

There is a demand to introduce multi-diet feeding strategies for greenlip abalone by optimising the dietary protein level for each age class and water temperature throughout the production cycle. However, the optimal dietary protein level for greenlip abalone in the production cycle is not clear. Abalone at 20 °C exhibited significantly superior growth, protein deposition, and feed conversion ratios (FCR) to those at 14 °C. Dietary protein level did not affect abalone growth. Faster growing animals at 20 °C up-regulated feed intake when fed low protein diets to increase protein intake and maximise growth. We recommend that greenlip abalone be fed at ~35% dietary protein at 20 °C. In contrast, there were no apparent benefits by feeding high protein diets to abalone at 14 or 17 °C. We recommend that 6-month old greenlip abalone be fed with 29% dietary protein at <17 °C to improve growth and feed efficiency.

Diet changes could alter digestive enzyme activities, but the response of digestive enzymes to the level of protein in formulated diets is not clearly understood. The digestive enzyme activities of juvenile (1-year old) and sub-adult (2-year old) greenlip abalone fed crude protein from 24 to 36% at 14, 18 and 22 °C were investigated. Trypsin, α-amylase and lipase activities were influenced differently by abalone age, water temperature and dietary

protein levels. The trypsin activity down-regulation by age suggests that dietary protein for 2-year-old abalone could be lower than for 1-year old greenlip abalone.

Although greenlip abalone are fed formulated diets on-farm, in the wild they predominantly consume macroalgae. In Australia, feeding macroalgae to greenlip abalone was previously limited due to the prohibition of wild macroalgae collection on mainland Australia. Recently however, there has been interest to develop an Australian macroalgae aquaculture industry, which would be capable of supplying high quality feed for farmed abalone. While *Ulva* sp. stimulated abalone feeding activity, animals had lower nutrient intake than those fed formulated diets, due to the high moisture content in fresh algae.

To improve protein density, the effect of nitrogen enrichment and the type of macroalgae species on the growth and feed utilisation of greenlip abalone were investigated. Abalone fed *G. cliftonii* outperformed those fed *Ulva* sp., but the benefit of nutrient enrichment on macroalgae was species-dependent. While abalone fed enriched *Ulva* sp. exhibited superior growth to those fed non-enriched *Ulva* sp., abalone fed non-enriched and enriched *G. cliftonii* exhibited similar growth. Abalone fed non-enriched *G. cliftonii* had higher protein deposition and protein efficiency ratio than those in other dietary treatments. Feeding *Ulva* sp. and *G. cliftonii* together had a positive synergistic effect on abalone growth. However, abalone fed commercial formulated diets exhibited faster growth than those fed either type of fresh macroalgae.

While feeding fresh macroalgae to abalone led to sub-optimal growth, dried macroalgae meals were promising ingredients for abalone diets. Abalone fed *Gracilaria* sp. meal. outperformed those fed *Ulva* sp. meal. Abalone fed 5% *Gracilaria* sp. meal or *Ulva* sp. meal exhibited superior growth to abalone fed 0%. Increasing dietary *Gracilaria* sp. to >10% led to further growth improvements, but reduced protein and energy retentions. In contrast, abalone fed 10 and 20% *Ulva* sp. exhibited similar growth to those fed 0 and 5% *Ulva* sp. We

recommend a dietary inclusion of 10% *Gracilaria* sp. or 5% *Ulva* sp. to improve abalone growth.

This research provides new knowledge of the protein requirements in juvenile greenlip abalone. The changes of digestive enzyme activities in abalone depend on age, water temperature and dietary protein. Furthermore, this study indicates that greenlip abalone fed fresh macroalgae had slower growth compared with those fed formulated diets, but inclusions of dried macroalgae improve abalone growth. This research contributes to the improvement of dietary formulations and improved abalone production in Australia.

# 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.

Matthew Bansemer

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# Chapter 1: General introduction

## 1.1 Introduction

In Australia, abalone aquaculture is one of the fastest growing sectors, and in the past decade the industry value has increased from <$1 million in 1998-99, to ~$34 million in 2013, and is expected to increase further over the next decade (Stone et al., 2014a). Two abalone types, the greenlip abalone (*Haliotis laevigata*) and hybrid abalone (*H. laevigata* × *Haliotis rubra*) are currently cultured in land-based systems throughout southern Australia (Stone et al., 2014a). Formulated diets are crucial to the success of Australian abalone aquaculture, culture systems are typical designed to suit the physical characteristics of the diet chips and the nutritional profile of formulated diets can be manipulated to suit the optimal nutritional requirement of the animal (Stone et al., 2013).

In a separate review (Bansemer et al., 2014a), the nutritional requirements of abalone and the use of macroalgae as a feed ingredient for cultured abalone have been addressed. Based on this review, two major issues were identified to impede the further development of Australian abalone aquaculture: nutrition and health. The main thrust of this thesis is focused on improving the nutritional knowledge for greenlip abalone. The main aim of this research is the improved diet formulations and feeding practices for Australian abalone, and the new knowledge of water temperature dependent growth in Australian abalone, which will ultimately improve the production of abalone in Australia.

Feed costs account for up to 30% of the production costs for Australian cultured abalone (Stone et al., 2014a). Protein is an expensive macronutrient in abalone formulated diets (Fleming and Hone, 1996; Britz and Hecht, 1997; Shipton and Britz, 2001). Numerous investigations into the optimal dietary protein level in formulated diets with the ultimate aim of reducing farm expenses and increasing abalone production have been performed (Mai et al., 1995; Britz, 1996a; Britz and Hecht, 1997; Bautista-Teruel and Millamena, 1999; Coote et al., 2000; Dunstan, 2010; Stone et al., 2013). There is an increasing demand to introduce

multi-diet feeding strategies for greenlip abalone. Multi-diet feeding strategies (prestarter, starter, grower and finisher diets) have been successful for other aquaculture species including rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and Nile tilapia (*Oreochromis niloticusa*) (Ng and Romano, 2013; Sarker et al., 2013). In order for multi-diet feeding strategies to be successfully implemented into the feeding regime of cultured greenlip abalone there is a need to improve our understanding of the effects of seasonal water temperatures on the growth of greenlip abalone. Australian abalone farms experience geographical and temporal water temperature fluctuations, which can range from < 10 °C in Tasmania during winter to > 24 °C in South Australia during summer (Stone et al., 2013; Bansemer et al., 2015b). Water temperature affects almost every aspect of farmed abalone production including survival and growth and nutritional requirements (Britz et al., 1997; Steinarsson and Imsland, 2003; Stone et al., 2013). Stone et al. (2013) reported the optimal dietary protein level for greenlip abalone increased with increasing water temperature, but differed between age classes. As water temperature increased from 14 to 18 to 22 °C, the optimal dietary crude protein levels for 1-year old abalone increased from ~29.0 to 32.2 to 34.7% crude protein (CP), while the optimal protein level for 2-year old abalone increased from 24 to 34 and 34% CP (Stone et al., 2013). The optimal dietary protein levels for greenlip abalone throughout their whole production cycle are not clear. Further research focused on the nutritional requirements of greenlip abalone soon after weaning (~6-month old abalone) at different water temperatures is crucial to introduce multi-diet feeding strategies for greenlip abalone.

In addition to understanding the growth and feed utilisation response in greenlip abalone to dietary and temperature manipulation, understanding of the digestive physiology of greenlip abalone at different ages, water temperatures and dietary protein levels improves our knowledge on the digestive capacity of abalone. The utilisation of dietary protein, and

other macronutrients, by abalone is dependent on digestive enzyme activities and digestive enzyme-substrate contact time in the gastrointestinal tract, both of which limits the digestive capacity of aquatic animals (Fountoulaki et al., 2005; Currie et al., 2015). Previous research has identified the independent effects of age, water temperature and diet dependent digestive enzyme activities in abalone (Knauer et al., 1996; Erasmus et al., 1997; Edwards and Condon, 2001; Johnston et al., 2005). However, the interactive effects between age, water temperature and dietary protein level are not clearly understood.

During summer in southern Australia, sunset is not until ~20:30 h, and total darkness may not occur until ~21:30 h (Geoscience Australia 2014). On farm, abalone are typically fed before 17:00 h, and provided with feed throughout the night. Nutrient leaching loss from formulated diets during extended periods of immersion is a major concern to the abalone industry (Fleming et al. 1996, Ruff et al. 2014). Due to the nocturnal and slow feeding activity of abalone, understanding the nocturnal feeding patterns of abalone is essential to successfully manage on-farm feeding practices (Fleming et al., 1996; Buss et al., 2015).

There is also a dearth of information on the growth and feed utilisation of greenlip abalone fed live macroalgae compared to current commercial formulated diets. While Australian abalone farmers primarily feed formulated diets to cultured abalone, live macroalgae form a major dietary component of wild abalone. Fresh macroalgae are utilised for abalone feed in numerous countries including China, Korea and Chile. In Australia, while feeding fresh macroalgae to abalone was previously limited due to the prohibition of wild macroalgae collection on mainland Australia, Australia is in the early stages of developing a macroalgae aquaculture industry (Lorbeer et al., 2013; Nayar and Bott, 2014). The digestive system of abalone is anatomically and biochemically suited to digest macroalgae (Erasmus et al., 1997; Harris et al., 1998a; Takami et al., 1998). The composition of dietary ingredients currently utilised in commercially formulated abalone diets is distinctly different to

macroalgae (reviewed by Bansemer et al., 2014a). There are also numerous other benefits of feeding macroalgae to abalone, including growth, feeding attraction and stimulation, health and product quality, compared to abalone fed a formulated diet (reviewed by Bansemer et al., 2014a).

Previous studies investigating the growth performance of abalone fed live macroalgae and commercial formulated diets are limited and often conflicting (Naidoo et al., 2006; Hernández et al., 2009; Dang et al., 2011a; Mulvaney et al., 2013a). In addition, the preference of abalone for macroalgae is species specific and it is important to consider the specific response of greenlip abalone. International abalone species including blackfoot abalone (*Haliotis iris*; Cornwall et al., 2009), South African abalone (*Haliotis midae*; Britz, 1996a), red abalone (*Haliotis rufescens*; Flores-Aguilar et al., 2007) and Pacific abalone (*Haliotis discus hannai*; Flores-Aguilar et al., 2007) generally prefer brown macroalgae species. In contrast, temperate Australian abalone, blacklip abalone and greenlip abalone, generally prefer red macroalgae and *Ulva* spp. (Shepherd & Steinberg 1992; Fleming 1995a). Caution needs to be taken when extrapolating the results of feeding live macroalgae to other abalone species to greenlip abalone. Live macroalgae is a promising dietary ingredient for greenlip abalone.If live macroalgae are fed to cultured greenlip abalone, growth needs to be on-par or exceed current on-farm formulated diets. This area has not been explored thoroughly in past research.

Dried macroalgae meals are also promising dietary ingredients for abalone, and may be more practical than feeding live macroalgae to abalone. Two recent studies have investigated the growth performance of abalone fed dietary inclusions of dried macroalgae meal in formulated diets exist (O'Mahoney et al., 2014; Viera et al., 2015). O'Mahoney et al. (2014) reported similar growth, but superior feed efficiency for Pacific abalone (*Haliotis discus hannai*) when fed a formulated diet with dietary inclusions of dried macroalgae meal with a

combination of dried oarweed (*Laminaria digitata*), dulse (*Palmaria palmate*) and sea lettuce (*Ulva lactuca*), compared to abalone fed fresh *L. digitata*. In contrast, Viera et al. (2015) reported inferior growth rates for abalone (*H. tuberculata coccinea*) fed a formulated diet with a 43% dietary inclusion of mix dried macroalgae meal (*U. rigida, G. cornea, L. digitata, P. palmata*) compared to abalone fed live macroalgae alone. These studies may have had some limitations due to feeding regime or diet stability problems. If these problems are accounted for in the design of future experiments, there is a considerable scope to improve the growth of cultured abalone with macroalgae as a supplement.

## 1.2 Overall study objectives

The two main objectives of this study were to improve the knowledge of greenlip abalone nutrition pertaining to the interaction between age, seasonal water temperature fluctuations and dietary protein and to provide a fundamental understanding and practical and cost effective means to use macroalgae as feed for cultured Australian abalone.

In a separate review (Bansemer et al., 2014a), the nutritional requirements of abalone and the use of macroalgae as a feed ingredient for cultured abalone were assessed. Based on the information from the literature review, the following research aims were identified:

1. To identify the optimal protein level for post-weaned greenlip abalone at different seasonal water temperatures.
2. To identify changes to the digestive enzyme activities in greenlip abalone throughout the production cycle, with regards to age, water temperature and dietary protein level.
3. To understand feeding behaviour, growth and feed utilisation of abalone fed fresh macroalgae and formulated diets.
4. To identify the type and amount of dried macroalgae meal required to achieve the "algal effect" in formulated abalone feeds.

Five trials were conducted to address these aims. The experimental trials were:

1. Trial 1: Growth and feed utilisation of juvenile greenlip abalone in response to water temperatures and increasing dietary protein levels. This trial addressed aim 1, and is presented in Chapter 3.
2. Trial 2: Age-dependent response of digestive enzymes to dietary protein level and water temperature in greenlip abalone. This trial addressed aim 2, and is presented in Chapter 4.
3. Trial 3: Temperature-dependent feed consumption patterns for greenlip and hybrid abalone fed fresh macroalgae or a formulated diet. This trial addressed part of aim 3 and results are presented in Chapter 5.
4. Trial 4: Growth and nutrient utilisation of greenlip abalone fed nitrogen enriched macroalgae. This trial addressed aim 3, and results are presented in Chapter 6.
5. Trial 5: Dried macroalgal meal inclusions in formulated diets for greenlip abalone. This trial addressed aim 4, and result are presented in Chapter 7.

## 1.3 Thesis outline

This thesis is presented in eight chapters, a general thesis introduction, a literature review, five data chapters and a general discussion. Chapters 3 - 7 have been published, or are currently under review in peer-reviewed journals.

Chapter 1 is a general introduction to this thesis that outlined the major nutritional gaps in knowledge for abalone aquaculture. While there is a demand to introduce multi-diet feeding strategies for greenlip abalone by optimising the dietary protein level for each age class and water temperature throughout the production cycle, the optimal dietary protein level for greenlip abalone throughout their whole production cycle is not clear. Moreover, the

effect of manipulating the dietary protein level at different life stages and water temperatures on the digestive physiology of greenlip abalone is not well understood. In addition, while formulated diets are seen as crucial to the Australian abalone aquaculture industry, feeding macroalgae has the potential to improve production. A more detailed review of the nutritional requirements and use of macroalgae as ingredients in abalone feed are covered in Chapter 2.

In Chapter 2, the nutritional requirements and use of macroalgae as ingredients in abalone feed is reviewed. This chapter has been published in Reviews in Aquaculture (Bansemer et al., 2014a). In this review, the optimal dietary protein and energy for abalone is discussed and dietary ingredients that are currently used in commercial formatted diets that are fed on-farm in Australia. A number of benefits to the health, feeding behaviour and product quality of abalone fed macroalgae compared to formulated diets were identified. Based on this information and the identified knowledge gaps, the first experiment was designed to investigate the interaction between dietary protein level and water temperature on the growth performance and feed utilisation of post-weaned greenlip abalone.

In Chapter 3, the influence of the interaction between dietary protein level and water temperature on the growth performance and feed utilisation of post-weaned greenlip abalone were investigated. This chapter has been published in Aquaculture (Bansemer et al., 2015b). This chapter addressed aim 1, based on Trial 1. In this chapter, I aimed to identify the optimal protein level for post-weaned greenlip abalone at different water temperatures.

Chapter 4 is a sister chapter to Chapter 3. As the digestive capacity of abalone is dependent on the quantity and the types of digestive enzyme, in this chapter the effects of age, water temperature and dietary protein level on digestive enzyme activities in greenlip abalone were investigated. This chapter has been accepted for publication in Aquaculture, and is currently undergoing revisions. This chapter addresses aim 2, based on Trial 2. The digestive enzyme activities of juvenile (1-year old) and sub-adult (2-year old) greenlip

abalone fed crude protein levels ranging from 24 to 36% at 14, 18 and 22 °C were collected from a growth study published by Stone et al. (2013). Results from this study provide an improved understanding of the digestive physiology of cultured greenlip abalone throughout their production cycle at different seasonal water temperatures when fed increased dietary protein levels.

Chapter 5 has been accepted for publication in the Journal of Shellfish Research, and is currently in press. This chapter addresses part of aim 3 of this thesis, based on data from Trial 3. In Chapter 5, the apparent feed consumption at night for greenlip abalone and hybrid abalone fed fresh *Ulva* sp. or a commercial formulated diet at 18 or 22°C was investigated. This study aimed to understand the nocturnal feeding patterns of abalone to improve farmed abalone feed management and productivity. As a follow up trial, the growth performance and feed utilisation of greenlip abalone fed live macroalgae were investigated.

In Chapter 6, the growth and feed utilisation of greenlip abalone fed cultured, fresh, nutrient enriched macroalgae were investigated. This chapter addresses aim 3, based on data from Trial 4. This chapter has been submitted to Aquaculture, and is currently under review. Two macroalgae were selected as excellent feed for abalone, *Ulva* sp. and *Gracilaria cliftonii*. The performance of abalone fed live macroalgae was compared to abalone fed the three commercial formulated diets, which were being fed to cultured Australian abalone at the time of the study.

In Chapter 7, the growth, feed utilisation and digestive enzyme activity of greenlip abalone fed dietary inclusions of dried enriched macroalgae meal (*Ulva* sp. and *Gracilaria cliftonii*) were investigated. This chapter addressed aim 4, based on data from the Trial 5. This chapter is currently under review in Aquaculture.

Chapter 8 is the general discussion of this thesis, where all major research outcomes are compiled and summarised. Final recommendations to the abalone industry to improve

production are also given in this chapter. Further research, not investigated in this thesis, is recommended.

## 1.4 Publications

### *1.4.1 Co-authorship of chapters*

Chapter 2, 3, 4, 5, 6 and 7 are presented in stand-alone manuscript format suitable for a journal in the field of aquaculture. As a result, there is unavoidable repetition between chapters, particularly in methods and background. I wrote all chapters, but each chapter is co-authored due to contributions from other people. Each chapter is co-authored by Professor Jian Qin and Associate Professor David A.J. Stone, due to major contributions to experimental design, sample analysis and the preparation of each manuscript. Associate Professor James O. Harris is co-author for Chapters 2, 3, 4, 6 and 7 due to major contributions to experimental design, sample analysis and the preparation of each manuscript. Professor Gordon S. Howarth is co-author for Chapters 2, 4, 6 and 7 due to his contribution towards the digestive enzyme component and manuscript preparation. Dr. Louise Adams is a co-author for Chapter 3, due to her contribution towards the design and running of the study, and also aided in sample and data analysis. Mr. Duong N. Duong is a co-author for Chapters 3, 6 and 7 due to his contribution towards running the study and sample analysis. Mrs. Thanh Hai Hoang is a co-author for Chapter 6 due to her contribution of running the study. Mrs. Elise Schaefer, Miss Georgia Mercer and Mrs. Hanru Wang are co-authors for Chapter 4 due to their contribution running the study. Miss Krishna-Lee Currie is a co-author for Chapters 5 and 7 due to her contribution in designing and running these studies.

### *1.4.2 Thesis publications*

***Chapter 2:*** Matthew S. Bansemer, Jian G. Qin, James O. Harris, Gordon S. Howarth and David A.J. Stone (2014). Nutritional requirements and use of macroalgae as ingredients in abalone feed. Reviews in Aquaculture, doi: 10.1111/raq.12085.

***Chapter 3:*** Matthew S. Bansemer, James O. Harris, Jian G. Qin, Louise R. Adams, Duong N. Duong and David A.J. Stone (2015). Growth and feed utilisation of juvenile greenlip abalone (*Haliotis laevigata*) in response to water temperatures and increasing dietary protein levels. Aquaculture, 436, 13-20.

***Chapter 4:*** Matthew S. Bansemer, Jian G. Qin, James O. Harris, Elise N. Schaefer, Hanru Wang, Georgia J. Mercer , Gordon S. Howarth and David A.J. Stone (2016). Age-dependent response of digestive enzyme activities to dietary protein level and water temperature in greenlip abalone (*Haliotis laevigata*). Aquaculture, 451, 451-456.

***Chapter 5:*** Matthew S. Bansemer, Jian G. Qin, Krishna-Lee Currie and David A.J. Stone (2015). Temperature-dependent feed consumption patterns of greenlip (*Haliotis laevigata*) and hybrid (*H. laevigata* × *Haliotis rubra*) abalone fed fresh macroalgae or a formulated diet. Journal of Shellfish Research, 34 (3), 885-892.

***Chapter 6:*** Matthew S. Bansemer, Jian G. Qin, James O. Harris, Duong N. Duong, Thanh Hai Hoang, Gordon S. Howarth and David A.J. Stone (2016). Growth and feed utilisation of greenlip abalone (*Haliotis laevigata*) fed nutrient enriched macroalgae. Aquaculture, 452, 62-68.

***Chapter 7:*** Matthew S. Bansemer, Jian Qin, James O. Harris, Duong N. Duong, Krishna-Lee Currie, Gordon S. Howarth and David A.J. Stone (Under review; submitted 9/11/2015). Dietary inclusions of dried macroalgae meal in formulated diets

improve the growth of greenlip abalone (*Haliotis laevigata*). Journal of Applied Phycology, JAPH-D-15-00577.

# Chapter 2: Nutritional requirements and use of macroalgae as ingredients in abalone feed

Matthew S. Bansemer, Jian G. Qin, James O. Harris, Gordon S. Howarth and David A.J. Stone (2014). Nutritional requirements and use of macroalgae as ingredients in abalone feed. Reviews in Aquaculture, doi: 10.1111/raq.12085.

## 2.1 Abstract

Temperate abalone species in aquaculture have a grow-out period of approximately 3 years because of their slow and heterogeneous growth rate. Abalone aquaculture is still a developing industry, and at least two major issues, nutrition and health, impede its development. Abalone are fed macroalgae on-farm in a number of countries, including China, Korea, South Africa and Chile. Formulated diets are crucial to the success of abalone aquaculture in countries where abalone do not grow readily when fed brown macroalgae or when access to macroalgae is insufficient for culture. Typically, the growth rate of abalone fed formulated diets is higher than abalone fed live macroalgae. Due to a long production period, diets for abalone not only need to sustain high growth rates, but also maintain optimal health. Feeding live macroalgae improves the feeding activity, health and marketability of abalone. In this review, the nutritional requirements of abalone and dietary ingredients used in formulated diets will be discussed, together with the use of dried macroalgal meal as a dietary ingredient in formulated diets to improve the feeding activity, health and marketability of abalone in commercial production.

## 2.2 Introduction

The global demand for abalone (*Haliotis* spp.), primarily by Asian countries, has far out-weighed market supply since the late 1990s (Cook and Gordon, 2010). The high demand for abalone has caused a decline of wild stocks, but at the same time has stimulated the growth of global abalone aquaculture (Gordon and Cook, 2004; Cook and Gordon, 2010). Global abalone production increased by 270% to 86,455 metric tonnes per year from 2007 to 2011 (FAO, 2013). Abalone aquaculture is advantageous over the wild abalone fishery, as farms are not subject to size or quota limitations and can increase production without adding

pressure on wild abalone stocks. However, at least two issues, nutrition and health, impede the development of abalone aquaculture.

The digestive system of abalone is anatomically and biochemically suited to their predominantly macroalgae-based diet (Erasmus et al., 1997; Takami et al., 1998; Harris et al., 1998a; García-Carreño et al., 2003). Abalone are fed macroalgae on-farm in a number of countries, including China, Korea, South Africa and Chile (Daume, 2006; Kirkendale et al., 2010; Fotedar and Phillips, 2011). Over the past 10 years, there has been a rapid increase in macroalgae cultivation (Lee, 2008; FAO, 2013). The species of macroalgae cultured is vast and includes nori (*Porphyra* spp.), wakame (*Undaria pinnatifida*), kombu (*Laminaria japonica*), the red alga (*Gracilaria* spp.) and the sea lettuce (*Ulva* spp.) (Reviewed by Wikfors and Ohno, 2001). The largest proportion of the macroalgae aquaculture industry supplies macroalgae for human food in Asia (Wikfors and Ohno, 2001). However, a number of cultured macroalgae species, including *Gracilaria* spp. and *Ulva* spp., are excellent abalone feed (Shpigel et al., 1999; Naidoo et al., 2006; Daume et al., 2007; Viera et al., 2011).

Formulated diets are crucial to the success of abalone aquaculture in countries where abalone do not grow readily when fed brown macroalgae or are cultured in land-based systems. Abalone are also fed formulated diets on-farm during times of macroalgae shortages or to enhance growth rates (Fleming and Hone, 1996; Bautista-Teruel et al., 2011). Abalone diets not only need to sustain high growth rates, but also maintain optimal health and result in a superior product. Feeding abalone live macroalgae has been reported to improve health, product quality and feeding behaviour, compared to feeding formulated diets (Allen et al., 2006; Brown et al., 2008; Dang et al., 2011a; Chojnacka et al., 2012; Stone et al., 2014b). Feeding live macroalgae to abalone however, can be problematic due to the high moisture content and low nutrient density, inconsistent supply and the introduction of diseases, pests,

predators and competitors to culture systems (Bautista-Teruel et al., 2011). Recently, there has been increasing interest to utilise dried macroalgae meal as a dietary ingredient in formulated diets for abalone, and may be a more practical option to gain the benefits of feeding macroalgae to abalone (Viera et al., 2012; O'Mahoney et al., 2014; Stone et al., 2014b).

A number of publications relating to abalone nutrition are available in the existing literature. For example, Fleming et al. (1996) reviewed the development of artificial diets for abalone with the aim to identify future directions for research. Kawamura et al. (1998) reviewed the feeding and growth of post larval abalone, focusing on post settlement. Daume (2006) later reviewed the bottleneck in supplying feed to post-larval abalone. More recently, Hannon et al. (2013) reviewed the technical challenges facing aquaculture of the green ormer (*Haliotis tuberculata* L.) in Ireland and identified settlement cues, feeding transitions and artificial feeds as major challenges for the future development of this species. Research on the potential benefits of using macroalgae and macroalgae meal as a feed ingredient is increasing. However, there is a distinct deficiency in the literature in terms of a review on the use of macroalgae and their meal in abalone farming. Therefore, this review covers: (i) the nutritional requirements of abalone, (ii) the use of macroalgae as abalone feed, and (iii) the benefits of dried macroalgae inclusions as a dietary ingredient and identification of directions for future research.

## 2.3 Nutritional requirements of abalone in aquaculture

### *2.3.1 Dietary protein*

Protein is an expensive dietary component and plays a major role in the nutritional value of abalone feed. The availability of dietary protein initially limits protein deposition, in turn, affecting optimal growth of abalone (Fleming and Hone, 1996; Britz and Hecht, 1997;

Shipton and Britz, 2001). However, deamination of excess dietary protein occurs to supply energy for metabolism rather than tissue growth, resulting in increased feed costs and ammonia excretion (Chaitanawisuti et al., 2011). Many studies have investigated the optimal dietary protein level in formulated diets to reduce farm expenses and increase abalone production (Mai et al., 1995; Britz, 1996a; Britz and Hecht, 1997; Bautista-Teruel and Millamena, 1999; Coote et al., 2000; Dunstan, 2010; Stone et al., 2013).

Digestive enzymes degrade dietary protein to dipeptides and amino acids, which can then be absorbed, transported and ultimately used for protein deposition, energy and growth. There are 22 natural amino acids, of which ten cannot be synthesised by animals, termed essential amino acids. In addition to a diet that is digestible, it is important to supply the right balance of essential amino acids in the diet to ensure optimal protein deposition and growth (Fleming et al., 1996). Fleming et al. (1996) reviewed the amino acids requirement of abalone and concluded that due to the difficulty in formulating diets to determine the requirement of individual amino acids by abalone, applying the ideal protein concept is an acceptable alternative. This concept relies on the basis that the requirement of dietary amino acid composition is similar to the soft tissue of the animal. The requirement of each amino acid is calculated based on the lysine level (5% of the total soft tissue protein in greenlip abalone (*Haliotis laevigata* Donovan) (Coote et al., 2000), which is the most limiting amino acid in many aquaculture species, so that all other amino acids are provided in excess (Fleming et al., 1996). Shipton et al. (2002) investigated the dietary lysine requirement of the South African abalone (*Haliotis midae* L.), but was unsuccessful using microencapsulation of crystalline lysine to retard the leaching loss. Shipton et al. (2002) suggested alternative methods, including post-prandial tissue or serum free amino acid studies and serum oxidation studies, to determine the dietary lysine requirements of abalone. Providing abalone with the optimal

balance of dietary essential amino acids may result in improvements to abalone growth, compared to using the ideal protein concept.

**Table 2.1**. The optimal protein level for different abalone species at different water temperature and animal size from selected studies.

| Abalone species | Water temperature (°C) | Average starting weight (g) | Optimal dietary protein level (%) | Specific growth rate (% body weight day$^{-1}$) | Main protein source | References |
|---|---|---|---|---|---|---|
| South African abalone (*Haliotis midae*) | 21.7 | 1.46 | 47 | 1.1 | Fish meal | Britz (1996a) |
| | 18 | 0.2 - 1 | 34 | 2.2 | Fish meal | Britz and Hecht (1997) |
| | 18 | 7 - 14 | 44 | 0.5 | Fish meal | Britz and Hecht (1997) |
| Greenlip abalone (*Haliotis laevigata)* | 20 | 0.81 | 27 | 1.0 | Semolina and Casein | Coote et al. (2000) |
| | 14 | 1.8 | 29 | 0.7 | Solvent-extracted soybean meal, lupins and fish meal | Stone et al. (2013) |
| | 22 | 1.8 | 35 | 1.4 | Same as above | Stone et al. (2013) |
| | 14 | 22.9 | 24 | 0.3 | Same as above | Stone et al. (2013) |
| | 22 | 22.9 | 34 | 0.5 | Same as above | Stone et al. (2013) |
| Blacklip abalone (*Haliotis rubra*) | 17 | 0.04 | 45 | 5.1 | Defatted soya flour and fish meal | Dunstan (2010) |
| Green Ormer (*Haliotis tuberculata*) | 13 - 15 | 0.183 | 35 | 1.1 | Casein and gelatin with crystalline amino acids | Mai et al. (1995) |
| Pacific abalone (*Haliotis discus hannai***)** | 13 - 15 | 0.378 | 35 | 0.9 | Same as above | Mai et al. (1995) |

The reported optimal dietary crude protein levels for abalone growth range from 24% crude protein for *H. laevigata* (Stone et al., 2013) to as high as 47% crude protein for *H. midae* (Britz, 1996a; Table 2.1). However, limited studies have been conducted on the effect of biotic and abiotic factors, such as animal age and water temperature, on the protein requirements of abalone (Britz and Hecht, 1997; Stone et al., 2013). When studies have focused on these factors, the nutritional requirements differ between species (Table 2.1). For example, as water temperature increased from 14 to 18 to 22 °C, the optimal dietary crude protein level for 1-year old *H. laevigata* (1.8 g) increased from ~29.0 to 32.2 to 34.7% (Stone et al., 2013). Moreover, the authors suggested similar or slightly lower optimum protein levels for larger 2-year old *H. laevigata,* but the optimal dietary crude protein level similarly increased from 24 to 34 and 34% as water temperature increased from 14 to 18 to 22 °C. The higher dietary protein requirements of smaller *H. laevigata* or as water temperature increased may have been due to the higher scope for growth during these periods. For example, the specific growth rate (SGR) of 1-year old *H. laevigata* was 300% greater than the SGR of 2-year old abalone (Stone et al., 2013; Table 2.1). Moreover, the SGR of 1-year old *H. laevigata* was significantly 140% superior at 22 °C compared to 14 °C (Stone et al., 2013). In contrast, the optimal protein level for growth of large *H. midae* (7.0 - 14.0 g) was 44% crude protein, whereas the optimal protein level for growth of smaller (0.2 - 1.0 g) abalone was 34% crude protein (Britz and Hecht, 1997). These discrepancies could have been due to a number of reasons including size differences, water temperature differences and variation in response between species or difference in diet formulation. Diets used by Britz and Hecht (1997) were formulated on a crude basis, whereas diets used by Stone et al. (2013) were formulated on a digestible basis, which also likely influenced the results. When designing future experiments, researchers should account for all of these factors.

Water temperature governs the body temperature of poikilothermic organisms and significantly influences metabolic rate, enzymatic activity, growth and survival (Britz et al., 1997; Edwards and Condon, 2001; Hochachka and Somero, 2002; Stone et al., 2013). Protease activity in blacklip abalone (*Haliotis rubra* Leach) is dependent on temperature and significantly increased by 75% from 9 °C to 24 °C (Edwards and Condon, 2001). Increased protease activity in *H. rubra* might increase dietary protein utilisation at higher water temperatures and contribute to higher growth rates when the optimal water temperature of 17 °C is reached (Gilroy and Edwards, 1998). Stone et al. (2013) observed an increased utilisation of dietary protein by 1-year old *H. laevigata* (1.8 g) at higher temperatures and recommended a crude dietary protein level of 34.7% at 22 °C, whereas the authors recommend a dietary protein level of 29% at 14 °C (Table 2.1).

The abalone industry is relatively new compared to other aquaculture sectors such as fish culture, and is still developing. The development of pre-starter, starter, grower and finisher diets in other aquaculture sectors, including rainbow trout (*Oncorhynchus mykiss* Walbaum), Atlantic salmon (*Salmo salar* L) and Nile tilapia (*Oreochromis niloticusa* L.), has been successful (Ng and Romano, 2013; Sarker et al., 2013). Further improvements to abalone diet formulation, concentrating on optimising dietary protein level for environmental change and life stages, will ultimately lead to increased production and reduced expenses.

### *2.3.2 Dietary energy*

Typically, the dietary gross energy in formulated diets for abalone is ~16 - 17 MJ $kg^{-1}$ (Mai et al., 1995; Bautista-Teruel and Millamena, 1999; Coote et al., 2000). If dietary protein and amino acid are not limiting, dietary energy is the next limiting factor. If the dietary energy level does not meet the energy requirement of abalone, a reduction in growth, feed conversion and survival occurs (Green et al., 2011). However, if dietary energy is in excess

of the requirement of the animal, the feed consumption is affected. For example, the feed consumption rate of *H. midae* fed high-energy diets was significantly lower than abalone fed low-energy diets, and therefore, has implications on the intake of other nutrients, namely protein and amino acids, and vitamins and minerals (Green et al., 2011). The optimal dietary energy level is dependent on a number of factors including the digestibility of ingredients, levels of dietary lipid, carbohydrate and protein (Britz and Hecht, 1997; Bautista-Teruel and Millamena, 1999), animal size (Britz and Hecht, 1997) and the temperature-dependent energy requirement of the animal (Green et al., 2011).

Wild abalone are adapted to require low levels of dietary lipids and have a barely detectible lipase activity when fed a formulated diet with 6% dietary crude lipid (Britz et al., 1996). The *H. midae* fed a dietary inclusion of 10% crude lipid resulted in reduced growth rates, protein deposition, and protein efficiency ratio compared to abalone fed diets containing 2 - 6% crude lipid (Britz and Hecht, 1997). The authors concluded that 10% dietary crude lipid was too high to maintain normal growth in abalone (Britz and Hecht, 1997). Similarly, Dunstan et al. (2000) suggested that *H. laevigata* require ~3.5% dietary crude lipid.

To reduce feed costs, carbohydrates should preferably satisfy the energy requirements of abalone (Dunstan, 2010). Wild abalone predominately consume carbohydrate-rich macroalgae, as such, abalone are able to digest the majority of carbohydrates in macroalgae. Abalone and gut-associated bacteria are able to hydrolyse both structural and reserve carbohydrates with a number of different carbohydrases (e.g., agarase, carrageenase, carboxymethylcellulase, alginate lyase, laminarinase, α-amylase, maltase, sucrase, and β-galactosidase) (Britz et al., 1996; Knauer et al., 1996; Erasmus et al., 1997; Harris et al., 1998b; Takami et al., 1998; Enríquez et al., 2001; Kemp, 2001). Due to the high carbohydrase activity in the gastrointestinal system, the energy digestibility coefficient for

numerous dietary ingredients used in formulated diets is high. For example, the energy digestibility coefficient for dehulled narrow-leaved lupin (*Lupinus angustifolius* L.), whole pale yellow lupin (*Lupinus luteolus* Kellogg), casein, skim milk powder, whey powder and pre–gelatinised maize starch is above 75% (Fleming et al., 1998; Vandepeer, 2002; Vandepeer and van Barneveld, 2003).

### *2.3.3 Dietary ingredients*

Ideally, formulated diets should contain ingredients that are palatable, digestible, provide adequate nutrients and have a balanced amino acid profile (Britz, 1996b; Bautista-Teruel and Millamena, 1999; Bautista-Teruel et al., 2003). Among others, fish meal, shrimp meal, poultry meal, casein, meat and bone meal, defatted soybean meal, corn gluten meal, *Spirulina* spp. and torula yeast have been investigated in experimental diets for abalone (Britz, 1996b; Cho, 2010; Bautista-Teruel et al., 2003). Fish meal, cereal grains, oilseeds and pulses are commonly used as major protein source in commercial formulated diets for abalone (Britz, 1996b; Bautista-Teruel and Millamena, 1999; Bautista-Teruel et al., 2011; Stone et al., 2013). However, there are potential ecological, economical or nutritional problems associated with these ingredients.

Fish meal, as an exclusive protein source (44% dietary inclusion), supports sufficient growth for *H. midae* (Britz, 1996b).The aquaculture industry is tending to reduce its reliance on fish meal, primarily due to a substantial increase in price and a reduction in availability, and is turning to more economical and ecologically sustainable protein sources (Hardy and Tacon, 2002). In addition, feeding fish meal-based formulated diets compared to plant-based formulated diets may affect the taste of abalone, as the use of fish oil in formulated diets has been suggested to result in a “fishier” flavour in cultured abalone (Dunstan et al., 1996).

The economical and ecologically sustainable plant protein sources currently used in commercial formulated diets include soybean meal and lupin meal. Further research is required, as these ingredients result in growth reductions or are detrimental to the health of abalone. For example, soybean meal may not provide the optimal essential amino acids for abalone growth, resulting in an overestimation of the optimal protein level (Coote et al., 2000). In addition, soybean meal contains a number of antinutrients including oligosaccharides, β-conglycinin, glycinin, lectins, phytic acid-P, saponins and trypsin inhibitors (Francis et al., 2001; Gatlin et al., 2007). Antinutrients in soybean meal have been reported to cause a reduction to the protective distal intestine mucus layer in yellowtail kingfish (*Seriola lalandi* Valenciennes), which subsequently resulted in the first stages of sub-acute enteritis (Bansemer et al., 2015a). Moreover, sensitive fish species, such as *O. mykiss* and *S. salar*, also exhibit sub-acute enteritis when fed soybean meal (van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996). Despite detrimental effects of feeding soybean meal to fish, the effect of antinutrients, in soybean meal and other terrestrial meals, on the gastrointestinal tract of abalone, is unknown. Schaefer et al. (2013) reported significant reduction to the crop epithelial thickness in 1-year old *H. laevigata* fed 33% dietary crude protein level compared to 24% dietary crude protein, corresponding to 24% and 16% dietary solvent extracted soybean meal, respectively. The authors suggested that this might have been a compensatory change, due to a thinning of stomach epithelium at increased water temperatures, by reducing epithelial thickness, thereby increasing crop surface area and digestive capacity when fed higher dietary inclusions of soybean meal (Schaefer et al., 2013). Antinutrients such as polyphenolic compounds, namely phlorotannins (tannins) in brown macroalgae species, such as kelp (*Ecklonia radiata* C. Agardh), were suggested to result in a reduced preference and feeding behaviour by *H. rubra*, which may have implications for dietary inclusions of soybean meal (Fleming et al., 1995a). The concentration of

phlorotannins in *E. radiata* is up to 15% (dry weight) (Steinberg, 1989), while the concentration of phlorotannins in soybean meal is 0.84% (Collins et al., 2013).There are also numerous other antinutrients in cereal grains, oilseeds and pulses used in formulated diets for abalone on the animals and the effect these have on the feeding behaviour of abalone is unknown. In addition to future investigation into the potential damage to the gastrointestinal tract, it would also be beneficial to investigate the effect of antinutrients in dietary ingredients in formulated diets on the feeding activity and dietary preference of abalone.

## 2.4 Macroalgae as a dietary ingredient

### *2.4.1 Nutritional characteristics of macroalgae*

Macroalgae are fast growing multicellular, photosynthetic organisms, classified into three major groups: brown algae Phaeophyta, red algae Rhodophyta, and green algae Chlorophyta (Lee, 2008). The reserve and structural carbohydrates in macroalgae make up to 50% of the dry weight (Table 2.2), and differ between the major groups (Table 2.3). The digestive system of abalone is biochemically adapted to digest both reserve and structural carbohydrates (Britz et al., 1996; Knauer et al., 1996; Erasmus et al., 1997; Harris et al., 1998b; Takami et al., 1998; Kemp, 2001; Enríquez et al., 2001). There is limited information available on the digestibility of reserve and structural carbohydrates in macroalgae. In contrast, a number of studies have investigated the digestibility of terrestrial plant ingredients used in formulated diets, which have observed distinct differences between the digestibility of reserve and structural carbohydrates.

The major reserve carbohydrate in terrestrial plants is starch, which consists of glucose repeating units with α-(1,4) glycosidic linkages. The apparent gross energy digestibility (ADC%) of pregelled starch fed to *H. laevigata* was 93% (Vandepeer et al., 2003), indicating that abalone are able to effectively digest and utilise terrestrial reserve carbohydrates as an

energy source. In contrast, abalone have a limited ability to digest terrestrial cellulose, the major structural carbohydrate in terrestrial plants. Cellulose consists of glucose repeating units, but differs from starch by β-(1-4) glycosidic linkages between glucose units. The apparent gross energy digestibility of whole *L. angustifolius* by *H. laevigata* was 50%, while the apparent gross energy digestibility of dehulled *L. angustifolius* was 83% (Vandepeer et al., 2002). The hull of *L. angustifolius* is composed of up to 58% cellulose. Removing the hull of *L. angustifolius* reduces the cellulose concentration, thereby, increasing the concentration of reserve carbohydrates (galactose, arabinose and uronic acid) and subsequent apparent gross energy digestibility (Vandepeer et al., 2002). The authors suggested that although cellulase activity occurs in the abalone gut, cellulose found in the hulls of *L. angustifolius* might be difficult to hydrolyse compared to substrates used in cellulase assays, carboxymethylcellulose, due to the complexity of untreated cellulose and the presence of lignin, which may act as a cellulase inhibitor (Vandepeer et al., 2002).

**Table 2.2** Proximate composition of macroalgal diets and commercial formulated diets fed to abalone from selected studies

| Proximate composition (g $100g^{-1}$ dry matter) | Moisture (%) | Crude Protein | Lipid | Total Carbohydrate | Energy (MJ $kg^{-1}$) | References |
|---|---|---|---|---|---|---|
| *Diet* | | | | | | |
| Commercial formulated diet (Australia) | 12.7 | 32.8 | 5.4 | 56.0 | 19.3 | Mulvaney et al. (2013a) |
| Commercial formulated diet (South Africa) | 10.0 | 35.0 | 5.0 | 43.0 | NR[4] | Troell et al. (2006) |
| *Macrocystis pyrifera* | 91.4 | 11.7 | 0.6 | NR[4] | 11.1 | Hernandez et al. (2009) |
| *Ulva rigida* (Non-enriched)[1] | 82.1 | 16.6 | 3.7 | 56.4 | 14.7 | Viera et al. (2012) |
| *U. rigida* (Protein-enriched)[2] | 82.0 | 33.8 | 4.4 | 40.5 | 16.3 | Viera et al. (2012) |
| *Gracilaria cornea* (Non-enriched)[1] | 83.9 | 11.27 | 5.4 | 58.0 | 15.1 | Viera et al. (2012) |
| *G. cornea* (Protein-enriched)[2] | 84.9 | 29.4 | 7.2 | 31.8 | 15.1 | Viera et al. (2012) |

[1] Supplied with fresh seawater
[2] Enriched with sediment free, effluents from fishponds
[3] Enriched with inorganic nutrients (disodium phosphate and ammonium sulphate)
[4]NR: Not reported

**Table 2.3** Reserve and structural carbohydrates in macroalgae.

| Macroalgae group | Reserve carbohydrates | Structural carbohydrates | References |
|---|---|---|---|
| Brown algae (Phaeophyta) | Laminarin | Phycocolloids, Cellulose, Alginic acid (Alginate) and Fucoidan | McCandless (1981); Evans (1989); Seigler 1998 |
| Red algae (Rhodophyta) | Floridean starch | Cellulose, Agar and Carrageenan | McCandless (1981); Evans (1989); Seigler (1998) |
| Green algae (Chlorophyta) | Amylose and Amylopectin | Cellulose and Ulvan | McCandless 1981; Lahaye & Robic (2007) |

Some macroalgae species also contain cellulose (Table 2.3), but the composition is different from terrestrial plants (Knoshaug et al., 2013). Cellulose from macroalgae and terrestrial plants crystallise in different arrangements with different dimensions. The cellulose in macroalgae is dominated by the Iα allomorph which has a triclinic unit cell containing one chain, while the cellulose in terrestrial plants is dominated by the Iβ allomorph which has a monoclinic unit cell containing two parallel chains (Knoshaug et al., 2013). The digestibility of cellulose from terrestrial sources and macroalgae may differ due to these differences and the specificity of enzymes, but is currently unknown. In addition to cellulose, macroalgae also contain a number of other structural carbohydrates (Table 2.3). While Fleming (1995b) did not report the apparent gross energy digestibility of macroalgae by *H. rubra*, the apparent dry matter digestibility was as high as 78% for the red alga (*Laurencia botryoides* C. Agardh). It seems that while abalone have a limited ability to digest terrestrial cellulose, they may be able to utilise a reasonable proportion of the structural carbohydrates in macroalgae.

### *2.4.2 Feeding abalone live macroalgae*

Abalone are fed macroalgae on-farm in a number of countries, including China, Korea, South Africa and Chile (Daume, 2006; Kirkendale et al., 2010; Fotedar and Phillips, 2011; Park and Kim, 2013). However, due to conflicting results in the literature pertaining to

feeding live macroalgae, it is difficult to differentiate the effect of feeding live macroalgae or formulated diets on the growth rate of abalone (Table 2.4).

**Table 2.4** Results from selected growth studies on the effect of feeding abalone live macroalgae as compared to formulated diets.

| Abalone species | Diets tested | Biological effect | References |
|---|---|---|---|
| South African abalone (*Haliotis midae*) | Formulated diet; *Ecklonia maxima*; *E. maxima* + formulated diet; *E. maxima* with epiphytes; and a mixed diet (*Gracilaria gracilis*, *Ulva lactuca*, and *E. maxima*). | The growth of abalone fed a mixed diet (*G. gracilis*, *U. lactuca*, and kelp) was superior to other diets tested, abalone fed a formulated diet grew poorly. | Naidoo et al. (2006) |
| | Five formulated diets with different protein sources (casein, *Spirulina* spp., soya, fish meal, torula yeast); and *Plocamium corallorhiza*. | Abalone fed fishmeal and *Spirulina* spp.-based diets grew significantly faster than abalone fed other diets. Growth of abalone fed *P.corallorhiza* was significantly lower than abalone fed formulated diets. | Britz (1996b) |
| Red abalone (*Haliotis rufescens*) | Formulated diet; *Macrocystis pyrifera*, *Porphyra columbina*; and formulated diet + *P. columbina.* | Abalone grew best when fed *P.columbina*. | Hernandez et al. (2009) |
| | Two formulated diets (25 and 38% crude protein) and *M. pyrifera*. | The growth of abalone fed either formulated diet was significantly higher than when fed *M. pyrifera*. | Garcìa-Esquivel and Felbeck (2009) |
| Green ormer (*Haliotis tuberculata coccinea*) | Eight macroalgal treatments, four non-enriched and four enriched macroalgal species (*Ulva rigida, Hypnea spinella, Gracilaria cornea,* and an equal part mixture of the three. | The growth of abalone fed enriched macroalgae was superior to abalone fed non-enriched macroalgae. The growth of abalone fed mixed diets was superior to single species diets. No formulated diets were tested | Viera et al. (2011) |
| Greenlip abalone (*Haliotis laevigata)* | Formulated diet; *U. lactuca*; *Spyridia filamentosa* and Formulated diet + live *U. lactuca* + live *S. filamentosa.* | Abalone grew faster when fed the formulated diet compared to either macroalgae diets. | Dang et al. (2011a) |
| | Three commercial formulated diet, three treatments of non-enriched and enriched live macroalgae (*Gracilaria cliftonii, U. lactuca* and a 1:1 mix). | The growth of abalone fed the formulated diet was superior to abalone fed either macroalgae treatments. Abalone fed mixed enriched macroalgae outperformed abalone fed other macroalgae treatments. | Bansemer et al. (2014b) |
| Hybrid abalone (*H. laevigata* x *Haliotis rubra*) | Two formulated diets; six live macroalgae treatments (different proportions of three species: *Grateloupia turuturu*, *Ulva australis* and/or *Ulva laetevirens*). | Best growth when fed protein enhanced *U. laetevirens* and *G. turuturu*, abalone fed either formulated diet had depressed growth rates. Access to formulated diets may have been limited. | Mulvaney et al. (2013a) |

**Table 2.5** Essential amino acid composition of selected macroalgal species

| *Essential amino acids (g 100g$^{-1}$ protein)* | Protein (g 100g$^{-1}$ dry weight) | Arginine | Histidine | Isoleucine | Leucine | Lysine | Methionine | Phenylalanine | Threonine | Trytophan | Valine | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Macroalgae species | | | | | | | | | | | | |
| Green algae (Chlorophyta) | | | | | | | | | | | | |
| *Ulva lactuca* | 7.3 | 3.6 | 1.8 | 3.7 | 6.7 | 4.2 | 1.6 | 4.0 | 4.7 | NR | 6.2 | Shuuluka *et al.* (2013) |
| Red algae (Rhodophyta) | | | | | | | | | | | | |
| *Gracilaria salicornia* | 9.6 | 7.6 | 1.4 | 3.0 | 7.7 | 7.7 | 7.8 | 3.3 | 3.3 | NR | 4.1 | Tabarsa *et al.* (2011) |
| *Gelidium pusillum* | 11.3 | 6.3 | 0.5 | 4.2 | 7.5 | 4.8 | 1.6 | 3.2 | 5.2 | NR | 4.5 | Siddique *et al.* (2013) |
| *Porphyra purpurea* | 16.8 | 9.0 | 2.2 | 3.4 | 5.3 | 2.9 | 1.4 | 7.8 | 5.0 | NR | 4.8 | Taboada *et al.* (2013) |
| Brown algae (Phaeophyta) | | | | | | | | | | | | |
| *Undaria pinnatifida* | 33.2 | 8.8 | 1.7 | 5.1 | 8.6 | 4.0 | 0.1 | 4.8 | 2.9 | NR | 5.8 | Taboada *et al.* (2013) |

NR: Not reported

Wild abalone are opportunistic feeders, and consume a mixture of macroalgal species (Shepherd, 1973). In captivity, the growth rate of abalone is superior when fed a mixture of macroalgal species compared to single macroalgal diets. The amino acid composition of macroalgal species is varied (Table 2.5). Essential amino acids may be limiting to the growth of abalone when fed as single macroalgal diets. A superior balance of essential nutrients and amino acids is achieved by feeding a mixed macroalgal diet, which may be the cause of superior growth compared to abalone fed single macroalgal species (Shpigel et al., 1999; Naidoo et al., 2006; Daume et al., 2007; Viera et al., 2011).

In addition, feeding macroalgae cultured in a nitrogen/protein enriching media to abalone results in improved growth rates (Table 2.4). The green ormer (*Haliotis tuberculata coccinea* Reeve) fed enriched sea lettuce (*Ulva rigida* J. Agardh), the red alga (*Gracilaria cornea* J.Agardh) or the red alga (*Hypnea spinella* C. Agardh), had significantly higher growth rates compared to corresponding non-enriched algae treatments (Viera et al., 2011). Similarly, *H. tuberculata* and the Pacific abalone (*Haliotis discus hannai* Ino) grew at a significantly greater rate when fed enriched sea lettuce (*Ulva lactuca* L) than when fed non-enriched *U. lactuca* (Shpigel et al., 1999). The superior growth of abalone fed nitrogen/protein enriched macroalgae is not surprising, as dietary protein and amino acid availability initially limit protein deposition and are a major limiting factor in the optimal growth of abalone (Fleming and Hone, 1996; Britz and Hecht, 1997; Shipton and Britz, 2001).

The growth rates and meat yield of abalone fed formulated diets are typically higher than abalone fed live macroalgae as feed producers are able to formulate diets to contain optimal dietary protein and energy, and provide a suitable amino acid profile for growth (Kemp et al., 2015; Table 2.4). Formulated diets are critical in countries where abalone do not grow readily when fed live brown macroalgae, or when macroalgae are scarce and are

also fed in conjunction with macroalgae to enhance growth rates (Fleming and Hone, 1996; Bautista-Teruel et al., 2011).

Although cultured abalone fed macroalgae may not reach the maximal growth potential, there are a number of benefits to the health, marketability and feeding activity by feeding macroalgae to abalone. Feeding live macroalgae to abalone can be problematic compared to formulated diets. The high moisture content of macroalgae results in reduced nutrient density, which may make it difficult for abalone to consume a sufficient amount to achieve a comparative nutrient intake. In addition, the low nutrient density and quality, particularly the concentration of protein and essential amino acids, results in slow protein deposition and growth depression (Tables 2.2 and 2.5). The inconsistent supply and the introduction of diseases, pests, predators and competitors to culture systems also pose biosecurity complications to feeding live macroalgae on-farm (Britz, 1996a; Chen and Lee, 1999; Bautista-Teruel and Millamena, 1999; Dang et al., 2011a; Bautista-Teruel et al., 2011). Therefore, to gain the benefits of high growth rates, coupled with increased health, marketability and feeding activity, dried macroalgal meal inclusions into nutritionally balanced formulated diets may be one solution, providing there is no compromise to nutrient composition and quality.

### *2.4.3 The use of dried macroalgae meal in formulated diets*

In contrast to the numerous studies investigating the use of live macroalgae as feed for abalone, to the best of our knowledge, only two studies have investigated the growth performance of abalone fed dietary inclusions of dried macroalgae meal in formulated diets. Feeding abalone live macroalgae improves the feeding activity, health and product quality compared to feeding formulated diets (Allen et al., 2006; Brown et al., 2008; Dang et al., 2011a; Chojnacka et al., 2012; Stone et al., 2014b). In addition, as the diet of wild abalone is

predominately consists of macroalgae, dried macroalgae meal is a promising dietary ingredient for formulated diets and a more practical way to gain the benefits of feeding macroalgae to abalone.

The growth rate of *H. discus hannai* when fed a formulated diet with dietary inclusions of a combination of oarweed (*Laminaria digitata* Hudson), dulse (*Palmaria palmata* L.) and *U. lactuca* was similar to *H. discus hannai* fed fresh *L. digitata*. However, the feed efficiency of *H. discus hannai* fed a formulated diet with dietary inclusions of dried macroalgae meal was significantly superior to *H. discus hannai* fed fresh *L. digitata* (O'Mahoney et al., 2014). The authors noted that this was the first time a novel feed resulted in higher feed efficiency compared to fresh *L. digitata.* Nonetheless, O'Mahoney et al. (2014) only fed abalone every 3 - 4 days, which resulted in up to 27% dry matter loss of formulated diets. Britz et al. (1996) previously recommended a daily feeding frequency for abalone fed formulated diets. Therefore, further improvements to feeding frequency in future studies investigating dried macroalgae meal might result in further improvements to feed utilisation and growth performance.

In contrast, in a preliminary study, the growth of *H. tuberculata coccinea* fed a formulated diet with a 43% dietary inclusion of mix dried macroalgae meal (*U. rigida, G. cornea, L. digitata, P. palmata*) was significantly reduced compared to abalone fed live macroalgae (Viera et al., 2012). It is not clear however, whether the reduced growth rate of abalone fed dietary inclusions of macroalgae meal was due to nutritional problems or the poor water stability of the formulated diets tested, as diets test had a dry matter loss of up to 63% after 17 h (Viera et al., 2012).

Numerous studies have investigated the use of macroalgae meal as a dietary ingredient, and the response appears to be species-dependent (Satoh et al., 1987; Mustafa et al., 1995; Kut Güroy et al., 2007). For example, the growth of fish can be enhanced when fed

macroalgae. For example, *O.niloticusa,* an omnivorous fish, had similar (Kut Güroy et al., 2007) or significantly greater growth rate (Ergün et al., 2009) when fed a 5% dietary inclusion of dried macroalgae meal (*U. rigida*) compared to a control diet. In contrast, *O. mykiss* fed a 10% dried macroalgae meal diet (*U. lactuca* or sea lettuce *Enteromorpha linza* L.) had significantly reduced growth performance, feed utilisation and protein efficiency compared to fish fed a control diet (Yildirim et al., 2009). In this particular case, dried macroalgae meal may have reduced diet palatability or may have been less digestible by this species, due to their carnivorous nature (Yildirim et al., 2009). Moreover, red sea bream (*Pagrus major* Temminck and Schlegel), a carnivorous species, had significantly greater disease resistance and phagocytosis activity when fed a 5% dietary inclusion of macroalgae meal (*Ulva pertusa*), compared to fish fed a control diet (Satoh et al., 1987).

The inherent composition of some green macroalgae species would suggest that high dietary inclusions of dried macroalgae meal might inhibit pellet binding and be detrimental to diet stability (Table 2.3). In contrast, among other commonly used binders in formulated diets for abalone, agar or carrageenan in red macroalgae and alginate in brown macroalgae may improve pellet binding and increase diet stability (Chao et al., 2010; Stone et al., 2013). Future studies should improve on feeding practices and diet stability, as reduced diet stability is detrimental to abalone performance, to achieve improved growth rates, feeding activity, health and product quality associated by feeding macroalgae to abalone (Allen et al., 2006; Brown et al., 2008; Hernández et al., 2009; Dang et al., 2011a; Chojnacka et al., 2012; Stone et al., 2014b).

## 2.5 Benefits of macroalgae as feed

### *2.5.1 Feeding stimulant*

Abalone rely on tactile and chemosensory cues to detect food (Allen et al., 2006). When waiting to catch drift macroalgae, abalone extend cephalic tentacles and forepart of their foot to detect macroalgae fragments (Shepherd, 1973; Jan et al., 1981; Allen et al., 2006). Chemosensory cues in macroalgae also result in a feeding response by abalone. Abalone (*Haliotis diversicolor supertexta* Reeve) immediately displayed feeding behaviour, despite no *Ulva* spp. present in the system when exposed to the *Ulva* spp. extract (25 g of dried *Ulva* spp. in 1 L of seawater for 1 h, then filtered) (Jan et al., 1981). The feeding behaviour of abalone when fed macroalgae is distinctly different from when fed formulated diets. *Gracilaria* spp. acted as an effective feeding stimulant compared to the commercial formulated diet when offered to *H. iris* (Allen et al., 2006). When *Gracilaria* spp. particles were present in the water, abalone spent more than 80% of their time feeding. In contrast, when offered the formulated diet, abalone spent most of the time sedentary (Allen et al., 2006). On-farm, shell growth rate was 15% higher in abalone fed the commercial formulated diet with *Gracilaria* spp. particles present compared to the abalone fed a control diet without *Gracilaria* spp. particles were present. In the laboratory however, no differences in growth rates were observed between diets with or without *Gracilaria* spp. particles (Allen et al., 2006). The authors suggested that this might have occurred due to the hydrodynamic conditions on-farm, as the particles were effectively suspended and circulated within the tank, compared to laboratory conditions (Allen et al., 2006).

The increased feeding activity and growth in abalone, when provided with tactile or chemical stimuli from macroalgae, are commercially important and deserve further investigation. Although no control diet without dried macroalgae meal was used, Garcìa-Esquivel and Felbeck (2009) recommended dietary inclusions of dried macroalgae as the

authors reported improved consumption and digestibility of formulated diets with dried inclusions of giant kelp (*Macrocystis pyrifera* L.). This has commercial implication, as a South African feed company has offered an optional low dried inclusion level of sea bamboo (*Ecklonia maxima* Osbeck) (5% wet weight; 0.88% dry weight), primarily as a feed attractant, for *H. midae* in their 34% crude protein formulated diet (Personal communication, Kurt Mätschke, Marifeed, Western Cape, South Africa).

The feeding preference for macroalgal species differs between abalone species and depends on the nutrient levels, particularly digestible nitrogen content and the presence of chemical defences, such as tannins that deter grazing (Fleming, 1995b; Cornwall et al., 2009). While Australian abalone, *H. rubra* and *H. laevigata*, generally prefer red macroalgae (Shepherd and Steinberg, 1992; Fleming, 1995a), other abalone species including *H. iris* (Cornwall et al., 2009), *H. midae* (Britz, 1996b), red abalone (*Haliotis rufescens* Swainson) (Flores-Aguilar et al., 2007) and *H. discus hannai* (Flores-Aguilar et al., 2007) generally prefer brown macroalgae species. A number of abalone species also have a high preference for *Ulva* spp. (Fleming, 1995b; Cornwall et al., 2009). Further studies are required to optimise the macroalgae type and inclusion level to improve feeding behaviour, as the macroalgae preference differs between abalone species (Fleming, 1995a; Cornwall et al., 2009).

As the preference for macroalgae species differs, further research on the most preferred macroalgae species, in addition to determining the optimal inclusion level for macroalgae meal, is required for different abalone species to maximise feeding activity and subsequent growth.

### *2.5.2 Health*

Temperate abalone species take approximately 3 years to reach a marketable size (Hahn, 1989). Due to the slow growth rate of abalone, diets not only need to provide sustainable growth but also maintain the optimal health of the animal. Commercial formulated diets usually contain a vitamin and mineral premix. Despite this, abalone are still susceptible to health implications and immunosuppression on-farm including infections by herpesvirus (Hooper et al., 2007) and *Vibrio* spp. (Travers et al., 2009; Dang et al., 2011b), high water temperatures (Vandepeer, 2006; Travers et al., 2009), exposure to anaesthetic and physical damage during handling (Hooper et al., 2011).

Chojnacka et al. (2012) reviewed the biologically active compounds in macroalgae extracts and their prospects of application in pharmaceuticals, animal feeds and fertilisers. The authors identified a myriad of biologically active compounds in macroalgae including polysaccharides, proteins, pigments and polyphenols, which have beneficial activity in animals during biotic and abiotic stress. Subsequently, galactans, fucoidan, laminarin and alginates were suggested to be the most important carbohydrates in macroalgae, as they display both growth promoting and health-improving activity as prebiotics, and have antimicrobial, antiviral, anti-infection and antioxidant activity (Luo et al., 2009; Chojnacka et al., 2012). The protein in macroalgae similarly exhibits antibacterial, antiviral and anti-inflammatory activities (Chojnacka et al., 2012). Carotenoids (β-carotene, fucoxanthin and antheraxanthin) and phycobiliproteins are the most important pigments in macroalgae and have considerable antioxidant, anti-inflammatory and antiviral activities (Schubert et al., 2006; Sachindra et al., 2007; Chojnacka et al., 2012). In addition, polyphenols (phenolic acids, flavonoids, isoflavones, cinnamic acid, benzoic acid, quercetin and lignans) also have considerable antioxidant activity, up to 10 times higher than ascorbic acid and α-tocopherol (Shibata et al., 2008; Chojnacka et al., 2012). Therefore, it is likely that feeding abalone

macroalgae, or extracted compounds from macroalgae, may reduce the vulnerability of abalone to health implications, immunosuppression, high water temperatures, and exposure to anaesthetic and physical damage during handling on-farm.

Feeding a crude extract of fucoidan, a biologically active compound in boto-boto (*Sargassum polycystum* C. Agardh) significantly affected the survival of black tiger shrimp (*Penaeus monodon* Fabricius), after a white spot syndrome virus (WSSV) challenge (Chotigeat et al., 2004). Shrimp fed a control diet and challenged with WSSV died within 5 days, while the survival rate of shrimp fed a diet with a crude extraction of fucoidan mixed with the control diet was 46% and 93% for 5 - 8 g and 12 - 15 g animals, respectively (Chotigeat et al., 2004).

It is likely that the use of crudely extracted biologically active compounds in macroalgae might also improve the health of abalone and enhance pathogen resistance (Dang et al., 2011a). Despite *H. laevigata* having a significantly lower growth rate when fed macroalgae (*U. lactuca* or hairy basket weed *Spyridia filamentosa* Wulfen) compared to a control formulated diet, these two macroalgae species increased antiviral activity in the digestive system lipophilic extract of abalone digestive gland against herpes simplex virus -1 (Dang et al., 2011a).

Feeding macroalgae may be particularly important during periods of high water temperature on farm. Considerable stock loss of abalone occur at high water temperatures, as this might reduce water quality and increase the susceptibility to *Vibrio* spp. infection (Vandepeer, 2006; Travers et al., 2009). Vandepeer (2006) hypothesised that increased intestinal microbial proliferation occurs at higher water temperatures, resulting in increased fermentation and gas production. Given the potential health benefits of feeding macroalgae to abalone, macroalgae meal inclusion in formulated diets may alleviate animal mortality on-

farm by enhancing dietary antimicrobial, antiviral, anti-inflammatory, antioxidant and health promoting activities (Dang et al., 2011a; Chojnacka et al., 2012).

Feeding live macroalgae to *H. laevigata* improved the survival of *H. laevigata* at 26 °C in a summer mortality model (Stone et al., 2014b). The survival rate of abalone fed live *U. lactuca* and a commercial formulated diet was ~95% and ~50%, respectively (Stone et al., 2014b). Abalone fed the commercial formulated diet were 20.5 times more likely to die than abalone fed live *U. lactuca* (Stone et al., 2013). In a parallel study, feeding dried macroalgae meal to *H. laevigata* also improved the survival of *H. laevigata* compared to a commercial formulated diet at 26 °C (Lange et al., 2014). The survival of *H. laevigata* fed a commercial formulated diet and a diet containing 30% dried macroalgae meal (*U. lactuca*) was ~50% and ~80%, respectively. Abalone fed the commercial formulated diet were 1.2 times more likely to die than abalone fed a 30% inclusion of dried macroalgae meal (Lange et al., 2014). Although not significant, due to high variability, the survival of abalone fed 30% *U. lactuca* was numerically higher than abalone fed live *U. lactuca*, 80% and ~95%, respectively (Lange et al., 2014).

The improved survival of *H. laevigata* fed live *U. lactuca* compared to dried *U. lactuca* inclusions into formulated diets may have occurred due to a number of reasons. Stone et al. (2014b) suggested that live *U. lactuca* might act as a bio-filter via photosynthesis through uptake of carbon dioxide and the production of oxygen. Live *U. lactuca* also has the ability to remove dissolved nitrogenous products and other potential harmful products from the culture system, unlike dried macroalgae (Shpigel et al., 1999; Viera et al., 2011; Stone et al., 2014b). In addition, the drying process may also be one of the causes for the difference in survival rate between live *U. lactuca* and 30% *U. lactuca* (Lange et al., 2014). The effect of dried macroalgae on the nutritional value is relatively unknown. The drying process requires serious consideration, as there are a number of negative impacts to protein quality from

thermal treatment including crosslinking and oxidation of sulphur containing amino acids, and the reduced bioavailability of essential amino acids, such as lysine through the Maillard reaction (Friedman, 1996; Meade et al., 2005; van Rooijen et al., 2013). In addition, the prebiotic and probiotic effects of macroalgae to abalone may be compromised by drying (Kemp et al., 2015). Additional research may facilitate the use of dietary inclusions of dried macroalgae meal to further increase the survival of abalone during periods of stress and immunosuppression. It would be beneficial for the abalone aquaculture industry to build on results by Stone et al. (2014b) and Lange et al. (2014) to improve the health of cultured abalone. Further research focused on optimising the dietary inclusion level of *U. lactuca* and other macroalgae species would also be beneficial to improve health and survival, as long as growth is not compromised (Viera et al., 2012; O'Mahoney et al., 2014).

### *2.5.3 Product quality*

Abalone command a high market price, particularly in Asian countries. The quality and marketability are due to the unique texture, taste and colour, which can be manipulated through dietary alterations (Oakes and Ponte, 1996; Smit et al., 2007; Brown et al., 2008; Kemp et al., 2015). The nutritional profile of feed ingredients currently used in formulated diets for abalone is distinctly different from their natural diet, which significantly affects the product quality, taste and appearance of abalone (Smit et al., 2007). Dietary manipulation may allow farms to tailor abalone to meet specific market needs (Oakes and Ponte ,1996).

Even on a dry weight basis, the lipid content of macroalgae is low (Table 2.2). Abalone fed formulated diets with excess dietary lipids result in reduced growth and increased lipid deposition (Britz and Hecht, 1997). Increased lipid deposition, and the type and concentration of long-chain polyunsaturated fatty acids, distinctly influence meat flavour and deserve

attention in regards to consumer acceptance of farmed abalone (Dunstan et al., 1996; Britz and Hecht, 1997).

The concentrations of free amino acids, glycine and glutamate, and the nucleotide adenosine monophosphate (AMP) affect abalone taste (Brown et al., 2008). Compared to abalone fed an artificial diet, those fed *Gracilaria* spp. contained lower taurine and arginine levels, but higher levels of glycine, glutamic acid, alanine, serine, proline, AMP and glycogen (Brown et al., 2008). Differences in these constituents may have caused the increased chemosensory preference of abalone fed an artificial diet compared to abalone fed *Gracilaria* spp. (Chiou and Lai, 2002). Smit et al. (2010) however, reported a higher preference for wild abalone compared to cultured animals fed macroalgae. This result is interesting, and needs further investigation, as other factors, other than diet, might also influence the sensory preference (Smit et al., 2010).

Foot colour is particularly important to market and consumer acceptance. Generally, consumers prefer abalone with lighter pigmentation, as they do not require trimming, washing or bleaching (Oakes and Ponte, 1996). Wild *H. iris* has a distinctive dark epidermal foot colouration. In captivity, the distinct dark foot is maintained when fed macroalgae, *Gracilaria* spp. or *M. pyrifera*, but becomes pale when fed a commercial formulated diet (Allen et al., 2006). This has implications for marketability, as abalone with lighter pigmentation command a higher price (Oakes and Ponte, 1996). On the other hand, if abalone aquaculture attempts to fill the market void of the wild fishery, it may be beneficial to revert to feeding macroalgae on-farm, in order for the cultured product to mimic the wild product. Similarly, the distinctive green mantle in wild *H. laevigata* is difficult to maintain in aquaculture when fed formulated diets (Personal communication, Nick Savva, AbTas, Tasmania, Australia). If the optimal diet constituents are present, such as pigments or

pigment precursors, the mantle may return to the wild coloration, leading to the increased marketability of farmed *H. laevigata*.

Investigating macroalgae species that promote superior chemosensory preference and flesh colouration of cultured abalone would be beneficial as this may increase the market appeal of cultured abalone (Brown et al., 2008). In addition, isolating the pigments, or pigments precursors in macroalgae and adding to commercial formulated diets may be beneficial as a finishing diet, fed for a specific period at the end of the culture cycle. If this is successful, further research is required to determine the optimal duration that abalone require dietary pigment-enhancing components in order to promote optimal colouration and possibly improve market acceptability.

## 2.6 Conclusions

In this review, the nutritional requirements of abalone have been examined and the benefits of feeding live macroalgae or dried macroalgae meal inclusions in formulated diets to abalone have been highlighted. Feeding live macroalgae to abalone can be problematic due to the high moisture content, low nutrient density and quality. Dried macroalgae meal inclusions in nutritionally balanced formulated diets, despite the shortage of studies investigating dried macroalgae meal, may also have the potential to enhance the feeding activity, health and marketability of farmed abalone.

Abalone rely on both tactile and chemosensory cues to feed. Macroalgae acts as a feeding stimulant to improve the feeding activity of abalone. This may improve feed intake, in turn, resulting in a significant increase in growth. Additionally, due to the long grow-out period of abalone, diets need to maintain the optimal health of the animal. While formulated diets contain a vitamin and mineral premix, dried macroalgae meal could provide many biologically active compounds that have potent antimicrobial, antiviral, anti-inflammatory,

antioxidant and health promoting activities to reduce the vulnerability to health problems and immunosuppression.

The quality and marketability of abalone depends on the unique texture, taste and colour of abalone. The colour of the foot is a particularly important aspect to marketability. Dietary manipulation may allow farms to tailor abalone products to meet specific market needs. The dietary inclusion of dried macroalgae meal may also offer a useful approach to increase marketability of farmed abalone.

The manufacturing process is critical to include macroalgae meal as a dietary ingredient for abalone feed. If macroalgae meal is a suitable dietary ingredient for abalone diets, the following four factors during the manufacturing process require consideration.

1. It is important to maintain product quality and the activity of biologically important compounds when drying macroalgae, as this may affect the bioactive substances therein.
2. Prior to drying, certain species of macroalgae should be cultured in a nitrogen/protein enriched medium as abalone fed non-enriched macroalgae have reduced growth, likely due to protein limitations.
3. The inclusion of certain dried macroalgae meals may affect diet pellet stability, which may have a negative impact on abalone performance and nutrient retention capacity.
4. To achieve the desired results of improved growth, feeding activity, health, and marketability the dietary inclusion level and type of dried macroalgae meal needs further investigation for different abalone species.

# Chapter 3. Growth and feed utilisation of juvenile greenlip abalone (*Haliotis laevigata*) in response to water temperatures and increasing dietary protein levels

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

## 3.1 Abstract

In this 91-day study, the interaction between four dietary crude protein (CP) levels (27, 30, 33 and 36% CP) and three water temperatures (14, 17 and 20 °C) on the growth and feed utilisation of 6-month old greenlip abalone (*Haliotis laevigata*) (0.91 g) were investigated. Diets were formulated to be isoenergetic (12.5 MJ $kg^{-1}$ digestible energy), containing a lipid level of ~3.6% and digestible protein from 17.99 to 28.57%. Abalone were fed to excess at 16:00 h daily, and uneaten feed was collected the following day. The specific growth rate (SGR) of abalone improved significantly as water temperatures increased from 14 to 17 to 20 °C. In addition, apparent protein deposition was significantly higher in abalone at 17 and 20 °C compared to abalone at 14 °C. There was no significant effect of dietary protein level on SGR, but faster growing abalone at 20 °C compensated by consuming more feed when fed low dietary protein levels. In contrast, a significant positive relationship was observed between dietary protein level and feed consumption rate in slower growing abalone at 14 and 17 °C. A non-significant tendency for the apparent feed conversion ratio (FCR) to improve was observed in abalone fed high protein diets at 20 °C, while at 14 °C, abalone had a significantly poorer FCR, especially when fed high dietary protein levels. Based on results from the current study, it is plausible to heat land-based nursery systems in order to gain accelerated growth of juveniles before transfer to grow-out systems. Additionally, no benefits were apparent by feeding abalone high protein diets at 14 or 17 °C, and we therefore recommend a dietary protein level of 29% CP at 14 and 17 °C. While the SGR of abalone at 20 °C was not significantly influenced by dietary protein, the feed consumption rate decreased and there was a tendency for FCR to improve as dietary protein level increased. Therefore, it may be beneficial for abalone to be switched to a diet containing ~35% CP at water temperatures > 20 °C.

## 3.2 Introduction

Greenlip abalone (*Haliotis laevigata*) are primarily grown in land-based systems throughout southern Australia. The water temperature during grow-out affects almost every aspect of on-farm production (Britz et al., 1997), and can range from below 10 °C in Tasmania during winter to above 24 °C in South Australia during summer. The optimal water temperature for growth for a Tasmanian greenlip abalone strain (82 mm shell length [SL]) was 18.3 °C (Gilroy and Edwards, 1998), while the optimal water temperature for growth for a South Australian greenlip abalone strain (23 mm SL) was 22 °C (Stone et al., 2013). The temperature dependent response in abalone growth may be attributed to genetics or animal size differences, the latter of which has also previously been reported in red abalone (*Haliotis rufescens*) (Steinarsson and Imsland, 2003).

Once juvenile greenlip abalone are weaned off a microalgae diet, they are fed a formulated diet for approximately three years until they reach market size. Dietary protein plays a major role in the nutritional value of formulated diets, as optimal growth is dependent on maximising protein deposition, which is limited by dietary protein availability (Fleming and Hone, 1996; Britz and Hecht, 1997; Shipton and Britz, 2001). The optimal dietary protein level is dependent on a number of factors including the abalone species, abalone size, water temperature, ingredient digestibility and dietary energy level (Bautista-Teruel and Millamena, 1999; Stone et al., 2013; Bansemer et al., 2014a). The abalone industry is relatively new compared to other aquaculture sectors such as fin fish, which have successfully developed pre-starter, starter, grower and finisher diets for different grow-out stages (Ng and Romano, 2013; Sarker et al., 2013). There is an increased demand to introduce multi-diet feeding strategies for greenlip abalone by optimising the dietary protein level for each age class and water temperature throughout the production cycle. However, the optimal dietary protein level for greenlip abalone throughout their whole production cycle is not clear. Prior to 2013,

Australian abalone diets were formulated to contain ~27% crude protein (CP) based on a growth trial for juvenile greenlip abalone (0.55 - 0.94 g) at 20 °C (Coote et al., 2000). Currently, Australian abalone feed contains 30 to 35% CP as suggested by recent research for greenlip abalone (1.75 g) at 22 °C (Stone et al., 2013), but the authors also reported the optimal dietary protein level is dependent on both age (1- and 2-year old abalone) and water temperature (14, 18 and 22 °C). Further research focused on the nutritional requirements of greenlip abalone soon after weaning (~6-month old abalone) is required to improve our understanding on feed formulation for juvenile abalone.

In this study, our aim was to identify the optimal dietary CP level for post-weaned greenlip abalone (6-month old) at 14, 17 and 20 °C. On-farm, in land-based facilities throughout southern Australia, the water temperature fluctuates throughout the grow-out period; the water temperatures selected in the current study represent the temperature range typically occurring from autumn, through winter, to early summer experienced by post-weaned juvenile greenlip abalone. The nominal dietary CP levels used in this study were 27, 30, 33 and 36%. These levels are considered to be commercially applicable to land-based abalone production in southern Australia. Diets used in this study were formulated on a digestible protein basis and contained highly palatable and digestible ingredients at realistic inclusion levels, using protein and energy digestibility data reported for greenlip abalone (Fleming et al., 1998; Vandepeer, 2005). Diets were formulated using the "ideal protein concept," such that the ratio of each essential amino acid to lysine was equal to, or greater than, the soft tissue amino acid values for greenlip abalone (Coote et al., 2000). Diets contained ~3.6% lipid (Van Barneveld et al., 1998; Dunstan et al., 2000), and ~17. 4 MJ $kg^{-1}$ crude and ~12.5 MJ $kg^{-1}$ digestible energy levels. The results of this study will contribute towards the development of diets suitable for early weaned abalone at different water

temperatures and the formulation of appropriate abalone diets for each grow-out stage throughout the abalone production cycle.

## 3.3 Methods

### *3.3.1 Experimental animals and system*

Greenlip abalone (weight 0.91 ± 0.00 g; shell length 19.46 ± 0.02 mm; $n$ = 864) were purchased from South Australian Mariculture (Port Lincoln, South Australia, Australia) in April 2013. Prior to stocking, abalone were held in a flow through seawater system at South Australia Research and Development Institute Aquatic Science Centre (SARDI ASC) (West Beach, South Australia, Australia) for two weeks and fed a commercial diet (~30% CP; Eyre Peninsula Aquafeed Pty Ltd, Lonsdale, SA, Australia).

The experiment was conducted in a photoperiod and temperature controlled laboratory described in Stone et al. (2013). The photoperiod was 12 h low intensity fluorescent lighting at 3.4 lux: 12 h dark. The air temperature was adjusted based on the incoming water temperature and ranged from 16.0 to 19.3 °C. Three identical culture systems (14, 17 or 20 °C) were supplied with 30 μm sand-filtered, UV treated seawater (Model 025120-2, 120w, Emperor Aquatics, Pottstown, PA, USA). Sixteen 12.5 L blue plastic culture units (Nally IH305, Viscount Plastics Pty Ltd.; 39.2 × 28.8 × 11.0 cm) per system were each supplied with flow-through seawater (300 mL $min^{-1}$). Water depth was held at 2.5 cm using a standpipe with a mesh screen (0.8 mm) on the outlet to retain uneaten feed. 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).

### *3.3.2 Stocking*

Abalone were gently prised from the substrate using a spatula. Eighteen animals were weighed, measured and stocked into one of four replicate culture units per treatment combination. Animals were acclimated to the system for 16 days and fed their respective diets. After seven days the water temperature was either lowered or raised slowly (1 °C $day^{-1}$) to the desired water temperatures (14, 17 or 20 °C) and was maintained at these levels (± 1 °C) throughout the remainder of the 75 day experiment. Dead abalone during the experiment were measured, weighed, recorded, and replaced with abalone of a similar weight and size that had been held at each respective water temperature and fed the commercial formulated diet.

### *3.3.3 Diets and feeding*

At each temperature, animals were fed with one of four dietary protein levels (27, 30, 33 and 36% CP, Table 3.2). The proximate composition of the ingredients was analysed prior to diet formulation. Diets were formulated on a digestible protein and isoenergetic basis, based on data reported for greenlip abalone (Fleming et al., 1998; Vandepeer, 2005). Solvent-extracted soybean meal, de-hulled lupins, casein and fish meal were used as the main dietary protein source, while fish oil and de-hulled lupins were used as the main dietary lipids source. Diets were also formulated, using book values, so that the ratio of each essential amino acid to lysine was equal to, or greater than that analysed for the soft body tissue of greenlip abalone (Coote et al., 2000). Due to the difficulty to determine the amino acid requirement of abalone (Shipton et al., 2002), applying the "ideal protein concept" was concluded to be an acceptable alternative (Fleming et al., 1996). Diets were cold-pressed into flat pellets (4 × 3 × 2 mm thick) using a commercial pasta machine (La Prestigiosa medium; IPA. Vicenza. Italy). 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, by 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. Abalone were fed to excess of their daily requirements (4% of the abalone biomass $day^{-1}$) at 16:00 h. Feed rates were maintained at these levels throughout the study based on monthly weight checks. Tanks were cleaned and uneaten feed was collected by sieving the entire tank contents through a fine mesh at 08:30 h and stored at -20 °C, and was later dried at 105 °C for 16 h. Daily feed consumption was estimated by the difference between feed offered and uneaten feed in dry weight. The proportion of uneaten feed lost between 08:30 to 16:00 h, from leaching and by sieving the entire tank contents through a fine mesh without animals in the tank, at the respective water temperatures, was used as a correction factor to calculate the apparent feed consumption rate.

### *3.3.4 Biochemical and water quality analysis*

At the commencement of the experiment, the soft tissue of 50 animals ($n$ = 4 replicates) were collected, shucked and stored at-20 °C to analyse the initial soft tissue proximate composition. At the conclusion of the experiment, 10 abalone from each tank were collected, shucked and stored at-20 °C. The abalone were later pooled for each tank for the analysis of soft tissue proximate composition. The proximate composition analyses of ingredients, diets, and whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (2000).

All data reported for animal performance were based on the pooled data from each tank. All calculations using abalone weight were based on wet values, while feed use values were based on dry values:

Biomass gain (g $tank^{-1}$) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)

Specific growth rate (SGR, % $day^{-1}$) = ([ln final weight - ln intial weight] / days) × 100

Shell growth rate (µm $day^{-1}$) = (final shell length - initial shell length) / days

Condition factor = 5575 × (weight [g] / length $[mm]^{2.99}$) (Britz and Hecht, 1997)

Apparent feed consumption = feed offered – uneaten feed collected – ([total feed offered × % leaching loss without animals] + [uneaten feed collected / % retained without animals × % leaching loss without animals]) / 2 (Stone et al., 2013)

Apparent feed conversion ratio (FCR) = feed consumed / abalone weight gain

Apparent protein efficiency ratio (PER) = abalone weight gain / protein consumed

Apparent energy efficiency ratio (EER) = abalone weight gain / energy consumed

Apparent protein deposition = ([final soft body protein-initial soft body protein] / protein intake) × 100

Apparent energy deposition = ([final soft body energy-initial soft body energy] / energy intake) × 100

Water quality parameters were measured daily and were maintained throughout the study at appropriate levels for the growth of abalone (Table 3.1). Water temperature was measured using a thermometer. Dissolved oxygen (mg $L^{-1}$ and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, IL, USA). Salinity (g $L^{-1}$) was measured using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, NH, USA). Light intensity was measured using a LI-COR 1400 Quantum light meter (LI-COR Environmental, Lincoln, NE, USA).

**Table 3.1** Summary of water quality for each water temperature system[1,2].

| Nominal temperature | Actual temperature (°C)[3] | Dissolved oxygen (mg $L^{-1}$)[4] | Dissolved oxygen (% saturation)[4] | pH[4] | Salinity (ppt)[4] |
|---|---|---|---|---|---|
| 14 °C | 14.0 ± 0.1 (13.8 - 14.1) | 8.0 ± 0.3 (7.1 - 8.5) | 99.3 ± 1.4 (95.8 - 103.0) | 8.14 ± 0.05 (7.95 - 8.26) | 35.7 ± 0.57 (34.0 - 38.0) |
| 17 °C | 17.0 ± 0.3 (16.1 - 17.9) | 7.6 ± 0.2 (7.0 - 8.0) | 98.2 ± 1.2 (94.5 - 101.8) | 8.15 ± 0.04 (7.99 - 8.26) | 35.7 ± 0.57 (34.0 - 38.0) |
| 20 °C | 19.9 ± 0.3 (19.0 - 20.9) | 7.3 ± 0.2 (6.9 - 7.7) | 97.2 ± 1.6 (91.3 - 102.0) | 8.15 ± 0.04 (8.02 - 8.26) | 35.7 ± 0.57 (34.0 - 38.0) |

[1] Values means ± standard deviation, values in parentheses represent the range of values.
[2] Data for DO, pH and salinity for entire experiment, while the data for water temperature is from end of temperature acclimation period.
[3] $n = 75$
[4] $n = 91$

### *3.3.5 Statistical analyses*

IBM SPSS, Version 20 for Windows (IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and the standardised residuals against the predicted mean plot, respectively. All percentage data was arcsine transformed before analyses. All variables were analysed using two-factor ANOVA, with water temperature as the first factor and dietary protein level as the second factor. When significant interactions were observed, post-hoc tests were used to detect significant differences between all treatment combinations (Student Newman-Keuls). Linear and second order polynomial regression analyses were also applied to SGR, feed consumption rate (mg $individual^{-1}$ $day^{-1}$) and FCR. A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated. If SE was < 0.01 it is reported as "0.00".

## 3.4 Results

### *3.4.1 General observations*

The analysed protein content of the diets was slightly higher than the formulated nominal values (Table 3.2). There were no significant differences in the initial weight and shell length between treatments ($P > 0.05$). The average initial weight and shell length was 0.91 ± 0.00 g and 19.46 ± 0.02 mm, respectively. The overall mortality for the study was 4.05%, but was a significantly higher at 14 °C (7.64%) compared to 17 °C (2.09%) and 20 °C (2.43%) ($P = 0.006$). Mortalities were not significantly influenced by dietary protein level ($P = 0.592$) or interaction between water temperature and dietary protein level ($P = 0.309$).

**Table 3.2** Ingredient and nutrient composition of experimental diets.

| | Nominal crude protein level (%) | | | |
|---|---|---|---|---|
| | 27 | 30 | 33 | 36 |
| *Ingredients (g 100g$^{-1}$ diet as fed)* | | | | |
| 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 |
| Salmon 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 100g$^{-1}$ diet as fed; 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)[1] | 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$^{-1}$)[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$^{-1}$)[1] | 16.57 | 19.06 | 20.79 | 22.80 |
| *Calculated amino acids (g 100g$^{-1}$)* | | | | |
| 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 |

[1]Digestible protein and energy values based on data reported by Fleming et al. (1998) and Vandepeer (2005); NFE=Nitrogen free extract=100 %-(protein % + lipid % + ash %); EPA. Eyre Peninsula Aquafeed Pty Ltd.

**Table 3.3** Growth performance, feed efficiency and nutrient retention of greenlip abalone at three water temperature fed four dietary protein levels (mean ± SE; $n$ = 4).

| Temperature (°C) | 14 | | | | 17 | | | | 20 | | | | SE | ANOVA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nominal protein (%) | 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 | |
| *Growth performance and mortality* | | | | | | | | | | | | | | | | | | | | | |
| Initial weight (g) | 0.91 | 0.91 | 0.92 | 0.92 | 0.91 | 0.91 | 0.92 | 0.91 | 0.91 | 0.91 | 0.92 | 0.91 | 0.00 | NS | | | NS | | | | NS |
| Final weight (g) | 1.59 | 1.47 | 1.53 | 1.49 | 1.92 | 1.97 | 1.95 | 1.96 | 2.58 | 2.50 | 2.57 | 2.47 | 0.06 | X | Y | Z | NS | | | | NS |
| Biomass gain (g tank$^{-1}$) | 11.74 | 10.19 | 10.49 | 10.20 | 18.18 | 18.97 | 19.02 | 18.80 | 29.97 | 28.40 | 29.81 | 28.03 | 1.16 | X | Y | Z | NS | | | | NS |
| SGR (% day$^{-1}$) | 0.62 | 0.53 | 0.56 | 0.54 | 0.82 | 0.86 | 0.82 | 0.84 | 1.15 | 1.11 | 1.14 | 1.10 | 0.04 | X | Y | Z | NS | | | | NS |
| Mortality (%) | 5.56 | 2.78 | 9.72 | 12.50 | 2.78 | 1.39 | 2.78 | 1.39 | 4.17 | 2.78 | 1.39 | 1.39 | 0.82 | Z | Y | Y | NS | | | | NS |
| *Somatic growth parameters* | | | | | | | | | | | | | | | | | | | | | |
| Initial shell length (mm) | 19.40 | 19.46 | 19.55 | 19.53 | 19.36 | 19.51 | 19.48 | 19.48 | 19.37 | 19.41 | 19.51 | 19.45 | 0.02 | NS | | | NS | | | | NS |
| Final shell length (mm) | 22.11 | 21.71 | 22.04 | 21.96 | 24.14 | 24.34 | 24.23 | 24.17 | 26.67 | 26.45 | 27.02 | 26.61 | 0.30 | X | Y | Z | NS | | | | NS |
| Shell growth rate (μm day$^{-1}$) | 29.98 | 24.96 | 27.57 | 26.60 | 52.65 | 53.17 | 52.61 | 55.89 | 80.27 | 78.21 | 83.41 | 79.12 | 3.34 | X | Y | Z | NS | | | | NS |
| Condition factor | 0.85 | 0.83 | 0.82 | 0.81 | 0.78 | 0.79 | 0.79 | 0.80 | 0.78 | 0.78 | 0.75 | 0.76 | 0.01 | Z | Y | X | NS | | | | NS |
| *Feed utilisation* | | | | | | | | | | | | | | | | | | | | | |
| Feed consumption rate (mg individual$^{-1}$ day$^{-1}$) | 15.66$^{de}$ | 14.60$^{e}$ | 16.16$^{de}$ | 18.44$^{cd}$ | 16.96$^{de}$ | 18.31$^{cd}$ | 18.60$^{cd}$ | 20.46$^{c}$ | 28.56$^{a}$ | 25.91$^{ab}$ | 25.84$^{ab}$ | 24.61$^{b}$ | 0.69 | * | * | * | * | * | * | * | * |
| Apparent FCR | 2.17 | 2.35 | 2.55 | 2.95 | 1.55 | 1.58 | 1.65 | 1.79 | 1.55 | 1.51 | 1.42 | 1.43 | 0.08 | Z | Y | Y | NS | | | | NS |
| *Nutrient retention* | | | | | | | | | | | | | | | | | | | | | |
| Apparent PER | 1.55 | 1.24 | 1.06 | 0.83 | 2.21 | 1.83 | 1.63 | 1.35 | 2.15 | 1.94 | 1.87 | 1.69 | 0.07 | Y | Z | Z | z | y | y | x | NS |
| Apparent PD | 17.90 | 14.55 | 12.40 | 10.25 | 24.56 | 16.41 | 16.77 | 12.41 | 23.90 | 18.58 | 20.97 | 15.29 | 0.84 | Y | Z | Z | z | y | y | y | NS |
| Apparent EER | 2.47 | 2.23 | 2.06 | 1.79 | 3.51 | 3.31 | 3.17 | 2.91 | 3.42 | 3.49 | 3.63 | 3.65 | 0.11 | X | Y | Z | NS | | | | NS |
| Apparent ED | 10.69 | 8.52 | 9.08 | 7.33 | 15.02 | 12.79 | 11.40 | 10.55 | 13.71 | 13.63 | 14.22 | 13.03 | 0.43 | Y | Z | Z | z | zy | zy | y | NS |
| *Proximate composition* | | | | | | | | | | | | | | | | | | | | | |
| Moisture (%) | 75.15 | 75.80 | 75.12 | 75.60 | 74.18 | 74.90 | 75.44 | 76.27 | 74.62 | 74.70 | 74.81 | 75.72 | 0.14 | NS | | | y | zy | zy | z | NS |
| Protein (% dry) | 50.95 | 54.77 | 51.51 | 54.51 | 52.03 | 46.77 | 53.44 | 49.58 | 53.35 | 48.65 | 55.20 | 49.12 | 0.92 | NS | | | NS | | | | NS |
| Lipid (% dry) | 5.49 | 5.06 | 5.17 | 5.40 | 5.29 | 5.08 | 5.18 | 4.91 | 4.69 | 4.95 | 4.51 | 4.20 | 0.07 | Z | Z | Y | NS | | | | NS |
| Ash (% dry) | 10.27 | 10.57 | 9.92 | 10.34 | 9.84 | 10.28 | 10.50 | 10.76 | 10.35 | 10.29 | 9.85 | 11.05 | 0.10 | NS | | | NS | | | | NS |
| Energy (MJ kg$^{-1}$ dry) | 19.92 | 20.02 | 19.94 | 20.11 | 20.02 | 19.56 | 19.97 | 19.62 | 19.88 | 19.65 | 20.04 | 19.38 | 0.07 | NS | | | NS | | | | NS |

SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; PD, protein deposition; EER, energy efficiency ratio; ED, energy deposition; Standard errors, SE, SE less than 0.01 are reported as "0.00".

Initial soft tissue content of greenlip abalone (dry): protein (46.79%), lipid (4.07%), ash (13.92%), and energy (18.71 MJ kg$^{-1}$ dry).

X, Y, Z: For variables with a significant effect of temperature and no interaction, values without a common upper case letter are different (Z indicates the highest value; $P < 0.05$); w, x, y, z: For variables with a significant effect of protein level and no interaction, values without a common lower case letter are significantly different (z indicates the highest value; $P < 0.05$); * denotes parameters with a significant interaction (A×B; $P < 0.05$)), difference in protein level are compared across all water temperatures (one-factor ANOVA, SNK test), values without a common superscript are significantly different ($^{a}$ indicates the highest value; $P < 0.05$); NS: denotes non significant differences ($P > 0.05$).

### *3.4.2 Growth performance*

Water temperature had a significant effect on the final weight and shell length of greenlip abalone ($P < 0.001$; 14 < 17 < 20 °C; Table 3.3). Final individual weight and shell length were not significantly affected by dietary protein level ($P = 0.801$ and $P = 0.965$, respectively) or by the interaction of these two factors ($P = 0.924$ and $P = 0.965$, respectively).

Biomass gain, SGR and shell growth rate were also significantly affected by water temperature ($P < 0.001$; 14 < 17 < 20 °C; Table 3.3). Dietary protein level had no significant effect on SGR ($P = 0.772$), biomass gain ($P = 0.799$) or shell growth rate ($P = 0.840$) and there were no significant interactive effects between water temperature and dietary protein level on biomass gain ($P = 0.958$), SGR ($P = 0.927$) or shell growth rate ($P = 0.989$). In addition, there was no significant linear or second order polynomial relationship between dietary protein level and SGR for abalone at 14, 17 or 20 °C ($P > 0.05$). Condition factor was significantly affected by water temperature ($P < 0.001$; 14 > 17 > 20 °C; Table 3.3), but not significantly affected by dietary protein level ($P = 0.472$), or the interactive effects between these two factors ($P = 0.732$).

### *3.4.3 Feed use*

Water temperature had a significant effect on feed consumption rate (mg abalone$^{-1}$ day$^{-1}$) ($P < 0.001$), while dietary protein level did not ($P = 0.184$). Feed consumption rate was significantly affected by the interaction between water temperature and dietary protein level ($P = 0.002$; Table 3.3). When compared to abalone fed other dietary protein levels at their respective water temperatures, the feed consumption rate by abalone fed 36% dietary CP level at 17 °C was significantly higher, while the feed consumption rate by abalone fed 36% dietary CP level at 20 °C was significantly lower. The feed consumption rates of abalone at

14 and 17 °C were similar when fed the same dietary protein level. The feed consumption rate of abalone at 20 °C was significantly higher than abalone at 14 and 17 °C (Table 3.3). In addition, regression analyses indicated that there were significant moderate positive second order polynomial and linear relationships between dietary protein level and feed consumption rate for abalone at 14 °C ($R^2$ = 0.536, $P$ = 0.007) and 17 °C ($R^2$ = 0.501, $P$ = 0.002), respectively. In contrast, regression analyses indicated that there was a significant moderate negative linear relationship between dietary protein level and feed consumption rate for abalone at 20 °C ($R^2$ = 0.501, $P$ = 0.002).

The apparent FCR was significantly affected by water temperature ($P$ < 0.001; 14 > 17 = 20 °C; Table 3.3), while dietary protein level had no significant influence on FCR ($P$ = 0.111) and there was no significant interaction between water temperature and dietary protein level ($P$ = 0.137). Regression analyses indicated that there was a significant moderate positive second order polynomial relationship between dietary protein level and FCR for abalone at 14 °C ($R^2$ = 0.406, $P$ = 0.034). While there were no significant relationships between dietary protein level and FCR for abalone at 17 or 20 °C ($P$ > 0.05), there were positive and negative tendencies, respectively.

### *3.4.4 Soft tissue composition*

Dietary protein level had a significant effect on the soft tissue moisture content ($P$ = 0.018). The soft tissue moisture content was significantly higher in abalone fed 36% CP compared to abalone fed 27% CP (Table 3.3). There were no significant differences between abalone fed other diets. Water temperature had no significant effect on soft tissue moisture content ($P$ = 0.364), and there were no significant interactions between water temperature and dietary protein level ($P$ = 0.441). Soft tissue protein content in greenlip abalone was not significantly affected by water temperature ($P$ = 0.556) or dietary protein level ($P$ = 0.637),

and there were no significant interactions between these two factors ($P$ = 0.606). Soft tissue lipid content was significantly influenced by water temperature ($P$ < 0.001; 14 = 17 > 20 °C), but was not significantly affected by dietary protein level ($P$ = 0.073), and there were no significant interactions between water temperature and dietary protein level ($P$ = 0.595). Soft tissue ash content was not significantly affected by water temperature ($P$ = 0.962) and dietary protein level ($P$ = 0.252), and there were no significant interactions between these two factors ($P$ = 0.211). Soft tissue energy was not significantly affected by water temperature ($P$ = 0.231) and dietary protein level ($P$ = 0.317), and there were no significant interactions between these two factors ($P$ = 0.519; Table 3.3).

### *3.4.5 Nutrient use*

The apparent PER was significantly affected by water temperature ($P$ < 0.001; 14 < 17 = 20 °C) and dietary protein level ($P$ < 0.001). The PER of abalone fed a diet containing 27% CP compared to all other diets was significantly superior to that in all other treatments, while the PER of abalone fed a diet containing 36% CP was significantly inferior to that in all other treatments. The PER of abalone fed diets containing 30 and 33% CP was not significantly different ($P$ > 0.05). There was no significant interaction between water temperature and dietary protein level for PER ($P$ = 0.771).

Water temperature had a significant effect on apparent protein deposition ($P$ < 0.001; 14 < 17 = 20 °C). Protein deposition was significantly influenced by dietary protein level ($P$ = 0.002), and was significantly superior in abalone fed a diet containing 27% CP compared to abalone fed other CP levels. The protein deposition of abalone fed 30, 33 or 36% CP was not significantly different ($P$ > 0.05). There was no significant interaction between water temperature and dietary protein level ($P$ = 0.567).

The apparent EER was significantly influenced by water temperature ($P < 0.001$; 14 < 17 < 20 °C). The EER was not significantly affected by dietary protein level ($P = 0.268$) or the interaction between water temperature and dietary protein level ($P = 0.429$). The apparent energy deposition was significantly affected by water temperature ($P < 0.001$; 14 < 17 = 20 °C; Table 3.3) and dietary protein level ($P = 0.013$). Abalone fed 27% CP had a significant superior energy deposition compared to abalone fed 36% CP (27 = 30 = 33% CP; 30 = 33 = 36% CP). There was no significant interaction between these two factors ($P = 0.451$).

## 3.5 Discussion

The experimental animals fed actively on diets throughout the study and growth rates were comparable to those observed in commercial facilities and other laboratory-based studies (Coote et al., 2000; Vandepeer, 2005; Stone et al., 2013). For example, Coote et al. (2000) reported a SGR for greenlip abalone (~1 g) of 1.03% $day^{-1}$ at 20 °C for 85 days (Coote et al., 2000), Vandepeer (2005) reported a SGR for greenlip abalone (2.3 g) of 1.05% $day^{-1}$ at 18 °C for 50 days, while Stone et al. (2013) reported a calculated peak SGR of 1.48% $day^{-1}$ for greenlip abalone (1.8 g) over 84 days at their optimal water temperature of 22 °C.

Water temperature is a key environmental variable that affects the survival, growth, feed consumption, nutritional requirements and digestive physiology of abalone (Britz et al., 1997; Edwards and Condon, 2001; Vandepeer, 2006; Schaefer et al., 2013; Stone et al., 2013; Stone et al., 2014b). In the current study, the SGR, biomass gain, apparent protein deposition, PER, shell growth and feed consumption rate of greenlip abalone all significantly increased with water temperature from 14 to 20 °C. Significantly improved SGR with increasing water temperature was previously reported for 1-year old greenlip abalone up to 22 °C (Stone et al., 2013), and similarly in South African abalone (*Haliotis midae*) up to 20 °C (Britz et al., 1997). In the current study, the improved growth and protein deposition of abalone as water

temperature increased may have occurred due to an increased efficiency at utilising dietary components, particularly protein, due to temperature dependent feed intake and digestive enzyme activity (Britz et al., 1997; Edwards and Condon, 2001; Hochachka and Somero, 2002). Edwards and Condon (2001) reported significantly higher (75%) protease activity as temperature increased from 9 to 24 °C in blacklip abalone (*Haliotis rubra*) and suggested that this would contribute to improved growth rates up to this species' optimal water temperature of 17 °C (Gilroy and Edwards, 1998).

The optimal water temperature reported for the growth of greenlip abalone differs between studies (Gilroy and Edwards, 1998; Stone et al., 2013). For example, Gilroy and Edwards (1998) reported a calculated optimal of 18.3 °C for Tasmania stock, while more recently Stone et al. (2013) reported an optimal of 22 °C for South Australian stock. Although 22 °C was not used in the current study, the SGR of abalone was significantly superior at 20 °C compared to 17 or 14 °C, providing further support for Stone et al. (2013). The possible discrepancy may have been due to genetic differences as Gilroy and Edwards (1998) worked with a Tasmanian strain, while in the current study and Stone et al. (2013), a more heat tolerant South Australian strain was used. Additionally, Gilroy and Edwards (1998) used larger greenlip abalone (82 mm SL) compared to smaller abalone (19 mm SL) in the current study. In addition, the optimal water temperature may also decline with size, previous research showed age-dependent differences when water temperature was raised from 18 to 22 °C; the growth rates of 1-year old greenlip abalone (23 mm SL) significantly increased, whereas the growth rate of 2-year old abalone (57 mm SL) did not (Stone et al., 2013). Steinarsson and Imsland (2003) reported similar size dependent optimal water temperature for *H. rufescens*, which peaked at 17.8°C for 44 mm SL abalone, and declined to 14.5 °C for 98 mm SL abalone. Lastly, the optimal water temperature reported by Gilroy and Edwards (1998) was not determined from growth studies, but was estimated from

behavioural studies on temperature preference. These discrepancies highlight the importance of species, strain and size-specific data from experiments concerning the variable of interest, and to not rely on models generated from other variables. The superior growth rate at high water temperatures observed in the current study further support the benefits of heating nursery systems during periods of low temperatures to improve growth. A cost-benefit analysis of implementing temperature controlled nursery systems is currently being undertaken on abalone farms throughout southern Australia. Further research investigating the optimal water temperature for different sized greenlip abalone, similar to Steinarsson and Imsland (2003), may be beneficial to increase on-farm production, especially with regards to implementing temperature controlled systems for other year classes of abalone.

To improve production without additional infrastructure, diet manipulation, particularly dietary protein levels, also leads to growth improvements (Britz, 1996a; Britz and Hecht, 1997; Coote et al., 2000; Stone et al., 2013). Protein is an expensive dietary component and plays a major role in abalone nutrition. The optimal dietary protein level has been the focus of numerous studies for a range of abalone species, including *H. midae* (Britz, 1996a; Britz and Hecht, 1997), *H. rubra* (Dunstan, 2010), green ormer (*Haliotis tuberculata*) (Mai et al., 1995), Pacific abalone (*Haliotis discus hannai*) (Mai et al., 1995) and greenlip abalone (Coote et al., 2000; Stone et al., 2013). The aims of these studies have been to reduce on-farm expense and increase production (Britz, 1996a; Fleming and Hone, 1996; Britz and Hecht, 1997; Shipton and Britz, 2001). The protein requirements of greenlip abalone have been the focus of two previous studies, and both studies formulated diets using the "ideal protein concept" (Coote et al., 2000; Stone et al., 2013). Coote et al. (2000) used highly digestible protein sources (casein and semolina) and reported an optimal dietary CP level of 27% for juvenile greenlip abalone (0.55 - 0.94 g) at 20 °C. A recent investigation by Stone et al. (2013) used highly digestible protein sources (solvent-extracted soybean meal, de-hulled

lupins, casein and fish meal) and reported water temperature- and size- dependent optimal dietary protein level for greenlip abalone. The optimal dietary CP level increased from ~29.0 to 32.2 to 34.7% CP for 1-year old abalone and from 24 to 34 and 34% CP for 2-year old abalone as water temperature increased from14 to 18 to 22 °C, respectively (Stone et al., 2013). Due to space limitations in Stone et al. (2013), the protein requirements of the younger age class of greenlip abalone (~6 month old) investigated in the current study could not be determined at the same time as 1- and 2- year old abalone. Although caution should be exercised when comparing between studies, the aim of the current study was to provide further information on the optimal dietary protein level for this age class of greenlip abalone. In the current study, the SGR of abalone was not significantly affected by dietary protein level. However, the benefit of high protein diets to SGR is masked by differences in feed consumption that also affected the FCR of abalone. As abalone were fed to satiation throughout the trial, the significant negative relationship between dietary protein level and feed consumption rate for abalone at 20 °C indicates that these faster growing abalone up regulated feed intake when fed low protein diets to increase protein intake and achieve near-maximum growth potential. In contrast, a significant positive relationship between feed consumption rate and dietary protein levels occurred in slow growing abalone at 14 and 17 °C. The positive relationship between dietary protein and feed consumption rate resulted in a significant positive relationship between dietary protein level and FCR for abalone at 14 °C. There were no significant relationships between dietary protein level and FCR for abalone at 17 °C and 20 °C, but a slight positive and negative tendency between dietary protein level and FCR, respectively. These results suggest that the interactive effects of water temperature and dietary protein on feed consumption rate and FCR may be influenced by increased digestive enzyme activity at warmer water temperatures (Edwards and Condon, 2001),

differences in the energetics of abalone at different water temperatures (Duong et al., 2014) or alterations to the gastrointestinal tract morphology (Schaefer et al., 2013).

Feeding abalone high levels of dietary protein, up to 36%, did not necessarily translate to increased soft tissue protein deposition. In contrast, superior protein deposition was previously reported in 1-year old greenlip abalone fed increasing dietary protein levels at 22 °C (Stone et al., 2013). At sub-optimal water temperatures, below 22 °C, abalone may deaminate excess protein to supply energy for metabolism rather than protein deposition and tissue growth, subsequently resulting in increased feed costs and ammonia excretion (Chaitanawisuti et al., 2011). A recent finding by Duong et al. (2014), from samples collected from the same animals used in the current study, indicated that ammonia excretion was significantly higher when abalone were fed diets containing 36% CP compared to abalone fed 27% CP, further supporting this hypothesis. While dietary ingredients used in the current study were selected due to their relatively high protein and energy digestible coefficient (Fleming et al., 1998; Vandepeer, 2005), to reduce feed costs the energy requirements of abalone should ideally be satisfied by dietary carbohydrates (Dunstan, 2010). Increasing the dietary digestible energy supplied by carbohydrates as dietary protein level increased may have reduced the use protein for energy and in turn, increased the utilisation of dietary protein for protein deposition and growth. In practical diet formulations, which were also utilised in the present study, there is limited room to economically manipulate the formulation to increase digestible carbohydrate levels. Lipid inclusion levels used in the current study are optimal for greenlip abalone (Dunstan et al., 2000), as such, are also a limited source of energy. It would be beneficial in future studies to investigate novel dietary ingredients, such as dried macroalgae, with particular consideration to the carbohydrate content, composition and carbohydrate digestibility, to achieve a protein sparing effect (Dunstan, 2010). This may also lead to further improvements to growth and reduction to feed cost.

Test diets were formulated with input from all of the Australian commercial abalone feed producers, and were designed to be isoenergentic, while maintaining the optimal crude lipid level of ~3.6% for greenlip abalone (Dunstan et al., 2000). Due to the inherent lipid content of the other dietary protein sources, fish oil levels decreased as dietary protein level increased. Fish oil, and the fish oil in fish meal, contain essential long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA): eicosapentaenoic acid (20:5n-3, EPA), docosapentaenoic acid (22:5n-3, DPA) and docosahexaenoic acid (22:6n-3, DHA) (Bautista-Teruel et al., 2011). LC n-3 PUFA are important for cellular membrane structure and function, controlling and regulating cellular metabolism, and many other aspects of animal physiology (Bautista-Teruel et al., 2011). The LC n-3 PUFA fatty acid profile of the salmon fish meal and salmon oil used in the current study were not analysed. Using the analysed crude lipid level of salmon fish meal and salmon fish oil (16 and 100%, respectively; Table 3.2), and the fatty acid levels of Atlantic salmon by-products (Nichols et al., 2002), the EPA, DPA, DHA and ∑LC n-3 PUFA levels were calculated to be lowest in the 36% CP diet, 0.071, 0.023, 0.108 and 0.202%, respectively. There are conflicting reports in the literature pertaining to the LC n-3 PUFA requirement of abalone. Dunstan et al. (2000) suggested the EPA and DHA requirement of greenlip abalone was 0.3% of the diet, especially at cooler water temperatures, which would suggest that the 36% CP diet in the current study may have been LC n-3 PUFA deficient. Dunstan et al. (2000) also concluded that maximum growth would be achieved, with no addition of fish oil, when the dietary fish meal inclusion was greater than 8% (fish meal lipid level of 8%; EPA= 0.014; DPA= 0.002; DHA = 0.011; ∑LC n-3 PUFA = 0.026%), which was far exceeded in all diets used in the current study. In addition, the gene expression of Δ-6 desaturase and elongase 2 and the bioaccumulation of LC n-3 PUFA demonstrated in hybrid abalone (*H. laevigata* × *H. rubra*) suggest that this closely related hybrid are able to desaturate and chain elongate the precursor α-linolenic acid

(18:3n-3, ALA) to EPA and DHA (Mateos et al., 2011). In the current study, the lipid fractions of de-hulled lupin meal, and to a lesser extent solvent extracted soybean meal, contained moderate levels of the precursor ALA (Chiofalo et al., 2012; Monteiro et al., 2012), and likely supplemented dietary LC n-3 PUFA. Moreover, Hernández et al. (2013) reported that if ALA is present in the diet, growth of abalone (*H. tuberculata*) is not compromised after 200 days compared to control animals fed a fish oil diet.

Throughout the study, no apparent gross disease symptoms, but a low number of mortalities were observed, which compared favourably with commercial facilities during routine tank harvesting of stocking procedures. However, significantly higher mortalities at 14 °C (7.64%) compared to 17 °C (2.09%) and 20 °C (2.43%) were observed. Stone et al. (2013) similarly observed significantly higher mortalities at 14 °C (6.60%), than 18 °C (1.25%) or 22 °C (1.25%). In contrast, summer mortality is the major concern to the abalone farmers in southern Australia during periods of high summer water temperatures (> 22 °C) where mortality rates can be up to 50% (Vandepeer, 2006). The moderate, yet significantly higher mortalities at low water temperatures observed in the current study might be easily overlooked on-farm. A higher amount of "walk-outs" during the dark period, particularly after monthly weight checks, occurred at 14 °C compared to 17 and 20 °C. These animals were typically found alive, and returned to their respective tank immediately. "Walk-outs" were also observed in *H. midae* when held at 12 - 20 °C, but they do not seem to be water temperature related (Britz et al., 1997). The reason for "walk-outs" and the higher mortalities at 14 °C deserves further investigation as it may be currently overlooked on-farm.

In conclusion, this study adds to the knowledge of the nutritional requirement of greenlip abalone throughout their production cycle, by investigating the optimal dietary protein level for 6-month old, post-weaned greenlip abalone at a more precise water temperature range. Based on results from the current study, it would be beneficial to take

results from the current study and investigate the effect of manipulating dietary protein level for greenlip abalone under culture conditions. When considering SGR, feed consumption rate, FCR and protein deposition, there were no apparent benefits to feed 6-month old greenlip abalone high protein diets at 14 or 17 °C. Therefore, we recommend a dietary protein level of 29% CP (21.9% digestible protein) with a digestible energy level of 12.5 MJ $kg^{-1}$at 14 and 17 °C, which was the minimum recommendation for 1-year old greenlip abalone by Stone et al. (2013). Although care must be taken when comparing between studies, as different sized animals and water temperatures were used Stone et al. (2013) previously recommended a dietary protein level of 34.7% CP (26.7% digestible protein) for slightly larger 1-year old greenlip abalone at 22 °C. In the current study, although dietary protein had no significant effect on SGR of abalone at 20 °C, abalone consumed less feed and there was a tendency for an improved FCR, but also a significantly lower PER, as animals were fed increased dietary protein level, therefore, we suggest that it may beneficial for greenlip abalone to be switched to a diet containing 34.7% CP (26.7% digestible protein) once the water temperature reaches 20 °C. The dietary protein recommendations presented in this study were derived using greenlip abalone that have been selected for growth and survival at higher summer water temperatures, such as those experienced in South Australian waters and Port Phillip Bay, Victoria. It may be beneficial to switch to higher protein diets at lower temperatures in areas where abalone have been selected to grow at lower water temperatures, such as Tasmania and coastal Victoria. Further research to determine the appropriate water temperature to switch to higher protein diets in Tasmania and coastal Victoria may be required to fine tune on-farm feeding practices. The current study and Stone et al. (2013) provide a much clearer understanding of the protein requirements for 6-month, 1-year and 2-year old greenlip abalone at a range of relevant water temperatures.

# Chapter 4. Age-dependent response of digestive enzyme activities to dietary protein level and water temperature in greenlip abalone (*Haliotis laevigata*)

Matthew S. Bansemer, Jian G. Qin, James O. Harris, Elise N. Schaefer, Hanru Wang, Georgia J. Mercer , Gordon S. Howarth and David A.J. Stone (2016). Age-dependent response of digestive enzyme activities to dietary protein level and water temperature in greenlip abalone (*Haliotis laevigata*). Aquaculture, 451, 451-456.

## 4.1 Abstract

Proteases, lipases and carbohydrases are digestive enzyme sub-classes that influence the digestive capacity of abalone. In a 12-week study, the effects of age, water temperature and dietary protein levels on digestive enzyme activity in greenlip abalone (*Haliotis laevigata*) were investigated. One- and 2-year old abalone were fed diets with crude protein (CP) levels from 24 to 36% (18.0 - 28.6% digestible protein) and cultured at 14, 18 and 22 °C. Diets were formulated to be isoenergetic (12.5 MJ $kg^{-1}$ digestible energy) and isolipidic (3.6% crude lipid). Trypsin, α-amylase and lipase activities were measured, and were influenced differently by abalone age, water temperature and dietary protein levels. Lipase and α-amylase activities significantly increased as water temperatures were raised. In contrast, trypsin activity was not affected by water temperature. Trypsin activity of 2-year old abalone were significantly lower (53%) than 1-year old abalone. The α-amylase activities of 1-year old abalone were significantly up-regulated as dietary protein level increased. In contrast, 2-year old abalone down-regulated α-amylase activity by 55% when fed 33% CP, compared to abalone fed 30% CP. The significant trypsin activity down-regulation in 2-year old abalone compared to 1-year old animals provides further support in reducing dietary protein for 2-year old abalone to optimise cultured greenlip abalone production. Significantly higher α-amylase activity in 1-year old abalone as starch levels were reduced indicates a compensatory effect in abalone fed carbohydrate deficient diets. Further research is recommended to optimise the protein to energy ratio for different age classes of greenlip abalone, especially when fed high dietary protein levels.

## 4.2 Introduction

Global abalone production increased by 270% to 86 455 metric tonnes per year from 2007 to 2011 (FAO, 2013). Greenlip abalone (*Haliotis laevigata*) are primarily cultured in land-based systems throughout southern Australia and fed formulated diets (Stone et al., 2013). Dietary protein is an expensive component of formulated diets, but also plays a major role in nutritional value (Bansemer et al., 2014). To reduce feed costs and increase abalone production efficiency, the optimal dietary crude protein (CP) level for growth has been the focus of studies encompassing a range of abalone species (Mai et al., 1995; Britz, 1996; Stone et al., 2013). Optimal growth is dependent on maximising soft tissue protein deposition, which is limited by dietary protein availability and digestion (Fleming and Hone, 1996; Britz and Hecht, 1997; Edwards and Condon, 2001). Digestion of macronutrients is influenced by a number of factors including feed consumption, digestive enzyme activities, digestive enzyme-substrate contact time in the gastrointestinal tract of abalone and the degree of extracellular versus intracellular digestion (Fountoulaki et al., 2005; Stone et al., 2013; Currie et al., 2015).

Moreover, digestive enzyme activity of abalone is significantly influenced by diet (Knauer et al., 1996; Erasmus et al., 1997; García-Carreño et al., 2003; Garcia-Esquivel and Felbeck, 2006). Juvenile South African abalone (*Haliotis midae*; 0.07 g) fed a formulated diet had significantly higher protease activity than abalone fed a diatom diet, which may be related to the higher CP level of the formulated diet (35%), compared to the diatom diet (5%; Knauer et al., 1996). Furthermore, abalone (*H. midae*; 35 mm shell length [SL]) fed *Ecklonia maxima* had higher alginate lyase, carboxymethylcellulase and laminarinase activities than abalone fed *Gracilaria uerrucosa*. In contrast, abalone fed *G. uerrucosa* had high agarase and carrageenase activity compared to abalone fed *E. maxima*. The regulation of carbohydrase activities in *H. midae* was associated with structural carbohydrate differences between

macroalgae species tested (Erasmus et al., 1997). Although numerous studies have investigated the optimal dietary protein level for abalone or diet-associated changes to digestive enzyme activity, the response of digestive enzymes to increasing dietary protein levels in formulated diets is not clearly understood.

The optimal water temperature for growth of a Tasmanian greenlip abalone strain (82 mm SL) was estimated to be 18.3 °C (Gilroy and Edwards, 1998), while the optimal water temperature for growth of a South Australian greenlip abalone strain (23 mm SL) was determined experimentally to be 22 °C (Stone et al., 2013). Under culture conditions, greenlip abalone are exposed to variations in water temperatures that range from 10 to 24 °C (Stone et al., 2013). Water temperature significantly influences the survival, growth, feed consumption and metabolism of abalone (Britz et al., 1997; Edwards and Condon, 2001; Stone et al., 2013; Bansemer et al., *in press*). Temperature also influences the digestive physiology of abalone, including gastrointestinal morphology (Schaefer et al., 2013), gastrointestinal evacuation time (Currie et al., 2015) and digestive enzyme activity (Edwards and Condon, 2001). Protease activity for blacklip abalone (*Haliotis rubra*) was significantly higher (75%) as temperature increased from 9 to 24 °C, which may improve protein utilisation and growth rates as temperature increases (Edwards and Condon, 2001).

Wild abalone have a distinct shift in feeding habit during their life cycle. Juvenile abalone (< 20 mm SL) initially graze higher protein microalgae (12 - 35% CP dry), before shifting to feeding on lower-protein macroalgae (11-19% CP dry) as sub-adults (Mai et al., 1994; Brown et al., 1997; Won et al., 2010). Under culture conditions, the optimal dietary CP level is slightly higher for 1-year old greenlip abalone (~29 - 34.7% CP), compared to larger 2-year old abalone (24 - 34% CP) (Stone et al., 2013). These observations suggest the digestive physiology of abalone may change over time. In a study that investigated radula structure and digestive enzyme activity in juvenile *H. rubra*, Johnston et al. (2005) reported

significant changes to the digestive physiology from 80 to 158 days post settlement. However, the grow-out period for temperate abalone is approximately two and a half to three years, and the ontogenetic development of digestive function for abalone throughout the remaining grow-out period is unknown.

The effect of abalone age, water temperature and dietary protein level on growth performance, feed utilisation and nutrient retention of greenlip abalone were reported by Stone et al. (2013). The authors identified and recommended different optimal dietary CP levels in formulated diets for 1-year and 2- year old greenlip abalone at different water temperatures (Stone et al., 2013). For example, as water temperature increased from 14 to 18 to 22°C, the optimal dietary crude protein level for 1-year old abalone increased from ~29.0 to 32.2 to 34.7%, and for 2-year old abalone increased from 24 to 34 and 34%, respectively (Stone et al. 2013). The digestive capacity of an aquatic animal is dependent on a number of factors, including the types and activities of digestive enzymes. In this study, we further sought to improve the understanding of the digestive physiology of cultured greenlip abalone, which may contribute to further diet development research based on the digestive capacity of greenlip abalone.

## 4.3 Methods

### *4.3.1 Experimental animals and system*

Abalone, diets and experimental design are described fully in Stone et al. (2013). In brief, two distinct year-class cohorts of greenlip abalone, which were spawned from the same broodstock line and cultured under commercial conditions, were purchased from South Australian Mariculture (Port Lincoln, South Australia). Abalone (1-year old: $n$ = 20 animals tank$^{-1}$; 2-year old: $n$ = 10 animals tank$^{-1}$) were stocked into one of four replicate culture units per treatment combination at the South Australian Research and Development Institute

(SARDI) Aquatic Science Centre (West Beach, South Australia). Abalone were held at 14, 18 and 22 °C (± 1 °C) for 84 d. One-year old greenlip abalone (1.75 ± 0.01 g; 23.31 ± 0.03 mm SL; *n* = 960 animals) were fed one of four nominal dietary protein levels (27, 30, 33 and 36% CP; Table 4.1). Two-year old greenlip abalone (22.93 ± 0.09 g; 56.64 ± 0.08 mm SL; *n* = 480 animals) were fed one of four nominal dietary protein levels (24, 27, 30 and 33% CP; Table 4.1). The different diet series were chosen according to the general understanding of the specific requirements of proteins for the small and large animals (Stone et al., 2013). Abalone were fed to excess of their daily requirements based on the total tank biomass (1-year old: 4 - 5%; 2-year old: 1 - 2% biomass $day^{-1}$) at 1600 h, which was based on the stocking biomass and adjusted monthly from weight checks. Tanks were cleaned and uneaten feed was collected the following day at 0830 h.

Water temperature, dissolved oxygen (mg $L^{-1}$ and % saturation), pH and salinity were measured daily, and were maintained at levels appropriate for greenlip abalone throughout the study (Table 4.2; Stone et al., 2013).

**Table 4.1** Ingredient and nutrient composition of experimental diets.

| | Nominal crude protein level (%) | | | | |
|---|---|---|---|---|---|
| | 24 | 27 | 30 | 33 | 36 |
| *Ingredients (g 100g$^{-1}$ diet as fed)* | | | | | |
| Salmon fish meal | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| Solvent extracted soybean meal | 16.40 | 18.90 | 21.40 | 23.90 | 26.47 |
| Lupins (de-hulled) | 17.95 | 20.80 | 23.60 | 26.40 | 29.14 |
| Waxy maize starch | 30.40 | 30.67 | 29.07 | 27.59 | 19.96 |
| Pregelatinised waxy maize starch | 15.40 | 10.00 | 5.62 | 1.15 | 0.00 |
| Wheat gluten meal | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Casein | 4.43 | 5.48 | 6.53 | 7.59 | 8.63 |
| Diatomaceous earth | 2.61 | 1.76 | 1.79 | 1.77 | 4.60 |
| Fish oil | 1.61 | 1.22 | 0.84 | 0.46 | 0.10 |
| EPA Vitamin/mineral premix | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Sodium alginate | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| Vitamin E | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| Calcium sulphate | 0.50 | 0.43 | 0.36 | 0.30 | 0.22 |
| Monosodium phosphate | 0.75 | 0.72 | 0.68 | 0.65 | 0.61 |
| Arginine | 0.27 | 0.31 | 0.37 | 0.41 | 0.46 |
| Threonine | 0.17 | 0.20 | 0.23 | 0.27 | 0.30 |
| *Ingredient composition (g 100g$^{-1}$ diet as fed), analysed and (calculated)* | | | | | |
| Moisture | 10.10 | 10.35 | 10.48 | 10.61 | 10.30 |
| Crude protein | 24.20 | 27.00 | 31.10 | 34.30 | 37.30 |
| Digestible protein (calculated) | 17.99 | 20.27 | 23.54 | 26.13 | 28.57 |
| Lipid | 3.60 | 3.60 | 3.60 | 3.70 | 3.50 |
| Gross energy (MJ kg$^{-1}$) | 17.46 | 17.00 | 17.25 | 17.64 | 17.27 |
| Digestible energy (MJ kg$^{-1}$) (calculated) | 12.72 | 12.24 | 12.35 | 12.57 | 12.53 |
| Ash | 5.68 | 5.02 | 5.31 | 5.24 | 8.17 |
| NFE (calculated) | 66.52 | 64.38 | 59.99 | 56.76 | 51.03 |
| Digestible CP:GE (g MJ$^{-1}$) | 14.14 | 16.57 | 19.06 | 20.79 | 22.80 |
| *Calculated amino acids (g 100g$^{-1}$)* | | | | | |
| Arginine | 1.95 | 2.22 | 2.50 | 2.77 | 3.04 |
| Histidine | 0.64 | 0.72 | 0.80 | 0.89 | 0.97 |
| Isoleucine | 1.13 | 1.28 | 1.44 | 1.59 | 1.74 |
| Leucine | 1.86 | 2.10 | 2.35 | 2.59 | 2.83 |
| Lysine | 1.34 | 1.52 | 1.71 | 1.90 | 2.09 |
| Methionine | 0.41 | 0.46 | 0.51 | 0.57 | 0.62 |
| Phenylalanine | 1.16 | 1.31 | 1.46 | 1.61 | 1.76 |
| Threonine | 1.07 | 1.22 | 1.37 | 1.53 | 1.67 |
| Tryptophan | 0.27 | 0.30 | 0.34 | 0.37 | 0.41 |
| Valine | 1.26 | 1.42 | 1.59 | 1.75 | 1.92 |

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

**Table 4.2** Summary of water quality parameters for each water temperature system[1,2].

| Nominal temperature | Actual temperature (°C)[3] | Dissolved oxygen (mg $L^{-1}$)[3] | Dissolved oxygen (% saturation)[3] | pH[3] | Salinity (ppt)[4] |
|---|---|---|---|---|---|
| 14 °C | 14.1 ± 0.2 (13.2 - 14.7) | 8.0 ± 0.2 (6.8 - 8.7) | 99.1 ± 1.6 (88.4 - 104.2) | 8.3 ± 0.05 (8.1 - 8.4) | 35.0 ± 0.6 (34.0 - 36.0) |
| 18 °C | 18.1 ± 0.2 (17.6 - 19.0) | 7.3 ± 0.3 (6.2 - 8.1) | 95.7 ± 3.00 (79.2 - 102.7) | 8.3 ± 0.04 (8.2 - 8.4) | 35.0 ± 0.6 (34.0 - 36.0) |
| 22 °C | 22.0 ± 0.4 (21.0 - 23.0) | 6.7 ± 0.4 (5.3 - 8.1) | 92.5 ± 4.5 (73.6 - 101.6) | 8.3 ± 0.1 (8.2 - 8.4) | 35.0 ± 0.6 (34.0 - 36.0) |

[1] Values means ± standard deviation, values in parentheses represent the range of values.
[2] Data for DO, pH and salinity for entire experiment, while the data for water temperature is from end of temperature acclimation period.
[3] $n = 83$
[4] $n = 12$

### *4.3.2 Sample collection and biochemical analyses*

At the end of the experiment, abalone were shucked and the gastrointestinal region (combined tissue and mucus) was carefully separated from the adductor muscle. Gastrointestinal samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C prior to the analysis of digestive enzyme activity.

Gastrointestinal samples from six 1-year abalone and three 2-year old abalone, per treatment replicate were partially thawed, weighed, pooled and homogenised in four volumes of distilled water (W/V) using a Dounce homogeniser. As each enzyme kit had set pH levels, the homogenate was resuspended in four volumes of buffer, supplied with each kit (V/V). The suspensions were centrifuged at an acceleration of 17 530 *g* for 20 min at 4 °C. The resulting supernatants were analysed in triplicate for trypsin, α- amylase and lipase activity at the corresponding temperature at which abalone had been held (i.e. 14, 18 or 22 °C) using spectrophotometric techniques and commercial enzyme test kits. Enzyme activities were measured in the linear reaction phase. Each specific enzyme kit included internal standard solutions.

Colourmetric analyses were used to determine trypsin (E.C 3.4.21.4) activity by reading the absorbance of samples at a wavelength of 405 nm at 0 and 1 h (Catalogue No. K771-100; Biovision, Inc., California, USA). Colourmetric analyses were used to determine α-amylase (E.C 3.2.1.1) activity by reading the absorbance of samples at a wavelength of 405 nm at 10 and 20 mins (Catalogue No. K711-100; Biovision). Fluorometric analyses were used to determine lipase (E.C 3.1.1.) activity by reading Ex/Em = 529/600 nm at 0 and 40 mins (Catalogue No. K724-100; Biovision). Total protein was determined using a bicinchoninic acid (BCA) protein assay kit with bovine serum albumin solution as the standard (Catalogue No. K813-2500; Biovision). Specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of one µ mole of substrate per minute per mg of protein (i.e. U mg soluble protein$^{-1}$) at the respective temperature (i.e. 14, 18, 22 °C).

### *4.3.3 Statistical analyses*

IBM SPSS, Version 22 for Windows (IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Homogeneity of variances and normality of data were assessed using Levene's test and Shapiro-Wilk test, respectively, and passed both tests in all cases. Data from each age class were analysed separately using two-factor Analysis of Variance (ANOVA) with water temperature as the first factor and dietary protein level as the second factor. In addition, data were also analysed using three-factor ANOVA (Factors: age [1- and 2-year old], water temperature [14, 18 and 22 °C] and dietary protein level [27, 30 and 33% CP]). Due to the different diet series chosen for 1- and 2-year old abalone, 1-year old abalone fed 36% CP and 2-year old abalone fed 24% CP were excluded from the three-factor ANOVA model. When significant interactions were observed, pairwise comparisons were used to determine significant differences between treatment combinations (Fisher's Least Significant Difference [LSD] test). A significance level of $P < 0.05$ was used for all statistical

tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

## 4.4 Results

### *4.4.1 Growth performance and feed utilisation*

Growth performance, feed utilisation, proximate composition and nutrient retention of 1- and 2-year old greenlip abalone were reported in Stone et al. (2013). In brief, the SGR of abalone was significantly superior as water temperatures were increased from 14 to 22 °C. Dietary protein level had no significant effect on SGR. Faster growing abalone at 22 °C compensated by consuming more feed when fed a low protein diet. This compensatory feed intake effect observed by abalone at 22 °C, significantly influenced the FCR of abalone, which were significantly increased as dietary protein decreased. Additionally, for both age classes of abalone, protein deposition increased as dietary protein level increased at 18 and 22 °C.

### *4.4.2 Digestive enzymes in 1-year old abalone*

Trypsin activity of 1-year old abalone was not affected by water temperature, dietary protein level, or the interaction between these two factors (Table 4.3). The α-amylase activity significantly increased as water temperature increased from 14 to 18 to 22 °C (Table 4.3). Moreover, α-amylase activity was also significantly influenced by dietary protein level (27 < 30 = 33 = 36% CP; Table 4.3). The α-amylase activity was not significantly affected by the interaction between water temperature and dietary protein level (Table 4.3). Lipase activity in 1-year old abalone was significantly lower at 14 and 18 °C than at 22 °C (Table 4.3). Moreover, lipase activity of abalone at 14 and 18 °C was not significantly different. Lipase

activity was not significantly affected by dietary protein level, or the interaction between water temperature and dietary protein level (Table 4.3).

### *4.4.3 Digestive enzymes in 2-year old abalone*

Trypsin activity of 2-year old abalone was not affected by water temperature, dietary protein level or the interaction between these two variables (Table 4.3). The α-amylase activity of abalone significantly increased as water temperature increased from 14 to 18 to 22 °C (Table 4.3). Dietary protein level also significantly influenced α-amylase activity (24 = 27 = 30 > 33% CP). However, α-amylase activity was not affected by the interaction between water temperature and dietary protein level (Table 4.3). Lipase activity was significantly higher in abalone at 22 °C, compared to abalone at 14 or 18 °C (Table 4.3). Lipase activities of abalone at 14 or 18 °C were similar. Lipase activity was not influenced by dietary protein level, or the interaction between water temperature and dietary protein level (Table 4.3).

### *4.4.4 Comparison between 1- and 2-year old abalone*

A three-factor ANOVA was used to analyse the interactive effects of water temperatures (14, 18 and 22 °C) and dietary protein levels (27, 30 and 33% CP) and age classes (1-year old and 2-year old) on digestive enzyme activities. Trypsin activity of 1-year old abalone was significantly higher than for 2-year old abalone (Table 4.4). Trypsin activity was not affected by water temperature, dietary protein level, or by any interaction combinations between age class, water temperature and dietary protein level (Table 4.4).

The α-amylase activity of abalone significantly increased as water temperature increased from 14 to 18 to 22 °C (Table 4.4). The α-amylase activity was not significantly influenced by age class, but was significantly affected by dietary protein level and the interaction between age class and dietary protein level (Table 4.4). The significant interaction

between age class and dietary protein level was due to the significant down-regulation of α-amylase activity for 2-year old abalone fed 33% CP, while the α-amylase activity of 1-year old abalone fed 33% CP was up-regulated, compared to abalone fed 27 or 30% CP ($P < 0.001$). The α-amylase activity of abalone was not affected by the interactions between age class and water temperature, water temperature and dietary protein level, and age class, water temperature and dietary protein level (Table 4.4).

Lipase activity was significantly influenced by water temperature (14 = 18 < 22 °C; Table 4.4), but was not significantly influenced by age class, dietary protein level or any interaction combinations between age class, water temperature and dietary protein level (Table 4.4).

**Table 4.3** Specific trypsin, α-amylase and lipase activities (U mg protein$^{-1}$) in the gastrointestinal tract of juvenile (1-year old) greenlip abalone fed four dietary protein levels (27, 30, 33, 36% crude protein) and sub-adult (2-year old) fed four dietary protein levels (24, 27, 30, 33% crude protein) at 14, 18 or 22 °C ($n$ = 4 replicates per treatment; mean ± SE).

| Temperature (°C) | 14 | | | | | 18 | | | | | 22 | | | | | ANOVA ($P$ value) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein level (%) | 24 | 27 | 30 | 33 | 36 | 24 | 27 | 30 | 33 | 36 | 24 | 27 | 30 | 33 | 36 | Temp (°C) (A) | Protein (%) (B) | A×B |
| *One-year-old* | | | | | | | | | | | | | | | | | | |
| Trypsin activities | NA | 0.41 ± 0.10 | 0.76 ± 0.12 | 0.53 ± 0.11 | 0.78 ± 0.32 | NA | 0.57 ± 0.17 | 0.44 ± 0.14 | 0.83 ± 0.13 | 0.52 ± 0.15 | NA | 0.48 ± 0.31 | 0.82 ± 0.44 | 0.35 ± 0.14 | 0.57 ± 0.09 | 0.913 | 0.725 | 0.524 |
| α-Amylase activities | NA | 54.59 ± 6.46 | 67.24 ± 11.03 | 77.21 ± 7.96 | 86.79 ± 6.93 | NA | 55.63 ± 17.69 | 94.87 ± 16.30 | 127.19 ± 19.77 | 139.93 ± 21.09 | NA | 125.50 ± 6.44 | 154.23 ± 12.44 | 146.80 ± 16.13 | 159.47 ± 12.48 | < 0.001 (14 < 18< 22 °C) | 0.001 (27 < 30 = 33 = 36) | 0.346 |
| Lipase activities | NA | 20.49 ± 1.77 | 17.70 ± 1.42 | 19.43 ± 2.03 | 18.53 ± 1.25 | NA | 21.37 ± 3.85 | 19.32 ± 4.28 | 24.67 ± 4.32 | 23.31 ± 3.05 | NA | 35.82 ± 5.15 | 32.66 ± 4.38 | 34.34 ± 2.77 | 34.13 ± 2.57 | < 0.001 (14 = 18 < 22 °C) | 0.655 | 0.988 |
| *Two-year-old* | | | | | | | | | | | | | | | | | | |
| Trypsin activities | 0.31 ± 0.15 | 0.30 ± 0.05 | 0.28 ± 0.07 | 0.18 ± 0.03 | NA | 0.25 ± 0.06 | 0.19 ± 0.05 | 0.41 ± 0.03 | 0.23 ± 0.09 | NA | 0.36 ± 0.17 | 0.11 ± 0.05 | 0.37 ± 0.12 | 0.31 ± 0.08 | NA | 0.928 | 0.172 | 0.552 |
| α-Amylase activities | 72.30 ± 3.54 | 54.54 ± 10.00 | 108.30 ± 22.68 | 51.02 ± 16.74 | NA | 115.79 ± 3.84 | 150.19 ± 15.58 | 150.32 ± 15.02 | 64.45 ± 2.56 | NA | 156.27 ± 18.66 | 189.16 ± 52.67 | 211.18 ± 57.52 | 96.63 ± 16.31 | NA | < 0.001 (14 < 18 < 22 °C) | 0.002 (24 = 27 = 30 > 33%) | 0.675 |
| Lipase activities | 24.36 ± 5.49 | 27.65 ± 6.14 | 20.19 ± 2.25 | 24.54 ± 5.28 | NA | 22.13 ± 4.46 | 22.03 ± 5.97 | 20.19 ± 5.09 | 21.63 ± 5.23 | NA | 31.31 ± 5.28 | 35.54 ± 6.62 | 35.31 ± 5.63 | 36.27 ± 7.51 | NA | 0.002 (14 = 18 < 22 °C) | 0.874 | 0.989 |

"NA" denotes not analysed

[1] A significance level of $P < 0.05$ was used for all statistical tests.

**Table 4.4** Three-factor ANOVA interaction effects between age class (1-year old [Y1] and 2-year old [Y2]), water temperature (14, 18 and 22 °C) and dietary protein level (27, 30 and 33% crude protein) on the specific trypsin, α-amylase and lipase activities (U mg protein$^{-1}$) in the gastrointestinal tract of greenlip abalone ($n$ = 4 replicates per treatment).

| | Age class (A)<br>*P*= | Water temperature (°C; B)<br>*P*= | Protein level (%; C)<br>*P*= | A×B<br>*P*= | A×C<br>*P*= | B×C<br>*P*= | A×B×C<br>*P*= |
|---|---|---|---|---|---|---|---|
| Trypsin activity | <0.001 (Y1 > Y2) | 0.910 | 0.172 | 0.965 | 0.967 | 0.533 | 0.192 |
| α-Amylase activity | 0.075 | <0.001 (14 < 18 < 22 °C) | 0.017 | 0.628 | <0.001 | 0.640 | 0.371 |
| Lipase activity | 0.346 | <0.001 (14 = 18 < 22 °C) | 0.454 | 0.560 | 0.976 | 0.971 | 0.971 |

[1] A significance level of $P < 0.05$ was used for all statistical tests. All significant interactions are compared using pairwise comparisons, and are explained in text (Fisher's Least Significant Difference [LSD] test).
[2] Refer to Table 4.3 for data.

## 4.5 Discussion

Optimal growth is limited by dietary protein availability and digestion (Fleming and Hone, 1996; Britz and Hecht, 1997; Coote et al., 2000). Trypsin, a sensitive serine protease, is important in protein digestion, and has been reported to be a useful indicator for fish growth (Lemieux et al., 1999; Rungruangsak-Torrissen et al., 2006). Trypsin activity was successfully detected in greenlip abalone in the current study. In contrast, trypsin activity was not detected in red abalone (*Haliotis rufescens*; Garcia-Esquivel and Felbeck, 2006), but has previously been reported in green abalone (*Haliotis fulgens*; Garcìa- Carreño et al., 2003), blue abalone (*Haliotis fulgens*; Hernández-Santoyo et al., 1998), and serine proteases (trypsin and chymotrypsin) in *H. rubra* (Edwards and Condon, 2001; Johnston et al., 2005).

During their life cycle, wild abalone shift from a benthic diet, to grazing on a predominantly macroalgae-based diet (Kawamura et al., 1998). The protein level (dry) of microalgae can be up to 35%, while the protein level of macroalgae is significantly lower (11-19%) (Mai et al., 1994; Brown et al., 1997; Won et al., 2010). This shift in feed strategy was suspected to result in a decreased protease activity in culture abalone throughout the production period. This hypothesis is supported by results from the current study. The trypsin activity of 2-year old greenlip abalone was significantly down-regulated by 53%, compared to 1-year old abalone. The significant down-regulation of trypsin likely reduced dietary protein utilisation in 2-year old abalone and provides further support to reduce dietary protein level for 2-year-old abalone compared to 1-year old greenlip abalone, as previously recommended by Stone et al. (2013). Uki and Watanabe (1992) previously investigated the digestibility of heat-treated fishmeal by small (13 mm) and large (55 mm) abalone (*Haliotis discus hannai*), and reported smaller abalone were more efficient at digesting heat-treated fishmeal, and suggested protease activity may decrease with age.

Previous research has identified the temperature dependent response of protease activity in abalone (Edwards and Condon, 2001). The authors reported significantly higher (75%) protease activity in *H. rubra* as the reaction incubation temperature increased from 9 to 24 °C. Although not investigated, Edwards and Condon (2001) also hypothesised that higher protease activity would contribute to superior growth rates at warmer water temperatures. Results from the current study do not support this hypothesis. While protein deposition and growth rates of abalone increased significantly as water temperature was raised from 14 to 22 °C (Stone et al., 2013), water temperature did not significantly influence trypsin activity. In addition, trypsin activity was not influenced by dietary protein levels in the current study. This is in contrast to significantly higher protease activity reported in abalone (*H. midae*; 0.07 g) fed a higher protein formulated diet (35% CP) compared to a lower protein diatom diet (5% CP) (Knauer et al., 1996). Other compensatory changes to the digestive physiology for 1- and 2-year old abalone, such as gastrointestinal evacuation time gastrointestinal morphology or other proteases, may play a greater role in protein utilisation in greenlip abalone (Schaefer et al., 2013; Currie et al., 2015). The gastrointestinal evacuation time for greenlip abalone was greater as water temperature decreased from 26 to 14 °C, which may increase the contact time between digesta and digestive enzymes to optimise nutrient utilisation (Currie et al., 2015). Furthermore, from samples collected from the same animals used in the current study, Schaefer et al. (2013) reported a significantly thicker stomach epithelial thickness in abalone at 14 °C compared to 22 °C, which was hypothesised to increase the stomach surface area and nutrient utilisation. In addition to morphological changes, trypsin may not be representative of protease in abalone, and other proteases, such as aminopeptidases and chymotrypsin, might be preferentially up-regulated in response to increasing water temperature and dietary protein levels. Aminopeptidases and chymotrypsin have been reported in other abalone species (Garcia-Esquivel and Felbeck, 2006), but were

not investigated in the current study. It would be beneficial in future studies to investigate aminopeptidases and chymotrypsin activity regulation as dietary protein levels and water temperatures are manipulated.

In contrast to trypsin activities, abalone up-regulated α-amylase and lipase activities as water temperature increased. The α-amylase activities of 1- and 2- year old abalone at 22 °C, compared to abalone at 14 °C, was up-regulated by 105% and 128%, respectively. Furthermore, lipase activities of 1- and 2-year old abalone at 22 °C were also significantly higher than abalone cultured at lower water temperatures. Significantly higher α-amylase and lipase activities were likely up-regulated to increase the utilisation of starch and lipids and may be important to the energy metabolism for abalone at warmer water temperatures as the energy expenditure for abalone (*Haliotis kamtschatkana*) is significantly higher as water temperature increases (Donovan and Carefoot, 1998).

In order to increase the dietary protein level, dietary non-nitrogen free extract and carbohydrate levels were unavoidably decreased. Waxy maize starch and pre-gelatinised waxy maize starch were used as the primary dietary carbohydrate energy sources. Starch is composed of α-(1,4) glycosidic linkages, which are efficiently hydrolysed by α-amylase during digestion by abalone (Britz et al., 1996; Vandepeer and van Barneveld, 2003). In the current study, as dietary protein level increased and dietary starch level decreased, the α-amylase activity of 1-year old abalone was significantly up-regulated. Clissold et al. (2010) suggested the first response of animals fed excess macronutrients is to decrease the secretion of enzymes that digest the respective macronutrient, while animals increase the secretion of enzyme for deficient macronutrients. In contrast however, the α-amylase activity in 2-year old abalone fed 33% CP was down-regulated by 55%, compared to abalone fed 30% CP. Prior to the commencement of the current experiment, Schaefer et al. (2013) reported signs of maturation with eggs observed in some of the 2-year old abalone. At the conclusion of the

current experiment, fully developed gonads were also observed in all sampled 2-year old abalone. In contrast, gonads were not observed in 1-year old abalone. Based on the optimal digestion theory, in order to not waste metabolic expense, the secretion of digestive enzymes by animals is positively correlated with substrate concentration (Sibly, 1981; Penry and Jumars, 1986). Results of the current study suggest that abalone shift from up-regulating α-amylase activity when fed carbohydrate deficient diets, to energy partitioning for reproductive maturation (Clissold et al., 2010). This hypothesis is supported by the energy budget for *Haliotis kamtschatkana* (Donovan and Carefoot, 1998). The authors reported a shift in energy allocation from somatic growth in non-mature abalone, to an increased proportion being allocated to gonad development in reproductive mature abalone (Donovan and Carefoot, 1998).

The growth of greenlip abalone does not appear to be impaired by feeding diets containing low levels of carbohydrates (Stone et al., 2013). However, an energy cost is likely associated with up-regulating α-amylase in response to deficient macronutrients in 1-year old abalone. It may be beneficial in further studies to investigate the protein to energy ratio for different age classes of greenlip abalone. Dietary energy should ideally be supplied by carbohydrates as greenlip abalone do not grow well when fed diets high in lipid (> 5%; Dunstan et al., 2000). Optimising the protein to energy ratio has been successful for other abalone species, including *Haliotis asinina* (Bautista-Teruel and Millamena, 1999) and *H. midae* (Britz and Hecht, 1997). However, the nutritional requirements differ between abalone species (reviewed by Bansemer et al., 2014), and caution should be exercised applying information from other abalone species to greenlip abalone.

In conclusion, we have successfully demonstrated that trypsin, α-amylase and lipase activities in greenlip abalone were influenced differently by abalone age, water temperature and dietary protein level. Lipase and α-amylase activities of abalone increased as water

temperature increased, while trypsin activities were not affected by water temperature. Trypsin activity in 2-year old abalone was significantly lower than in 1-year old abalone and further supports reducing the dietary protein level for 2-year old abalone, which was previously recommended by Stone et al. (2013). As dietary protein levels increased, and dietary starch levels were reduced, 1-year old abalone up-regulated α-amylase activity, while 2-year old abalone down-regulated α-amylase activity. Further research to optimise the protein to energy ratio for different age classes of greenlip abalone, especially when fed high dietary protein levels is recommended.

# Chapter 5. Temperature-dependent feed consumption patterns for greenlip (*Haliotis laevigata*) and hybrid (*H. laevigata* × *Haliotis rubra*) abalone fed fresh macroalgae or a formulated diet at night

Matthew S. Bansemer, Jian G. Qin, Krishna-Lee Currie and David A.J. Stone (2015). Temperature-dependent feed consumption patterns of greenlip (*Haliotis laevigata*) and hybrid (*H. laevigata* × *Haliotis rubra*) abalone fed fresh macroalgae or a formulated diet. Journal of Shellfish Research, 34 (3), 885-892.

## 5.1 Abstract

Due to the nocturnal and slow feeding activity of abalone, farmed abalone are typically provided with feed throughout the night. Understanding the nocturnal feeding patterns of abalone is fundamental to feed management and productive abalone farming. In this study, the apparent feed consumption for greenlip abalone (*Haliotis laevigata*) and hybrid abalone (*H. laevigata* × *Haliotis rubra*) fed fresh *Ulva* sp. or a commercial formulated diet at 18 or 22 °C were investigated at night. Abalone were exposed to low light intensity (3.4 Lux) from 07:00 to 19:00 h and darkness from 19:00 to 07:00 h. Abalone were fed to excess daily at 16:00 h and feed intake was determined at 19:00, 22:00, 01:00, 04:00 and 8:00 h. When *Ulva* sp. was added to the tank, greenlip and hybrid abalone immediately displayed a feeding response, which was not observed in abalone fed the formulated diet. Abalone consumed *Ulva* sp. at a linear rate from 16:00 to 08:00 h. In contrast, the apparent feed consumption rate of abalone fed the formulated diet was minimal from 16:00 to 19:00 h, and was highest between 19:00 and 01:00 h. Apparent feed consumption rate of abalone significantly increased as water temperature increased from 18 to 22 °C, but the effect was greater for hybrid abalone compared to greenlip abalone. The total apparent feed intake of both greenlip and hybrid abalone fed *Ulva* sp. was significantly greater than for both types of abalone fed the formulated diet. The total apparent feed intake on dry basis, and nutrient intake for abalone fed *Ulva* sp. was significantly lower than for abalone fed the formulated diet. This study indicates that the upper temperature range for feed intake in hybrid abalone is higher than in greenlip abalone. *Ulva* sp. can stimulate abalone feeding, though the high moisture content in algae can reduce nutrient intake.

## 5.2 Introduction

Macroalgae are the predominate diet of wild abalone, and are also fed to farmed abalone (Shepherd, 1973; Kirkendale et al., 2010). The nutrient intake and growth of abalone fed fresh macroalgae may be limited due to the high moisture content (~80%) and low nutrient density (Kirkendale et al., 2010; Bansemer et al., 2014a). In contrast, nutrient dense formulated diets are crucial to the success of land-based abalone culture as these diets contain an optimal nutritional profile for growth (Fleming and Hone, 1996; Bansemer et al., 2014a).

Abalone rely on chemical and tactile stimuli to detect macroalgae, which are typically limited in commercial formulated diets (Allen et al., 2006). For example, greenlip abalone (*Haliotis laevigata*) graze briefly on a formulated diet chip before moving on to graze on the next, but abalone fed macroalgae (*Ulva* sp. or *Gracilaria cliftonii*) consume the whole fragment before recommencing foraging (Buss et al., 2015). Moreover, blackfoot abalone (*Haliotis iris*) spend most of the time sedentary when fed a formulated diet, but when dried and mulched *Gracilaria* spp. particles (300 - 500 μm) are suspended in the culture system, abalone spend > 80% of their time feeding (Allen et al., 2006).

Due to the nocturnal and slow feeding activity of abalone, feed is typically provided to cultured abalone at night. Nutrient leaching loss from commercial formulated diets during extended periods of immersion is a major concern to the abalone industry (Fleming et al., 1996; Ruff et al., 2014). Binding agents such as agar, carrageenan or alginate are typically used in formulated diets to retard nutrient leaching (Bautista-Teruel et al., 2013). If no binders are used in a formulated diet, the dry matter leaching loss is ~80% after 24 h (Bautista-Teruel et al., 2013). Therefore, reduction of nutrient leaching from the diet has important implications on the nutrient intake and growth of abalone.

Understanding the nocturnal feeding patterns is fundamental to the development of successful feed management practices for abalone farming (Fleming et al., 1996). Various

methods have been used to investigate the peak feeding activity of abalone including the observation of gut fullness (Britz et al., 1996), feed intake rate (Tahil and Juino-Menez, 1999), video monitoring (Allen et al., 2006; Buss et al., 2014). Discrepancies between studies are apparent, likely due to a number of factors including methodological differences, species-specific responses, water temperature variations and differences in physical and biochemical characteristics of diets (Britz et al., 1996; Tahil and Juino-Menez, 1999; Buss et al., 2015).

Two abalone types are primarily cultured in land-based systems throughout southern Australia, the greenlip abalone and hybrid abalone (*H. laevigata* × *Haliotis rubra*). Greenlip abalone are more extensively farmed than hybrid abalone, but culturing hybrid abalone may be advantageous due their superior growth rate, market appeal and disease resistance compared to parental species (Fotedar and Phillips, 2011). Australian abalone farms experience water temperature variations from 10 to 24 °C (Stone et al., 2013), which influences almost every aspect of abalone production including growth and feed consumption (Britz et al., 1997; Bansemer et al., 2015b). A number of factors including species, genetic strain and size influence the optimal water temperature of abalone (Britz et al., 1997; Gilroy and Edwards, 1998; Stone et al., 2013; Bansemer et al., 2015b).

To improve our understanding of the differences in the nocturnal feed consumption patterns between the most commonly cultured abalone in Australia, our aim was to understand the impact of diet type and water temperature on the nocturnal feed consumption patterns for greenlip and hybrid abalone. To achieve this aim in this study, a factorial design was used with abalone type (greenlip or hybrid abalone), diet type (EPA “Abgrow premium” formulated diet [5mm chip] or fresh *Ulva* sp.) and water temperature (18 or 22 °C), resulting in a total of eight treatment combinations. The commercial formulated diet is routinely fed on-farm, while *Ulva* spp. has previously been reported to be excellent feed for abalone (Viera et al., 2011; Stone et al., 2014b). The water temperatures investigated in this study, represent

on-farm temperatures experienced during the period of high feed consumption for both types of abalone (Stone et al., 2013; Lange et al. 2014; Stone et al. 2014a). The knowledge on abalone feeding and feed consumption would provide insight into the improvement of management strategies in terms of time of feeding and the use of macroalgae for feeding stimulation.

## 5.3 Materials and methods

### *5.3.1 Experimental animals*

Greenlip abalone (weight 21.35 ± 0.30 g, shell length 55.50 ± 0.51 mm; $n$ = 100) and hybrid abalone (weight 25.50 ± 0.60 g, shell length 58.87 ± 0.42 mm; $n$ = 100) were purchased from South Australian Mariculture Pty. Ltd. (Port Lincoln, South Australia, Australia). Prior to the study, abalone were held at ambient water temperature in a 5000 L holding tank supplied with sand filtered, UV treated, flow-through seawater at the South Australian Research and Development Institute (SARDI) Aquatic Science Centre (ASC) and fed a commercial formulated diet *ad libitum* ("Abgrow premium" 5 mm chip; Eyre Peninsula Aquafeeds [EPA], Lonsdale, South Australia).

### *5.3.2 Experimental system*

The experiment was conducted in a temperature controlled system previously described in Stone et al. (2013). Briefly, two identical systems were utilised, each system consisted of twenty 12.5 L rectangular blue plastic tanks (Nally IH305, Viscount Plastics Pty Ltd). Each tank was supplied with temperature controlled, sand filtered, UV treated, flow-through seawater at a rate of 300 mL $min^{-1}$. Water temperature was held at 18 or 22 °C (± 1 °C) by 3kW immersion heaters (240V, 3kW, JQ20; Austin & Cridland, Carlton, NSW, Australia). A mesh screen (0.8 mm mesh size) on the outlet of each tank was used to retain uneaten feed.

The photoperiod was held constant at 12 h low intensity fluorescent lighting (3.4 Lux; 07:00 - 19:00 h):12 h dark (19:00 - 07:00 h).

### *5.3.3 Experimental stocking and animal acclimation*

At the commencement of the experiment, abalone were removed from the holding tank (water temperature 14 °C), weighed (± 0.01 g) and measured (± 0.01 mm), and five animals were systematically interspersed in to each experimental tank ($n$ = 5 tanks treatment$^{-1}$). Animals were acclimatised to the experimental system for seven days, and the water temperature was slowly raised (1 °C day$^{-1}$) to the desired temperature (18 or 22 °C). During the acclimation period, abalone were fed their respective diet to excess at 16:00 h (fresh Ulva sp. 4.3%, formulated diet 2.6 % of the abalone biomass day$^{-1}$). Tanks were cleaned at 08:00 h the following day. No feed was left in the tanks between 08:00 and 16:00 h.

### *5.3.4 Diets, feeding and sampling*

During the experimental period, abalone were also fed to excess at 16:00 h, at the same rate as used in the acclimation phase. During the experiment, each tank was systematically sampled five times, once at each sampling time (19:00, 22:00, 01:00, 04:00 and 08:00 h), over five consecutive days. Sampling involved collecting uneaten feed, by sieving the entire tank contents through a fine mesh screen (500 µm). To ensure that feed intake was not limited after the removal of feed at each sampling period, tanks were immediately re-fed to excess and were re-cleaned at 08:00 h the following morning. Abalone were not provided with feed between 08:00 h and 16:00 h each day.

### *5.3.5 Calculation of cumulative feed consumption and total feed intake*

Collected uneaten feed samples were stored at -20 °C, and were oven dried to a constant weight to obtain dry weights. To account for feed leaching losses (formulated diet) or growth (*Ulva* sp.), diets were added to tanks without animals present, and collected at each sampling period at 18 and 22 °C. This value was used as a correction factor to calculate the apparent feed consumption at each sampling period. The calculation for the apparent feed consumption for each sample time was based on dry values for feed intake and wet values for abalone weight. The apparent feed consumption for each collection period is expressed as:

Apparent feed consumption (g kg abalone$^{-1}$) = (feed offered - uneaten feed collected - ([total feed offered × % leaching loss without animals] + [uneaten feed collected / % retained without animals × % leaching loss without animals]) / 2) / tank biomass (Stone et al., 2013).

The total feed and nutrient intake was calculated using the apparent feed consumption formula (above) at 08:00 h (16 h post-feeding). The total feed and nutrient intake were based on wet or dry values for the feed consumption, and wet weight of abalone. The calculations for the total feed intake and nutrient intake are expressed as g kg abalone$^{-1}$ day$^{-1}$.

### *5.3.6 Biochemical and water quality analyses*

The proximate composition of the diets were analysed according to the methods of the AOAC International (1995) and are presented in Table 5.1. Moisture content was determined by oven drying samples to a constant weight. Crude protein (N × 6.25) was determined by the Kjeldahl method. Crude lipid was analysed using a Soxtherm rapid extraction system (Gerhardt GmbH & Co. KG, Königswinter, Germany) with petroleum liquid (BP 100 °C) as the extracting solvent. Total carbohydrate was determined by the Molisch test and a glucose

standard curve. Gross energy of the formulated diet was determined using a bomb calorimeter calibrated with benzoic acid. The gross energy content of the *Ulva* sp. was calculated using the values of 17.2, 23.6 and 39.5 MJ $kg^{-1}$ for carbohydrate, protein and lipid, respectively (NRC, 2011).

Water quality parameters are presented in Table 5.2. Salinity was measured at the start of the five day experimental period (35 g $L^{-1}$). The water temperature, dissolved oxygen (mg $L^{-1}$ and % saturation) and pH were measured daily and maintained at levels appropriate for abalone throughout the study (Hutchinson and Vandepeer, 2004).

**Table 5.1.** Analysed nutrient composition of test diets (g $100g^{-1}$ dry basis).

| Item | Commercial formulated diet[1] | Fresh *Ulva* sp. |
|---|---|---|
| Moisture | 10.66 | 84.15 |
| Crude protein | 34.12 | 18.36 |
| Crude carbohydrate | 58.06 | 25.42 |
| Crude lipid | 3.29 | 1.01 |
| Gross energy (MJ $kg^{-1}$) | 17.01 | 9.10[2] |

[1] Commercial formulated diet: Eyre Peninsula Aquafeeds; Lonsdale, South Australia.
[2] Gross energy was calculated using the values of 23.60, 17.20 and 39.50 MJ $kg^{-1}$ for protein, carbohydrate and lipid, respectively (NRC, 2011).

**Table 5.2.** Summary of water quality for each temperature controlled treatment[1].

| Temperature system | Temperature (°C) | Dissolved oxygen (mg $L^{-1}$) | Dissolved oxygen (% saturation) | pH |
|---|---|---|---|---|
| 18 °C | 18.2 ± 0.3 (17.5 - 18.6) | 7.78 ± 0.25 (7.33 - 8.08) | 101.1 ± 3.4 (95.4 - 109.6) | 8.11 ± 0.03 (8.06 - 8.15) |
| 22 °C | 21.8 ± 0.4 (21.3 - 22.5) | 7.10 ± 0.23 (6.54 - 7.53) | 98.6 ± 3.1 (90.5 - 103.6) | 8.10 ± 0.03 (8.04 - 8.17) |

[1] Values are mean ± standard deviation. Values in parentheses represent the range of values over the five day experimental period.

### *5.3.7 Statistical analyses*

IBM SPSS (version 20 for Windows, IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and the standardised residuals against the predicted mean plot, respectively. Apparent total feed intake (as fed and dry basis) and nutrient intake were analysed using three-factor analysis of variance (ANOVA) (Factors: abalone type [greenlip and hybrid abalone], diet type [fresh *Ulva* sp. and formulated diet] and water temperature [18 and 22 °C]). When significant interactions were observed, pairwise comparisons were used to determine significant differences between treatment combinations. Pearson's linear and second order polynomial regression analyses were also applied between time and apparent cumulative feed consumption. To determine differences between relationships, apparent cumulative feed consumption was analysed using three-factor analysis of covariance (ANCOVA) (Factors: abalone type, water temperature and diet type), with time included as a covariate [19:00, 22:00, 01:00, 04:00 and 08:00 h]). Data were log transformed to satisfy the assumption of ANCOVA. A significant level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean ($n = 5$), unless otherwise stated. If the SE was < 0.01 it is reported as "0.00".

## 5.4 Results

### *5.4.1 Apparent cumulative feed consumption*

Abalone fed actively on both diets throughout the study and immediately displayed an active feeding response post sample collection and re-feeding.

For greenlip abalone, regression analyses showed significant positive second order polynomial relationships between time and apparent cumulative feed consumption for abalone fed the formulated diet at 18 °C ($R^2 = 0.841$; $P < 0.001$; Fig. 5.1) and 22 °C ($R^2 =$

0.915; $P < 0.001$; Fig. 5.1). Due to the low $x^2$ value when a second order polynomial relationship was fitted to the data, the apparent cumulative feed consumption for abalone fed *Ulva* sp. was analysed using Pearson's linear regression analyses. There were significant positive linear relationships between time and cumulative feed consumption for greenlip abalone fed *Ulva* sp. at 18 °C ($R^2 = 0.730$; $P < 0.001$; Fig. 5.1) and 22 °C ($R^2 = 0.612$; $P < 0.001$; Fig. 5.1).

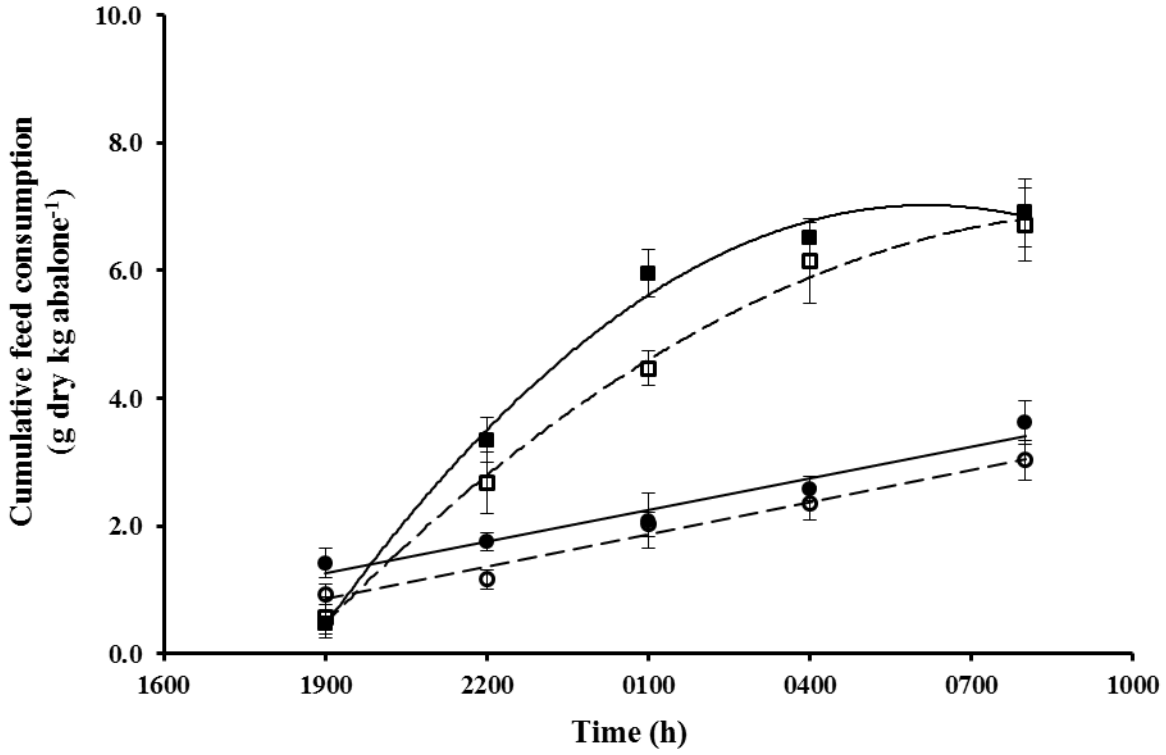


**Figure 5.1.** Cumulative feed consumption (dry basis, *y*) for greenlip abalone (*Haliotis laevigata*) fed the commercial formulated diet at 18 °C (□, dashed line) and 22 °C (■, solid line) or *Ulva* sp. at 18 °C (○, dashed line) and 22 °C (●, solid line) throughout the night ($n$ = 5 replicate tanks treatment$^{-1}$; mean ± SE, $x$). Second order polynomial relationships: commercial formulated diet at 18 °C, $y = -0.29x^2 - 1.030x - 2.356$, $R^2 = 0.841$, $P < 0.001$; and at 22 °C, $y = -0.53x^2 - 1.494x - 3.558$, $R^2 = 0.915$, $P < 0.001$. Linear relationships: *Ulva* sp. at 18 °C, $y = 0.168x - 0.350$, $R^2 = 0.730$, $P < 0.001$; and at 22 °C, $y = 0.165x - 0.771$, $R^2 = 0.612$, $P < 0.001$.

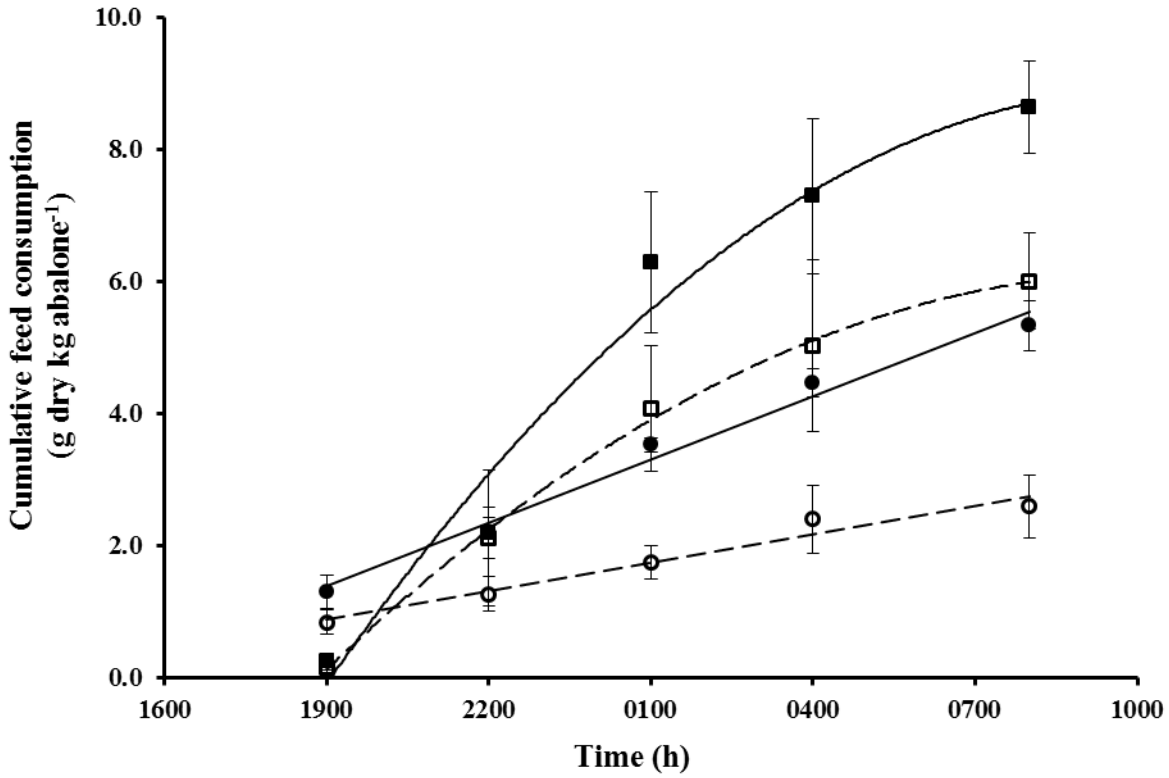


**Figure 5.2.** Cumulative feed consumption (dry basis, *y*) for hybrid abalone (*Haliotis laevigata* × *Haliotis rubra*) fed the commercial formulated diet at 18 °C (□, dashed line) and 22 °C (■, solid line) or *Ulva* sp. at 18 °C (○, dashed line) and 22°C (●, solid line) throughout the night ($n$ = 5 replicate tanks treatment$^{-1}$; mean ± SE, $x$). Second order polynomial relationships: commercial formulated diet at 18 °C, $y = -0.026x^2 + 0.939x - 2.468$, $R^2 = 0.566$, $P < 0.001$; and at 22 °C, $y = -0.038x^2 + 1.405x - 3.957$, $R^2 = 0.776$, $P < 0.001$. Linear relationships: *Ulva* sp. at 18 °C, $y = 0.144x - 0.445$, $R^2 = 0.440$, $P < 0.001$; and at 22 °C, $y = 0.321x + 0.410$, $R^2 = 0.883$, $P < 0.001$.

For hybrid abalone, regression analyses showed significant positive second order polynomial relationships between time post-feeding and cumulative feed consumption for abalone fed the formulated diet at 18 °C ($R^2$ = 0.566; $P$ < 0.001; Fig. 5.2) and 22 °C ($R^2$ = 0.776; $P$ < 0.001; Fig. 5.2). Due to reasons previously stated for greenlip abalone fed *Ulva* sp., the relationship between time and apparent cumulative feed consumption for hybrid abalone fed *Ulva* sp. was analysed using Pearson's linear regression analyses. There were significant positive linear relationships between time and cumulative feed consumption for hybrid abalone fed *Ulva* sp. at 18 °C ($R^2$ = 0.440; $P$ < 0.001; Fig. 5.2) and 22 °C ($R^2$ = 0.883; $P$ < 0.001; Fig. 5.2).

To determine treatment effects on the apparent cumulative feed consumption, data were analysed using ANCOVA, with log transformed time as a covariate to satisfy the linear assumption of ANCOVA. The apparent cumulative feed consumption was significant influenced by the covariate, time ($P$ < 0.001), and diet type (formulated diet > *Ulva* sp.; ANCOVA; Figs. 5.1 and 5.2). Apparent cumulative feed consumption was not significantly influenced by abalone type ($P$ = 0.468), but was significantly affected by water temperature ($P$ < 0.001), and the interaction between water temperature and abalone type ($P$ = 0.009; ANCOVA; Figs. 5.1 and 5.2). Apparent cumulative feed consumption significantly increased as water temperature increased from 18 to 22 °C, this response was significantly higher for hybrid abalone. The apparent cumulative feed consumption was not significantly affected by the interaction between diet type and abalone type ($P$ = 0.111), diet type and water temperature ($P$ = 0.990), or abalone type, water temperature and diet type ($P$ = 0.740).

**Table 5.3.** Apparent total feed and nutrient intake for greenlip abalone (*Haliotis laevigata*) and hybrid abalone (*H. laevigata* × *H. rubra*)[1,2].

| **Abalone type** | **Greenlip abalone** | | | | **Hybrid abalone** | | | | **ANOVA (*P* =)** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Diet type** | **Formulated diet** | | **Fresh *Ulva* sp.** | | **Formulated diet** | | **Fresh *Ulva* sp.** | | **Species (A)** | **Diet (B)** | **Temp (C)** | **A×B** | **A×C** | **B×C** | **A×B×C** |
| **Water temperature** | **18 °C** | **22 °C** | **18 °C** | **22 °C** | **18 °C** | **22 °C** | **18 °C** | **22 °C** | | | | | | | |
| Feed consumption (g as fed kg abalone$^{-1}$ day$^{-1}$) | 7.52 ± 0.65 | 7.73±0.59 | 19.11±1.99 | 22.82±2.18 | 6.72±0.82 | 9.67±0.78 | 16.33±2.99 | 33.64±2.37 | 0.078 | < 0.001 | < 0.001 | 0.179 | 0.003 | 0.001 | 0.039 |
| Feed consumption (g dry kg abalone$^{-1}$ day$^{-1}$) | 6.72±0.58 | 6.91±0.53 | 3.03±0.32 | 3.62±0.35 | 6.00±0.73 | 8.63±0.70 | 2.59±0.47 | 5.33±0.38 | 0.118 | < 0.001 (>) | < 0.001 | 0.858 | 0.003 | 0.722 | 0.846 |
| Crude protein (g kg abalone$^{-1}$ day$^{-1}$) | 2.29±0.20 | 2.36±0.18 | 0.56±0.06 | 0.66±0.06 | 2.05±0.25 | 2.95±0.24 | 0.48±0.09 | 0.98±0.07 | 0.204 | < 0.001 (>) | 0.001 | 0.813 | 0.010 | 0.445 | 0.346 |
| Crude carbohydrate (g kg abalone$^{-1}$ day$^{-1}$) | 3.90±0.34 | 4.01±0.31 | 0.77±0.08 | 0.92±0.09 | 3.49±0.42 | 5.01±0.40 | 0.66±0.12 | 1.36±0.10 | 0.069 | < 0.001 (>) | 0.006 | 0.344 | 0.035 | 0.608 | 0.529 |
| Crude lipid (g kg abalone$^{-1}$ day$^{-1}$) | 0.22±0.02 | 0.23±0.02 | 0.03±0.00 | 0.04±0.00 | 0.20±0.02 | 0.28±0.02 | 0.03±0.00 | 0.05±0.00 | 0.284 | < 0.001 (>) | 0.006 | 0.660 | 0.024 | 0.118 | 0.182 |
| Gross energy [3] (MJ kg abalone $^{-1}$ day$^{-1}$) | 0.11±0.01 | 0.12±0.01 | 0.03±0.00 | 0.03±0.00 | 0.10±0.01 | 0.15±0.01 | 0.02±0.00 | 0.05±0.00 | 0.235 | < 0.001 (>) | 0.002 | 0.754 | 0.008 | 0.338 | 0.400 |

[1]Values are mean ± SE; $n = 5$; SE < 0.01 is reported as "0.00".

[2]A significance level of $P < 0.05$ was used for all statistical tests, for variables with a significant effect of diet ($P < 0.05$) and no interaction, < or > denotes that the variable for abalone fed the formulated diet was significantly less than or greater than abalone fed *Ulva* sp. For variables with a significant interaction, differences between treatments were analysed using pairwise comparisons.

[3] Gross energy intake for abalone fed fresh *Ulva* sp. was calculated using the values of 23.60, 17.20 and 39.50 MJ kg$^{-1}$ for protein, carbohydrate and lipid, respectively (NRC, 2011).

### *5.4.2 Apparent total feed intake*

The apparent total feed intake was significantly influenced by the interaction between abalone type, water temperature and diet type ($P = 0.039$; Table 5.3). Thisinteraction was due to the significantly greater increase in apparent total feed intake for hybrid abalone as water temperature increased from 18 to 22 °C, compared to the response for greenlip abalone. Moreover, compared to greenlip abalone, a significantly greater increase in apparent total feed intake was observed for hybrid abalone fed *Ulva* sp. compared to hybrid abalone fed a formulated diet when water temperature increased from 18 to 22 °C.

The apparent total feed intake (dry basis) was significantly influenced by diet type (formulated diet > *Ulva* sp.; $P < 0.001$; Table 5.3). The apparent total dry mass feed intake was not significantly affected by abalone type ($P = 0.118$), but was by water temperature ($P < 0.001$) and the interaction between abalone type and water temperature ($P = 0.003$). The significant interaction between abalone type and water temperature for apparent total dry mass feed intake was due to the greater increase in feed intake for hybrid abalone as water temperature increased from 18 to 22 °C, compared to greenlip abalone.

Apparent protein, carbohydrate, lipid and energy intake of abalone was significantly influenced by diet type (formulated diet > *Ulva* sp.; $P < 0.001$; Table 5.3). The apparent protein, carbohydrate, lipid and energy intake was not significantly affected by abalone type ($P = 0.118$), but was by water temperature ($P < 0.001$) and the interaction between abalone type and water temperature ($P = 0.003$). The significant interaction between abalone type and water temperature for apparent protein, carbohydrate, lipid and energy intake occurred due to the significantly greater increase in feed intake for hybrid abalone as water temperature increased from 18 to 22 °C, compared to greenlip abalone.

## 5.5 Discussion

Australian abalone farms typically use one to two layers of shade cloth to reduce light in the culture units during the day (Stone et al., 2014a). This low level of daytime light intensity was mimicked in the current study by using low fluorescent lighting (07:00 - 19:00 h; 3.4 Lux; comparable to the dark limit of civil twilight under a clear sky). The apparent feed consumption for greenlip and hybrid abalone fed the formulated diet at either 18 or 22 °C was minimal between the introduction of feed (16:00 h) and the end of the light period (19:00 h). On farm, abalone are typically fed before 17:00 h, but during summer in southern Australia, sunset is not until ~20:30 h, and total darkness may not occur until ~21:00 h (Geoscience Australia, 2014; Stone et al., 2014a). The dry matter leaching loss from commercial formulated diets is relativity low (~15% after 24 h; Ruff et al., 2014), but highly water soluble nutrients, such as vitamins, minerals and free amino acids, may be leached between feeding and total darkness, before abalone have commenced feeding (Gadient and Schai, 1994; Fleming et al., 1996; Coote et al., 2000). It may be beneficial to feed cultured abalone closer to darkness, but this may not be economically viable due to the high cost of labour in Australia. Currently, some hybrid abalone farms culture animals in total darkness during the day (Stone et al., 2014a), and it may also be beneficial to culture greenlip abalone under the same conditions. This practice may not be feasible due to logistical problems associated with occupational health and safety and the practicality of employees working in darkness.

It would be favourable to entice abalone to feed during the light period. In the current study, greenlip and hybrid abalone immediately displayed a feeding response when *Ulva* sp. was added to the tank during the light period, and continued to consume *Ulva* sp. throughout the light period. This response was in contrast to abalone fed the formulated diet. *Ulva* sp. may act as an effective feeding stimulant to both greenlip and hybrid abalone. Previous

studies have reported that a combination of tactile and chemosensory cues in macroalgae, that are not present in formulated diets, which can stimulate a feeding response, and increase the feed intake and growth of abalone (Jan et al., 1981; Allen et al., 2006). For example, Jan et al. (1981) reported an immediate feeding response by abalone (*Haliotis diversicolor supertexta*) when a water soluble extract of *Ulva* spp. (25 g of dried *Ulva* spp. steeped in 1 L of seawater for 1 h, then filtered) was administrated to the tank, despite the absence of feed in the system. Moreover, numerically higher feed intake and significantly higher shell growth rates (15%) were reported for *H. iris* fed a formulated diet with dried, mulched *Gracilaria* spp. particles (300 - 500 μm) suspended in the system compared to abalone fed a control formulated diet alone (Allen et al., 2006). Understanding the mechanism how fresh *Ulva* sp. stimulates the feeding activity of abalone has the potential to improve feeding efficiency in abalone farming. It would be beneficial in future studies to investigate whether the presence of cues from *Ulva* sp. also stimulates greenlip abalone to consume a formulated diet, particular during the light period.

In the current study, the apparent cumulative feed consumption rate of abalone was dependent on diet type, abalone type and water temperature. The cumulative feed consumption rate for greenlip abalone fed the formulated diet at 22 °C was highest between 19:00 and 01:00 h, but abalone ceased feeding after 01:00 h. A similar peak in feed consumption rate was reported between 06:00 h and 12:00 h for *H. midae* fed a formulated diet at 20 °C (Britz et al., 1996). In contrast, the peak feeding activity, determined by ventral video monitoring, for greenlip abalone fed the same formulated diet as used in the current study was between 22:00 and 06:00 h at 22 °C (Buss et al., 2015). Differences may have occurred due to size differences or methodology difference employed by Buss et al. (2015) and to those used in the current study. Moreover, in the current study, feed consumption was prolonged, the apparent feed consumption rate for greenlip abalone at 18 °C and hybrid

abalone at 18 and 22 °C was highest between 19:00 and 16:00 h, but abalone continued to feed at a lower rate until 08:00 h. Nutrient leaching loss from commercial formulated diets is a major concern to the abalone industry (Fleming et al., 1996; Ruff et al., 2014). Based on the results from the current study, we recommend that diets should ideally retard nutrient leaching loss from the addition of feed until morning (> 16 h). Currently, commercial abalone feed companies exceed this recommendation and use binders and manufacturing processes to improve formulated diet stability and aim to minimise nutrient leaching loss for 24 h (Ruff et al., 2014).

The peak feed consumption rate for the ass's-ear abalone (*Haliotis asinina*) fed a mixed macroalgae diet (*A. specifera*, *H. valentiae* and *L. papillosa*) occurred between 18:00 and 02:00 h (Tahil and Juino-Menez, 1999), which is similar to the aforementioned studies where abalone were fed a formulated diet (Britz et al., 1996; Buss et al., 2015). In contrast, greenlip and hybrid abalone in the current study consumed *Ulva* sp. at a linear rate throughout the feeding period. While the feed intake for abalone fed *Ulva* sp. was significantly higher (~300%) than abalone fed the formulated diet, the energy and nutrient intake of abalone fed *Ulva* sp. were significantly lower. Abalone regulate feed intake to achieve energy satiation (Fleming et al., 1996). In the current study, the inherent high moisture content of *Ulva* sp. may have increased gut-fullness and inhibited feed intake up-regulation to achieve energy satiation (Fleming et al., 1996; Alcantara and Noro, 2005). It should be noted that in the current study, *Ulva* sp. was cultured in unenriched seawater. *Ulva* spp. are able to utilise inorganic nitrogen and synthesise amino acids and protein when cultured in a nutrient enriching medium (Viera et al., 2011; Nielsen et al., 2012). Abalone fed nutrient enriched *Ulva* spp. have higher feed intake and growth rates than those fed unenriched *Ulva* spp. (Shpigel et al., 1999; Viera et al., 2011; Bansemer et al., 2014b). The feeding patterns of greenlip and hybrid abalone in the current study might have differed if abalone had been fed

nutrient-enriched *Ulva* sp. that had higher protein and energy content. The feeding response of abalone fed nutrient-enriched macroalgae warrants further investigation.

Water temperature affects abalone growth, metabolic activity and feed consumption (Britz et al., 1997; Bansemer et al., 2015b). In the current study, regardless of diet, the effect of water temperature on the cumulative feed consumption rate and feed intake was dependent on abalone type. The negligible increase in cumulative feed consumption rate and feed intake for greenlip abalone as water temperature increased from 18 to 22 °C is supported by a previous study by Stone et al. (2013). Stone et al. (2013) used greenlip abalone of a similar size (57 mm SL) and strain (South Australian Mariculture Pty Ltd.) to those used in this study, and found that raising water temperature from 18 to 22 °C had no significant effect on growth and feed intake when fed a formulated diet (31 - 34% crude protein level). The optimal water temperature for hybrid abalone growth is unknown, but are generally cultured in cooler water compared to greenlip abalone (Stone et al., 2014a). It should be noted that Gilroy and Edwards (1998) reported that the optimal water temperature for a strain of Tasmanian greenlip abalone (82 mm SL) was 18.3 °C, while the optimum for blacklip abalone collected from a similar geographical location was 17.0 °C. The notion that hybrid abalone have a lower water temperature optimum than greenlip abalone was not supported in the current study. The high survival (100%) and feed intake for hybrid abalone at 22 °C suggest that the upper physiological range for hybrid abalone may be ≥ 22 °C. This was a short-term study, and the effect of chronic exposure to water temperature ≥ 22 °C is unknown. Moreover, cultured hybrid abalone may be exposed to sub-optimal conditions, such as high stocking densities, low flow rates and low dissolved oxygen levels, which may also influence the physiological temperature range and ultimately survival for hybrid abalone (Wassnig et al., 2010). Further research on the optimal and physiological water temperature range for hybrid abalone would be beneficial to the abalone industry.

In conclusion, *Ulva* sp. induced an immediate feeding response of hybrid and greenlip abalone during the light period. The feed consumption of abalone fed a formulated diet was minimal during the light period. The high moisture content of *Ulva* sp. may restrict the energy and nutrient intake by greenlip and hybrid abalone and may ultimately compromise growth. To increase the feed intake of abalone fed a formulated diet during the light period on-farm, the cues present in fresh *Ulva* sp. that induced abalone feeding deserves further investigation. Compared with greenlip abalone and blacklip abalone, their hybrid can tolerate a higher temperature (≥ 22 °C). This study is a step stone for further investigation on the use of live macroalgae and dried macroalgae meal in abalone diets.

# Chapter 6. Growth and feed utilisation of greenlip abalone (*Haliotis laevigata*) fed nutrient enriched macroalgae

Matthew S. Bansemer, Jian G. Qin, James O. Harris, Duong N. Duong, Thanh Hai Hoang, Gordon S. Howarth and David A.J. Stone (2016). Growth and feed utilisation of greenlip abalone (*Haliotis laevigata*) fed nutrient enriched macroalgae. Aquaculture, 452, 62-68.

## 6.1 Abstract

Wild greenlip abalone predominantly consume macroalgae, but are fed formulated diets under culture conditions. This study aims to (i) investigate the effect of nutrient enrichment (non-enriched and enriched) and fresh macroalgae type (*Ulva* sp., *Gracilaria cliftonii*, and an equal combination of *Ulva* sp. and *G. cliftonii*) on the growth and feed utilisation of greenlip abalone and (ii) compare the performance of abalone fed fresh macroalgae to those separately fed either one of the three commercial formulated diets. Abalone were fed to excess at 16:00 h daily, and uneaten feed was collected the following morning. Nutrient enrichment increased the protein level for *Ulva* sp. and *G. cliftonii* from 5.3 to 27.7% and 12.9 to 38.1%, respectively. The growth of abalone fed *G. cliftonii* was superior to animals fed *Ulva* sp. The effect of enrichment was macroalgae species-dependent. While abalone fed enriched *Ulva* sp. exhibited superior growth to those fed non-enriched *Ulva* sp., animals fed non-enriched and enriched *G. cliftonii* exhibited similar growth. However, abalone fed non-enriched *G. cliftonii* had superior apparent protein deposition and protein efficiency ratio, compared to animals fed enriched *G. cliftonii.* Feeding an equal mix of *Ulva* sp. and *G. cliftonii* had a positive synergistic effect on abalone growth, compared to animals fed mono-specific algae. Abalone fed each commercial diet exhibited improved growth and feed utilisation compared to animals fed fresh macroalgae. The results of the current study suggest the use of fresh *G. cliftonii* as a source of carbohydrates may spare protein when feeding formulated diets to abalone. When formulated diets are unavailable or are inappropriate to feed abalone, a mix of enriched *Ulva* sp. and *G. cliftonii* may be used. However, feeding fresh macroalgae alone to cultured greenlip abalone should be avoided, if growth is the parameter of interest and we recommend that commercial formulated diets be fed to cultured greenlip abalone.

## 6.2 Introduction

Greenlip abalone (*Haliotis laevigata*) are cultured throughout southern Australia in land-based systems and fed formulated diets until market size (Fleming and Hone, 1996; Stone et al., 2013; Bansemer et al., 2014). However, the predominant diet of wild greenlip abalone is macroalgae (Shepherd, 1973). Macroalgae are also utilised as feed for cultured abalone in numerous countries including China, Korea and Chile (Kirkendale et al., 2010). Macroalgae improve abalone health and marketability and may also stimulate abalone feeding activity, which may improve growth, compared to those fed formulated diets (Allen et al., 2006; Brown et al., 2008; Lange et al., 2014; Stone et al., 2014; Buss et al., 2015). In Australia, feeding macroalgae to greenlip abalone was previously limited due to the prohibition of wild macroalgae collection on mainland Australia. Recently however, there has been increasing research to aid the development of an Australian macroalgae aquaculture industry, which would be capable of supplying high quality feed for farmed abalone (Lorbeer et al., 2013).

Numerous macroalgae species are cultured globally and abalone accept a variety of macroalgae species. Two macroalgae genera, the red algae *Gracilaria* spp. and the green algae *Ulva* spp. have been identified as excellent candidates for abalone feed (Naidoo et al., 2006; Viera et al., 2011). Dietary protein is the first growth limiting macronutrient for abalone, and the optimal dietary protein level for abalone ranges from 24% to 47% (Bansemer et al., 2014). However, non-enriched macroalgae are generally low in protein (11-19% dry) (Viera et al., 2011). Two management options to overcome protein and nutrient limitations when feeding fresh macroalgae to abalone are recommended: (i) prior to feeding, macroalgae should be cultured in a nutrient enriching medium; and (ii) macroalgae should be fed as mixed macroalgae diets (Shpigel et al., 1999; Viera et al., 2011). Some macroalgae genera, including *Gracilaria* spp. and *Ulva* spp., are able to assimilate exogenous inorganic

nitrogen for amino acid and protein synthesis (Hernández et al., 2002; Taylor et al., 2006). Culturing macroalgae in a nutrient enriching medium can increase the protein level to >30% (Viera et al., 2011). Abalone fed nitrogen/protein-enriched macroalgae exhibited superior growth compared to those fed non-enriched macroalgae (Shpigel et al., 1999; Viera et al., 2011). In addition to nutrient enrichment, feeding mixed macroalgae to abalone is recommended as it provides a superior balance of essential nutrients, such as amino acids, compared to mono-specific macroalgal diets (Viera et al., 2011). Although nutrient enrichment and mixed macroalgae diets have been tested for a range of other abalone species, macroalgae preference and nutritional requirements of abalone are species-specific. For example, while most abalone species generally prefer brown macroalgae species (e.g. *Macrocystis* spp., *Ecklonia* spp. and *Laminaria* spp.), Australian abalone generally prefer red algae and *Ulva* spp., and avoid brown algae (Fleming, 1995; Flores-Aguilar et al., 2007; Cornwall et al., 2009). There is a need to investigate the growth and feed utilisation of greenlip abalone fed nutrient-enriched algae, before fresh macroalgae is incorporated into commercial on-farm feeding practices for greenlip abalone.

It is important to note that if macroalgae form part of the feeding regime for cultured abalone, growth should not be compromised compared to current formulated diets. The current literature relating to the growth of abalone fed fresh macroalgae and formulated diets is controversial and conflicting (Naidoo et al., 2006; Garcìa-Esquivel and Felbeck, 2009; Hernández et al., 2009; Mulvaney et al., 2013a). For example, red abalone (*Haliotis rufescens*) fed a formulated diet exhibited superior growth to abalone fed *Macrocystis pyrifera* (Garcìa-Esquivel and Felbeck, 2009). In contrast, Mulvaney et al. (2013a) reported significantly lower growth rates for hybrid abalone (*H. laevigata* × *Haliotis rubra*) fed a formulated diet, compared to abalone fed fresh macroalgae (*Ulva* spp. and *Grateloupia* spp.). Further research is required to investigate the growth and feed utilisation of abalone fed fresh

macroalgae and commercial diets. In this study, we aimed to: (i) investigate the effect of nutrient enrichment (non-enriched and enriched) and macroalgae type (*Ulva* sp., *Gracilaria cliftonii* and an equal mix of *Ulva* sp. and *G. cliftonii*) on growth performance and feed utilisation of greenlip abalone; and (ii) compare the growth performance and feed utilisation of abalone fed fresh macroalgae to those separately fed either one of the three commercial formulated diets.

## 6.3 Methods

### *6.3.1 Experimental animals and system*

Greenlip abalone (0.80 ± 0.01 g; shell length 17.94 ± 0.03 mm) were purchased from *Kangaroo Island Abalone* Pty Ltd (Smith Bay, SA, Australia). Prior to stocking, abalone were held in a flow-through seawater system at South Australia Research and Development Institute Aquatic Sciences (West Beach, SA, Australia), and fed a commercial formulated diet *ad libitum* ("Abgrow premium" 5 mm chip; Eyre Peninsula Aquafeed Pty Ltd., Lonsdale, SA, Australia).

The experiment was conducted in a photoperiod and temperature controlled laboratory described in Stone et al. (2013). Briefly, abalone were housed in one of 36 12.5 L blue plastic culture units (bottom surface area of 1129 $cm^2$), and were supplied with sand filtered, UV treated, flow-through seawater at a rate of 300 mL $min^{-1}$. Water depth was held at 2.5 cm (effective tank water volume of 2.8 L) using a standpipe with a mesh screen (0.8 mm) on the outlet to retain uneaten food. Water temperature was held at 22 °C by using a 3 kW immersion heater (240V, JQ20; Austin & Cridland, Carlton, NSW, Australia) in the system sump.

### *6.3.2 Stocking*

Abalone were gently prised from the holding tank using a spatula. Fifteen animals were weighed, measured and stocked into four replicate culture units per dietary treatment. Abalone were acclimated to the system for two weeks and were fed a commercial formulated diet ("Abgrow premium" 5 mm chip). After seven days the water temperature was slowly raised from 19 °C to the final temperature of 22 °C. Dead abalone were recorded, measured, weighed and replaced with abalone of a similar weight, which had been fed their respective diet at 22 °C.

### *6.3.3 Diets and feeding*

In this 93 day study, we utilised a 3 × 2 factorial design, three macroalgae types (Ulva sp., G. cliftonii, and a mixed algae diet consisting of an equal mixture of both species) were fed as either non-enriched or nutrient/protein enriched treatments. In addition, abalone were separately fed either one of the three commercial formulated diets, which acted as controls to compare with animals fed fresh macroalgae.

Fresh *Ulva* sp. and *G. cliftonii* were collected from intertidal sand-flats at Outer Harbor (Gulf St Vincent, SA, Australia), and cultured in four 4000 L tanks, under ambient sunlight. Non-enriched *Ulva* sp. and *G. cliftonii* were supplied with fresh seawater at a rate of 8 L $min^{-1}$. Enriched *Ulva* sp. and *G. cliftonii* were held in static seawater and enriched fortnightly with 8 L of modified F2 nutrient media (Guillard and Ryther, 1962; Lange et al., 2014). Tanks were provided with aeration through a bottom-central pipeline to keep macroalgae in motion. The *G. cliftonii* were covered with shade cloth (80% nominal shade) to reduce epiphytic growth, while *Ulva* sp. were exposed to direct sunlight. During the experimental period, macroalgae were sub-sampled weekly, and samples within a tank were pooled for proximate composition analyses. Three formulated diets (3 - 5 mm chip) were supplied by different feed

companies (Eyre Peninsula Aquafeed; Aquafeeds Australia [formally Adam and Amos], Mount Barker, SA, Australia; and Skretting Australia, Cambridge, TAS, Australia) and were stored at -20 °C prior to feeding. Proximate composition of macroalgae and commercial formulated diets is presented in Table 6.1.

Prior to feeding, macroalgae were spun dry in a salad spinner, weighed into individual feeding containers for each tank and topped up with seawater. Commercial formulated diets were fed as supplied. Abalone were fed to excess daily at 16:00 h. Feed rates were maintained at 14% and 4.5% abalone biomass $day^{-1}$ for macroalgae and commercial diet treatments, respectively, and were adjusted throughout the experiment based on monthly bulk weight checks.

Tanks were cleaned and uneaten feed was collected by sieving the entire tank contents through a fine mesh at 08:30 h daily. Uneaten macroalgae were spun dry in a salad spinner and weighed. Uneaten formulated diets were collected, stored at -20 °C and were later dried at 105 °C for 16 h. Daily feed consumption was calculated by the difference between feed offered and uneaten feed in dry weight. To account for macroalgae growth, *Ulva* sp. and *G. cliftonii* were added to tanks without animals present at 22 °C at 16:00 h daily, collected at 08:30 h, spun dry in a salad spinner and weighed. To account for the leaching loss of formulated diets, diets were added to tanks without animals present at 22 °C. The weight difference of feed measured between 16:00 h and 08:30 h was used as a correction factor to calculate the apparent feed consumption rate.

**Table 6.1** Nutrient composition of non-enriched, nutrient-enriched and mixed fresh macroalgae diets and commercial formulated diets (dry g $100g^{-1}$).

| | Non-enriched macroalgae | | | Enriched macroalgae | | | Commercial formulated diets | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Ulva* sp. | *G. cliftonii* | Mixed[1] | *Ulva* sp. | *G. cliftonii* | Mixed[1] | 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 $kg^{-1}$) | 14.17 | 16.19 | 15.18 | 16.91 | 16.23 | 16.57 | 16.83 | 16.89 | 17.01 |
| Ash | 27.7 | 27.7 | 27.7 | 24.3 | 28.9 | 26.6 | 7.3 | 6.9 | 8.3 |
| Carbohydrate[2] | 65.4 | 57.6 | 61.5 | 46.2 | 31.4 | 38.8 | 50.6 | 54.1 | 48.3 |
| *Amino acids* | | | | | | | | | |
| 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 | 3.47 | 9.14 | 6.31 | 18.58 | 19.47 | 19.03 | 29.23 | 26.99 | 27.54 |

[1] Composition of mixed macroalgae diet is calculated based on feeding an equal mix (1:1 by wet weight) of *Ulva* sp. and *Gracilaria cliftonii*.
[2] Carbohydrate = 100% - (protein % + lipid % + ash %)

### *6.3.4 Biochemical and water quality analysis*

The proximate composition analyses of diets and whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardisation (2000). At the commencement of the experiment, the soft tissue of 40 animals ($n$ = 4 replicates) were collected, shucked and stored at -20 °C to analyse the initial soft tissue protein and energy composition. At the end of the experiment, five abalone from each tank were collected, shucked and stored at -20 °C. Abalone were later pooled for each tank for the analysis of soft tissue protein and energy composition.

All data reported for animal performance were based on the pooled data from each tank. All calculations using abalone weight were based on wet values, while the feed values were based on an as fed and dry values:

Biomass gain (g $tank^{-1}$) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)

Specific growth rate (SGR, % $day^{-1}$) = ([ln final weight - ln initial weight] / days) × 100

Shell growth rate (µm $day^{-1}$) = (final shell length - initial shell length) / days

Apparent feed consumption = (feed offered - uneaten feed collected - ([total feed offered × % leaching loss without animals] + [uneaten feed collected / % retained without animals × % leaching loss without animals]) / 2) / tank biomass (Stone et al., 2013)

Apparent feed conversion ratio (FCR) = feed consumed / abalone weight gain

Apparent protein efficiency ratio (PER) = abalone weight gain / protein consumed

Apparent energy efficiency ratio (EER) = abalone weight gain / energy consumed

Apparent protein deposition = ([final soft body protein-initial soft body protein] / protein intake) × 100

Apparent energy deposition = ([final soft body energy-initial soft body energy] / energy intake) × 100

Water quality parameters were monitored daily. Water temperature was measured using an alcohol filled thermometer. Dissolved oxygen (mg $L^{-1}$ and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, IL, USA). Salinity (g $L^{-1}$) was measured using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, NH, USA).

### *6.3.5 Statistical analyses*

IBM SPSS (Version 22 for Windows; IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro–Wilk test, respectively. All percentage data were arcsine transformed before analyses. Initial weight, initial shell length and mortality were compared between all treatments and were analysed using a one-factor ANOVA. To assess the effect of nutrient enrichment (non-enriched and enriched) and macroalgae types (*Ulva* sp., *G. cliftonii* and mixed diet) on abalone performance, data were analysed using two-factor (2 × 3)ANOVA. When significant main effects were observed, Fisher's least significant difference post-hoc test was used to detect significant differences between treatment means. When significant interactions between macroalgae types and nutrient enrichment were observed, pairwise comparisons were used to determine significant differences between treatment combinations (Fisher's least significant difference). To correct for the experimentwise error rate for pairwise comparisons, a significance level of $P < 0.01$ was used. There were no significant differences in performance between abalone separately fed either one of the three commercial diets (one-factor ANOVA). Data for abalone fed the three commercial diets were pooled ($n = 12$), and used as a control to compare to each fresh macroalgae treatment ($n = 4$ 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. If the SE was < 0.01 it is reported as "0.01".

## 6.4 Results

### *6.4.1 General observations*

The average initial weight and shell length of abalone were 0.80 ± 0.01 g and 17.94 ± 0.03 mm, respectively, and were not significantly different between diets (one-factor ANOVA; $P = 0.329$ and 0.819, respectively). Throughout the study, water quality parameters were maintained at levels appropriate for greenlip abalone: water temperature (21.9 ± 0.4, 20.8 - 23.0 °C [mean ± standard deviation, range]), dissolved oxygen (97 ± 4, 88 - 107% saturation; 7.0 ± 0.5, 6.0 - 8.7 mg $L^{-1}$), pH (8.2 ± 0.1, 7.6 - 8.6) and salinity (35 ± 1, 34 - 36).

Animals exhibited normal signs of feeding behaviour and fed actively on all diets during the study. No gross signs of disease were observed in abalone. The overall mortality rate of abalone during the study was 0.76%, and was not affected by diet (one-factor ANOVA; $P = 0.845$). Nutrient enrichment of *Ulva* sp. and *G. cliftonii* increased dietary protein level (dry) from 5.3 to 27.7% and 12.9 to 38.1%, respectively (Table 6.1).

### *6.4.2 Interactive effects between macroalgae type and enrichment*

#### *6.4.2.1 Growth performance*

Biomass gain, SGR and shell growth rate of abalone were significantly affected by the interaction between enrichment (non-enriched and enriched) and macroalgae type (*Ulva* sp., *G. cliftonii* and mixed diet) (two-factor ANOVA; $P < 0.001$; Table 6.2). While abalone fed *G. cliftonii* exhibited superior growth to animals fed *Ulva* sp., abalone fed enriched mixed macroalgae exhibited significantly superior growth to those fed other macroalgae treatments.

The interaction between enrichment and macroalgae type was due to the significant superior growth performance for abalone fed enriched *Ulva* sp. and mixed macroalgae, while abalone fed enriched *G. cliftonii* exhibited inferior growth, compared to abalone fed respective non-enriched treatments ($P < 0.001$; Table 6.2).

#### *6.4.2.2 Feed use*

Feed consumption rate (g as fed kg abalone$^{-1}$ day$^{-1}$) was significantly influenced by the interaction between enrichment and macroalgae type ($P < 0.001$; Table 6.2). The significant interaction was due to the significant increase in feed consumption rate of abalone fed enriched *Ulva* sp. compared to abalone fed non-enriched *Ulva* sp., while abalone fed enriched *G. cliftonii* and mixed macroalgae diets had similar or significantly lower feed consumption rates compared to non-enriched treatments, respectively. Abalone fed *G. cliftonii* (non-enriched and enriched) had significantly higher feed consumption rates than abalone fed mixed macroalgae treatments, which were significantly higher than those fed *Ulva* sp. ($P < 0.001$; Table 6.2).

The apparent FCR (as fed) was significantly affected by the interaction between enrichment and macroalgae type ($P < 0.001$; Table 6.2). The interaction was due to the significantly superior FCR for abalone fed enriched *Ulva* sp. compared to abalone fed non-enriched *Ulva* sp., while feeding abalone enriched *G. cliftonii* and mixed macroalgae diets did not significantly influence FCR.

#### *6.4.2.3 Soft tissue composition and nutrient use*

The soft tissue composition (moisture, protein and energy) of abalone were not significantly influenced by macroalgae type, enrichment, and the interaction between the two factors ($P > 0.05$; Table 6.2).

Apparent protein efficiency ratio (PER) and apparent protein deposition of abalone were significantly influenced by the interaction between macroalgae type and enrichment ($P < 0.001$; Table 6.2). The interaction between enrichment and macroalgae type was similar for apparent PER and apparent protein deposition. The interaction was due to the significant increase in PER and protein deposition for abalone fed enriched *Ulva* sp. compared to non-enriched *Ulva* sp., while the apparent PER and protein deposition for abalone fed non-enriched *G. cliftonii* and mixed macroalgae diets were significantly higher than those fed enriched equivalent treatments. Abalone fed non-enriched *G. cliftonii* and non-enriched mixed macroalgae had significantly higher apparent PER than abalone fed other diets.

The apparent energy efficiency ratio (EER) was significantly affected by macroalgae type ($P = 0.014$; *Ulva* sp. < *G. cliftonii* = mixed) and enrichment ($P = 0.001$; non-enriched < enriched), but not by the interaction between these two factors ($P = 0.287$; Table 6.2). Apparent energy deposition of abalone was significant influenced by the interaction between enrichment and macroalgae type ($P < 0.001$). Energy deposition increased for abalone fed enriched macroalgae, although the effect was more pronounced for abalone fed *Ulva* sp., compared to abalone fed *G. cliftonii* and mixed macroalgae diets. Moreover, the increase of energy deposition was also more pronounced for abalone fed enriched mixed macroalgae diets, compared to enriched *G. cliftonii*.

**Table 6.2** Growth performance, feed efficiency and nutrient retention of greenlip abalone fed non-enriched and nutrient-enriched mono-and mixed-macroalgae diets and commercial diets [1]

| Enrichment | | Non-enriched macroalgae | | | Enriched macroalgae | | | Dunnet's test (*P* value)[2] | 2 factor ANOVA (*P* value)[3] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macroalgae | Control commercial diets | *Ulva* sp. | *G. cliftonii*. | Mixed | *Ulva* sp. | *G. cliftonii* | Mixed | | Macroalgae (A) | Enrichment (B) | A × B |
| *Growth performance* | | | | | | | | | | | |
| Biomass gain (g tank$^{-1}$) | 70.24 ± 2.07$^{a}$ | 4.29 ± 0.11$^{b}$ | 38.06 ± 1.69$^{b}$ | 29.85 ± 2.85$^{b}$ | 23.58 ± 2.80$^{b}$ | 37.14 ± 6.86$^{b}$ | 45.31 ± 2.27$^{b}$ | < 0.001 | < 0.001 | < 0.001 | 0.020 |
| SGR (% day$^{-1}$) | 2.07 ± 0.03$^{a}$ | 0.33 ± 0.01$^{b}$ | 1.55 ± 0.03$^{b}$ | 1.36 ± 0.07$^{b}$ | 1.10 ± 0.05$^{b}$ | 1.35 ± 0.02$^{b}$ | 1.70 ± 0.04$^{b}$ | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| Shell growth rate (µm day$^{-1}$) | 181.49 ± 3.34$^{a}$ | 23.56 ± 0.96$^{b}$ | 128.85 ± 4.09$^{b}$ | 104.65 ± 7.15$^{b}$ | 75.85 ± 1.64$^{b}$ | 109.63 ± 1.98$^{b}$ | 143.05 ± 3.04$^{b}$ | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| *Feed utilisation* | | | | | | | | | | | |
| Feed consumption rate (g as fed kg abalone$^{-1}$ day$^{-1}$) | 10.61 ± 0.45$^{b}$ | 25.42 ± 1.27$^{a}$ | 62.19 ± 0.80$^{a}$ | 51.72 ± 0.10$^{a}$ | 38.54 ± 0.59$^{a}$ | 61.38 ± 0.25$^{a}$ | 48.12 ± 1.22$^{a}$ | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| Apparent FCR (as fed) | 0.67 ± 0.03$^{b}$ | 7.78 ± 0.49$^{a}$ | 4.68 ± 0.13$^{a}$ | 4.35 ± 0.22$^{a}$ | 3.51 ± 0.37$^{a}$ | 4.48 ± 0.62$^{a}$ | 3.39 ± 0.12$^{a}$ | < 0.001 | 0.001 | < 0.001 | < 0.001 |
| *Nutrient retention* | | | | | | | | | | | |
| Apparent PER | 5.20 ± 0.22$^{b}$ | 3.41 ± 0.09$^{c}$ | 9.77 ± 0.20$^{a}$ | 10.93 ± 0.57$^{a}$ | 4.78 ± 0.35$^{b}$ | 3.66 ± 0.04$^{c}$ | 5.54 ± 0.19$^{b}$ | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| Apparent PD | 28.86 ± 1.56$^{b}$ | 6.37 ± 2.26$^{c}$ | 73.62 ± 3.47$^{a}$ | 74.68 ± 3.92$^{a}$ | 32.35 ± 0.88$^{b}$ | 27.15 ± 1.47$^{b}$ | 38.69 ± 2.04$^{a}$ | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| Apparent EER | 11.73 ± 0.45$^{a}$ | 5.23 ± 0.32$^{b}$ | 10.06 ± 0.27$^{a}$ | 9.95 ± 0.48$^{a}$ | 10.74 ± 1.24$^{a}$ | 12.23 ± 2.31$^{a}$ | 12.53 ± 0.42$^{a}$ | < 0.001 | 0.014 (U < G = M) | 0.001 (NE < E) | 0.287 |
| Apparent ED | 3.41 ± 0.17$^{a}$ | 0.90 ± 0.12$^{b}$ | 3.03 ± 0.14$^{a}$ | 2.90 ± 0.10$^{a}$ | 2.86 ± 0.19$^{a}$ | 3.08 ± 0.13$^{a}$ | 3.54 ± 0.13$^{a}$ | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| *Proximate composition* | | | | | | | | | | | |
| Moisture (%) | 77.24 ± 0.37 | 77.93 ± 0.09 | 77.15 ± 0.57 | 77.50 ± 0.27 | 76.96 ± 0.59 | 77.23 ± 0.53 | 78.12 ± 0.57 | 0.690 | 0.440 | 0.829 | 0.264 |
| Protein (% dry) | 38.99 ± 0.71$^{b}$ | 48.23 ± 1.70$^{a}$ | 50.32 ± 2.37$^{a}$ | 48.57 ± 2.96$^{a}$ | 47.10 ± 2.28$^{a}$ | 50.25 ± 2.45$^{a}$ | 49.41 ± 2.29$^{a}$ | < 0.001 | 0.505 | 0.949 | 0.917 |
| Energy (MJ kg$^{-1}$ dry) | 19.18 ± 0.33 | 19.38 ± 0.64 | 19.65 ± 0.26 | 19.72 ± 0.23 | 19.21 ± 0.14 | 20.41 ± 0.14 | 19.58 ± 0.16 | 0.337 | 0.091 | 0.578 | 0.268 |

SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; PD, protein deposition; EER, energy efficiency ratio; ED, energy deposition. U, *Ulva* sp.; G, *Gracilaria cliftonii*; M, mixed macroalgae diets; NE, non-enriched; E, enriched.

Initial soft tissue content: moisture (76.92%) protein (67.37% dry) and energy (20.06 MJ kg$^{-1}$ dry).

[1](mean ± SE; $n$ = 4). SE less than 0.01 are reported as "0.01". A significance level of $P < 0.05$ was used for all statistical tests.

[2]Abalone fed the three commercial diets were pooled ($n$ =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). [a,b,c] values without a common superscript compared to the control are significantly different ([a] indicates the highest value; $P < 0.05$).

[3]Where significant main effects were detected, post-hoc tests were used to determine differences between means (Fisher's Least Significant Differences; $P < 0.05$). For variables with a significant interaction between macroalgae type and enrichment, differences between treatments were analysed using pairwise comparisons and are explained in text (Fisher's Least Significant Differences [$P < 0.01$]).

### *6.4.3 Comparison between commercial diets and macroalgae*

#### *6.4.3.1 Growth performance*

The performance of abalone separately fed either one of the three commercial diets were analysed using one-factor ANOVA. There were no significant differences between abalone fed the three commercial diets. Data from abalone fed the three commercial diets were pooled ($n$ =12), and used as a control to compare against the abalone fed fresh macroalgae ($n$ = 4 per treatment; one-factor ANOVA; Dunnett's post-hoc test). Abalone fed commercial diets exhibited significantly superior biomass gain, SGR and shell growth rate than animals fed any of the six macroalgae diets (one-factor ANOVA; Dunnett's post-hoc test; $P < 0.001$; Table 6.2).

#### *6.4.3.2 Feed use*

Abalone fed commercial diets had significantly lower feed consumption rates (g as fed kg abalone$^{-1}$ day$^{-1}$) than those fed fresh macroalgae ($P < 0.001$; Table 6.2). On a dry basis however, the feed consumption rates of abalone fed commercial diets were similar to animals fed non-enriched and enriched *G. cliftonii* and mixed macroalgae treatments ($P > 0.05$). The feed consumption rates of abalone fed commercial diets were significantly higher than those fed non-enriched and enriched *Ulva* sp. ($P < 0.05$; Table 6.2).

Apparent FCR (as fed) for abalone fed commercial diets was significantly lower than for those fed all macroalgae diets ($P < 0.001$). On a dry basis however, the apparent FCR for abalone fed commercial diets was similar to abalone fed all macroalgae treatments, except for non-enriched *Ulva* sp. Abalone fed non-enriched *Ulva* sp. had significantly higher apparent FCR (dry) than abalone fed commercial diets ($P < 0.05$; Table 6.2).

#### *6.4.3.3 Soft tissue composition and nutrient use*

The soft tissue moisture and energy content of abalone fed commercial diets were not significantly different from abalone fed macroalgae treatments ($P > 0.05$; Table 6.2). In contrast, the soft tissue protein content of abalone fed commercial diets was significantly lower than abalone fed macroalgae ($P < 0.001$; Table 6.2).

Apparent PER of abalone fed commercial diets was significantly lower than abalone fed non-enriched *G. cliftonii* and non-enriched mixed macroalgae, statistically similar to abalone fed enriched *Ulva* sp. and enriched mixed macroalgae, and significantly superior to abalone fed non-enriched *Ulva* sp. and enriched *G. cliftonii* ($P < 0.001$; Table 6.2). Apparent protein deposition for abalone was significantly influenced by diet, and the effect was similar to apparent PER, but differed from abalone fed enriched *G. cliftonii* and enriched mixed macroalgae. Abalone fed enriched *G. cliftonii* and enriched mixed macroalgae had statistically similar and significantly superior protein deposition compared to abalone fed commercial diets, respectively (Table 6.2).

Apparent EER and apparent energy deposition for abalone fed commercial diets were similar to abalone fed all macroalgae treatments, except for non-enriched *Ulva* sp. Abalone fed non-enriched *Ulva* sp. had significantly higher EER and energy deposition than abalone fed commercial diets ($P > 0.05$; Table 6.2).

## 6.5 Discussion

Greenlip abalone (1-year old; 1.8 g) require ~35% dietary protein to achieve optimal growth at 22 °C (Stone et al., 2013). However, the protein level of non-enriched macroalgae is ~11-19% (dry) (Viera et al., 2011). In the current study, nutrient enrichment was successful, and increased protein levels of *Ulva* sp. and *G. cliftonii* from 5.3 to 27.7%, and from 12.9 to 38.1%, respectively, which is comparable to previous studies that utilised

nutrient/protein enriched macroalgae as feed for abalone (Shpigel et al., 1999; Viera et al., 2011; Mulvaney et al., 2013a). Furthermore, protein levels of enriched *Ulva* sp. and *G. cliftonii* approached or exceeded the optimal protein level for greenlip abalone at 22 °C (~35%; Stone et al., 2013). Abalone fed *G. cliftonii* outperformed those fed *Ulva* sp., which is likely related to the higher preference and intake for red macroalgae by wild greenlip abalone. However, the benefit of enrichment on abalone growth and feed utilisation was macroalgae species-dependent.

In the current study, abalone fed non-enriched *Ulva* sp. exhibited inferior growth, compared to those fed enriched *Ulva* sp. Previous studies reported greenlip abalone readily accepted and consumed non-enriched *Ulva* sp. (Stone et al., 2014; Bansemer et al., 2015b). In the current study however, abalone fed non-enriched *Ulva* sp. had depressed feed intake compared to those fed other diets. In addition to depressed feed intake, non-enriched *Ulva* sp. was low in protein (5.3 %) and energy (14.17 MJ $kg^{-1}$). Nutrient enrichment of *Ulva* sp. improved protein (27.7%) and energy (16.91 MJ $kg^{-1}$) levels and also improved the feed intake. The improved nutritional profile, particularly the protein level and amino acid composition, and feed intake likely caused superior growth for abalone fed enriched *Ulva* sp., compared to animals fed non-enriched *Ulva* sp. The growth benefit of feeding enriched *Ulva* sp. to abalone is consistent with previous abalone growth studies (Shpigel et al., 1999; Viera et al., 2011). In contrast to the current study, an equal mix of non-enriched *Ulva australis* and *Ulva laetevirens* promoted similar or superior growth for a closely related hybrid abalone (*H. laevigata* × *H. rubra* ), compared to those separately fed either one of the commercial diets, or enriched *U. laetevirens* and *Grateloupia turuturu* combinations (Mulvaney et al., 2013a). It should be noted that the authors collected *U. australis* and *U. laetevirens* from abalone nursery tanks and outflow drains, which were likely exposed to higher inorganic nitrogen

levels, evident by the higher protein level (>20% dry; Mulvaney et al., 2013a), than non-enriched *Ulva* sp. utilised in the current study.

In contrast to *Ulva* sp., abalone fed non-enriched *G. cliftonii* exhibited similar growth to animals fed enriched *G. cliftonii*. Although dietary protein is the first limiting macronutrient for abalone growth (Fleming and Hone, 1996; Britz and Hecht, 1997), other nutritional factors in *G. cliftonii* may be of equal importance for abalone growth. In the current study, nutrient enrichment increased the dietary protein level of *G. cliftonii*, but this was at the expense of carbohydrates. Red macroalgae species contain unique carbohydrates that are specific to the group, including agar, carrageenan and floridean starch. Greenlip abalone are anatomically and biochemically adapted to digest and utilise these unique carbohydrates, which may also be important to abalone growth (Shepherd, 1973; Harris et al., 1998). This notion is further supported by superior protein utilisation (apparent protein deposition and PER) observed in abalone fed non-enriched *G. cliftonii* to animals fed enriched *G. cliftonii*. While numerous studies have focused on optimising protein levels in abalone diets (Mai et al., 1995; Britz, 1996; Dunstan, 2010; Stone et al., 2013), abalone fed high protein diets (>35% crude protein) deaminate protein to supply energy for metabolism, rather than protein deposition and tissue growth (Bansemer et al., 2015a). To overcome this problem, carbohydrates in *G. cliftonii* may be an available energy source to spare protein and improve protein utilisation when abalone are fed high protein diets. However, the growth performance of abalone fed *G. cliftonii* was inferior to animals fed formulated diets. To improve growth and protein utilisation, feeding formulated diets in conjunction with fresh macroalgae (*M. pyrifera*, *Lessonia berteroana* or *Lessonia spicata*) also improved the PER of red abalone (*H. rufescens*; Kemp et al., 2015). Moreover, abalone (*Haliotis discus hannai*) exhibited superior feed efficiency when fed a formulated diet with dietary inclusions of dried macroalgae meal (combination of *Laminaria digitata, Palmaria palmate* and *Ulva lactuca*) compared to

animals fed fresh *L. digitata* alone (O'Mahoney et al., 2014). Based on results from the current study, we recommend further research to investigate the use of fresh *G. cliftonii* and formulated diet combinations, dried *G. cliftonii* meal inclusions or inclusions of carbohydrate extracts from *G. cliftonii*, which may ultimately improve greenlip abalone growth and nutrient utilisation.

Compared to abalone fed *G. cliftonii* and *Ulva* sp. separately, feeding enriched mixed macroalgae (*G. cliftonii* and *Ulva* sp.) had a synergistic effect on abalone growth and FCR. Abalone fed the enriched mixed macroalgae diet were supplied with higher protein level and amino acid levels, particularly lysine, the first limiting amino acid, compared to animals fed enriched *Ulva* sp. or *G. cliftonii*, respectively (Fleming et al., 1996). These results are consistent with previous studies that have utilised mono- and mixed-macroalgae diets. For example, Viera et al. (2011) reported superior growth performance for *Haliotis tuberculata coccinea* fed a diet consisting of mixed macroalgae species. The authors suggested that abalone fed mixed macroalgae were supplied with a superior balance of essential nutrients and amino acid profile to animals fed mono-specific macroalgal diets, and concluded that fresh macroalgae can be fed to abalone until market size (Viera et al., 2011).

However, formulated diets are currently fed to cultured greenlip abalone in Australia. If macroalgae is to be used as part of the feeding regime for cultured abalone, it is important that the growth and feed utilisation of animals is comparable to currently used commercially available feeds. In the current study, although fresh macroalgae supported excellent growth, abalone fed formulated diets exhibited superior growth to those fed fresh macroalgae. Commercial formulated diets contain highly palatable and digestible dietary ingredients, which includes fish meal, cereal grains, oilseeds and pulses, which are carefully formulated to optimise dietary energy, lipid, protein and amino acid levels, and essential vitamins and minerals for growth (Stone et al., 2013; Bansemer et al., 2014). While the protein level of

enriched macroalgae was similar to commercial diets, commercial diets had superior amino acid profiles compared to fresh macroalgae, which likely influenced abalone growth. Although numerous studies have focused on comparing the growth of abalone fed fresh macroalgae and formulated diets, results are conflicting. Formulated diets promoted superior growth rates for red abalone (*Haliotis rufescens*; Garcìa-Esquivel and Felbeck, 2009). However, South African abalone (*Haliotis midae*), *H. rufescens* and *H. laevigata* × *H. rubra* fed fresh macroalgae out performed those fed formulated diets (Naidoo et al., 2006; Hernández et al., 2009; Mulvaney et al., 2013a). In addition, Hernández et al. (2009) and Mulvaney et al. (2013a) used pre-weaned animals, and these results may differ if animals had been weaned on to formulated diets prior to the commencement of the study. This is supported by extremely poor growth for *H. laevigata* × *H. rubra* fed a commercial diet (0.39% $day^{-1}$; Mulvaney et al., 2013a), compared to the growth for greenlip abalone fed commercial diets in the current study (2.07% $day^{-1}$). However, further differences in feeding regimes and feed availability between studies may have also influenced results. This study highlights the importance of species-specific data, for both macroalgae species and abalone species, and not applying general information from one abalone or macroalgae species to another.

In conclusion, based on results from the current study, we recommend the use of formulated diets for cultured greenlip abalone, as they support excellent growth and feed utilisation. We recommend that greenlip abalone should not be fed fresh macroalgae alone, as this practice may lead to sub-optimal growth. Australian abalone farms are not currently set up to feed fresh macroalgae, which would require further additional infrastructure. Recently, there has been increased interest to culture Australian abalone in offshore sea-cage systems, where formulated diets may be inappropriate as abalone feed, due to diet stability problems

(Mulvaney et al., 2013b). Under these conditions, it may be beneficial to feed enriched-mixed macroalgae to greenlip abalone, although this would likely result in a longer grow-out period.

# Chapter 7. Dietary inclusions of dried macroalgae meal in formulated diets improve the growth of greenlip abalone (*Haliotis laevigata*)

Matthew S. Bansemer, Jian Qin, James O. Harris, Duong N. Duong, Krishna-Lee Currie, Gordon S. Howarth and David A.J. Stone (Under review; submitted 9/11/2015). Dietary inclusions of dried macroalgae meal in formulated diets improve the growth of greenlip abalone (*Haliotis laevigata*). Journal of Applied Phycology, JAPH-D-15-00577.

## 7.1 Abstract

Wild greenlip abalone predominantly consumes macroalgae, but under culture conditions in Australia are fed formulated diets. Dried macroalgae meals are promising ingredients for abalone diets. In this 92 day study, the growth, feed utilisation and digestive enzyme activities of greenlip abalone (*Haliotis laevigata*; 2.89 g) fed dried macroalgae meals (*Ulva* sp. meal or *Gracilaria cliftonii* meal [referred to as *Gracilaria* sp. meal]) in formulated diets were investigated. Seven experimental formulated diets, a basal diet (0% diet), and three inclusion levels of *Ulva* sp. meal (5, 10 and 20% inclusions) and *Gracilaria* sp. meal (5, 10 and 20% inclusions) were used. Diets were formulated to be isonitrogenous (35% crude protein), isolipidic (5% crude lipid) and isoenergetic (17.5 MJ $kg^{-1}$ gross energy). A commercial diet was also fed to abalone and compared to the 0% diet. Abalone were fed to excess at 16:00 h daily, and uneaten feed was collected the following day. Abalone fed diets with *Gracilaria* sp. or *Ulva* sp. meal inclusions immediately displayed feeding behaviours when feed was added to the tank in the light phase, but not when fed the control diets (0% diet or commercial diet). Growth and feed conversion ratio (FCR) of abalone fed the 0% diet and commercial diet were similar. Abalone fed 5% *Gracilaria* sp. meal or *Ulva* sp. meal exhibited superior growth to abalone fed 0%. Increasing dietary *Gracilaria* sp. meal inclusions (> 10%) led to further growth improvements, but impaired protein and energy retentions. In contrast, abalone fed > 10% *Ulva* sp. meal inclusions exhibited similar growth to those fed 0 and 5% *Ulva* sp. Although *Ulva* sp. and *Gracilaria* sp. meals are currently not commercially viable, this study clearly demonstrates the potential to develop abalone feeds with inclusions of dried macroalgae meal. We recommend a dietary inclusion of 10% *Gracilaria* sp. meal or 5% *Ulva* sp. meal to improve abalone growth.

## 7.2 Introduction

Greenlip abalone (*Haliotis laevigata*) are primarily grown in land-based systems throughout southern Australia, and fed formulated diets until market size (Stone et al., 2013). Formulated diets typically contain a range of palatable, digestible, nutritionally balanced and cost-effective ingredients, including fish meal, cereal grains, oilseeds and pulses (Stone et al., 2013). However, abalone are suited to digest and utilise their wild diet, which is primarily macroalgae-based (Shepherd, 1973; Erasmus et al., 1997; Harris et al., 1998a).

The nutritional composition of macroalgae, including carbohydrate, lipid and mineral, differs from formulated diet ingredients (Viola et al., 2001; Nelson et al., 2002; Yu et al., 2002). For example, the reserve carbohydrates of macroalgae and terrestrial ingredient are glucose polymers, but differ in chain length and branching degree, which may influence digestive enzyme activity and ingredient utilisation (Erasmus et al., 1997; Viola et al., 2001; Yu et al., 2002). There are also numerous benefits of feeding macroalgae to abalone including feeding stimulation, health improvements, and improved marketability (reviewed by Bansemer et al., 2014a). Macroalgae are fed to farmed abalone in a number of countries such as China, South Korea and Chile (Kirkendale et al., 2010; Bansemer et al., 2014a). However, greenlip abalone growth is superior when fed formulated diets compared to live macroalgae (Bansemer et al., 2014b).

Dried macroalgae meals are promising ingredients for abalone formulated diets, to benefit from feeding both formulated diets and live macroalgae. Two previous studies that investigated the growth performance of abalone fed dietary inclusions of dried macroalgae meal in formulated diets were promising (O'Mahoney et al., 2014; Viera et al., 2015). For example, abalone (*Haliotis discus hannai*) fed a formulated diet with dietary inclusions of dried macroalgae meal (combination of *Laminaria digitata*, *Palmaria palmate* and *Ulva lactuca*) exhibited similar growth to those fed fresh *L. digitata* (O'Mahoney et al., 2014).

However, the feed efficiency of *H. discus hannai* fed a formulated diet with dietary inclusions of dried macroalgae meal was superior to *H. discus hannai* fed fresh *L. digitata* (O'Mahoney et al., 2014). Macroalgae meal inclusions in diets for fish have also been successful. Ergün et al. (2009) reported improved growth for Nile tilapia (*Oreochromis niloticus*), an omnivorous fish, fed a 5% dietary inclusion of *U. rigida* meal, compared to fish fed a control diet. In contrast, rainbow trout (*Oncorhynchus mykiss*), a carnivorous fish, fed a 10% inclusion of *U. lactuca* exhibited inferior growth performance, feed utilisation and protein efficiency to a control diet (Yildirim et al. 2009). The effect of macroalgae meal inclusions appears to be species-dependent, but is also influenced by macroalgae species and inclusion level.

Although macroalgae meal inclusions in formulated diets for other abalone species were promising, the effect of dietary macroalgae meal on greenlip abalone is unknown. Numerous species of macroalgae are cultured globally and the diet of wild greenlip abalone is variable. Two macroalgae genera, *Gracilaria* spp. and *Ulva* spp., were identified and used in the current study due to their ease of culture and excellent nutritional profiles (Hernández et al., 2002; Martínez-Aragón et al., 2002; Naidoo et al., 2006; Viera et al. 2011). Therefore, in the current study, we aimed to investigate graded dietary inclusions of *Ulva* sp. meal and *Gracilaria cliftonii* meal [referred to as *Gracilaria* sp. meal] on the growth performance, feed utilisation and digestive enzyme activities of greenlip abalone.

## 7.3 Methods

### *7.3.1 Experimental animals and system*

Greenlip abalone (weight 2.89 ± 0.01 g; shell length 22.41 ± 0.06 mm; $n$ = 480) were purchased from South Australian Mariculture (Port Lincoln, SA, Australia). Prior to stocking, abalone were held in a flowthrough seawater system at South Australia Research and

Development Institute Aquatic Sciences (SARDI AS) (West Beach, SA, Australia) and fed a commercial diet *ad libitum* ("Abgrow premium" 5 mm chip; Eyre Peninsula Aquafeed Pty Ltd., Lonsdale, SA, Australia).

The experiment was conducted in a temperature-controlled system previously described in Stone et al. (2013). In brief, thirty two 12.5 L rectangular blue plastic tanks (Nally IH305, Viscount Plastics Pty Ltd) were supplied with sand filtered, UV treated, flow-through seawater at a rate of 300 mL $min^{-1}$. Water level was set at 2.5 cm using a standpipe with a mesh screen (0.8 mm nominal mesh size) on the outlet to retain uneaten food. Water temperature was held at 22 °C by using 3 kW immersion heaters (240V, A3122-1; Hotco, Williamstown, SA, Australia).

### *7.3.2 Stocking*

Abalone were gently prised from the substrate using a spatula. Fifteen animals were weighed, measured and stocked into one of four replicate culture units per dietary treatment. Abalone were stocked into the experimental system at 18 °C, acclimated to the experimental system for one week and fed their respective diets. After one week, water temperature was slowly raised (1 °C $day^{-1}$) to the final temperature of 22 °C. Dead abalone were measured, weighed, recorded, and replaced with abalone of a similar weight, fed their respective diet at 22 °C.

### *7.3.3 Diets and feeding*

Seven experimental formulated diets were investigated in the current study, a basal diet (0% control diet), and three inclusion levels of *Ulva* sp. meal (5, 10 and 20%) and *Gracilaria* sp. meal (5, 10 and 20%). In addition, the performance of abalone fed the 0% control diet was

compared to those fed a commercially available formulated diet (Abgrow premium" 5 mm chip).

Dried *Ulva* sp. meal was provided by Venus Shell Systems (Narrawallee, NSW, Australia). Dried *Gracilaria* sp. meal was produced at SARDI AS. Live *G. cliftonii* was collected from Outer Harbor (SA, Australia) and cultured in a 4000 L tank under ambient sunlight. One week prior to harvest, *G. cliftonii* was enriched with 8 L of F2 nutrient media (Guillard and Ryther, 1962). Live *G. cliftonii* was then harvested, sun-dried for ~4 h, oven-dried at 45 °C for ~72 h, and milled (particle size < 300 μm).

Proximate composition of ingredients was analysed prior to diet formulation. Macroalgae meals (*Ulva* sp. or *Gracilaria* sp.) were included into a basal 0% diet, formulated at SARDI AS at 5, 10 and 20% inclusion levels, by reducing solvent extracted soybean meal, wheat flour and de-hulled lupins levels. Diets were formulated to contain a 35% crude protein level, 5% crude lipid level and a gross energy content of 17.5 MJ $kg^{-1}$, based on the nutritional requirements reported for greenlip abalone (Stone et al., 2013; Bansemer et al., 2015b). The proximate composition, amino acid profile, fatty acid profile and mineral composition of test ingredients and experimental diets are displayed in Table 7.1.

Experimental diets were prepared by weighing the required dry ingredients, which were then mixed in a Hobart mixer (Hobart Corp., Troy, OH, USA) for 5 mins. Water (~30% of the total ingredient weight), fish oil, sodium alginate and calcium sulphate were then added to the dry ingredient mix and mixed for a further 5 mins. The diets were manufactured using a TR110 pasta machine (Macchine Per Pasta SRL, Molina Di Malo, VI, Italy), and dried at 45 °C for 48 h, to produce a flat, sinking pellet (4 × 3 × 2 mm).

Abalone were fed to excess of their daily requirements based on the total tank biomass (4% biomass $day^{-1}$) at 16:00 h. Feed rates were based on the stocking biomass, and adjusted from monthly weight checks. Tanks were cleaned the following day at 08:30 h, and uneaten

feed was collected by sieving the entire tank contents through a fine mesh. Collected feed was stored at -20 °C, and was later dried at 105 °C for 16 h. Daily feed consumption was estimated by the difference between feed offered (dry) and uneaten feed in dry weight. Feed consumption was corrected for leaching loss by calculating the feed lost between 08:30 to 16:00 h by immersing diets in water at 22 °C in experimental tanks for 16.5 h without animals, and sieved through a fine mesh net (500 μm), and dried to constant weight. This value was also used as the diet stability.

**Table 7.1** Nutrient composition of test ingredients and experimental diets.

| | Ingredients (meals) | | Diet | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Macroalgal species | *Ulva* sp. | *Gracilaria* sp. | NA | | *Ulva* | | | *Gracilaria* | | |
| Inclusion level (%) | | | EPA | 0 | 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 |
| Gross energy (MJ kg$^{-1}$) | 15.67 | 13.09 | 16.94 | 17.54 | 17.58 | 17.34 | 17.00 | 17.45 | 17.24 | 16.86 |
| 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 |
| *Amino acids (g 100g$^{-1}$ diet as fed)* | | | | | | | | | | |
| Alanine | 2.55 | 1.14 | 1.10 | 1.38 | 1.45 | 1.56 | 1.69 | 1.47 | 1.42 | 1.56 |
| Aspartic acid | 4.19 | 1.95 | 2.66 | 3.36 | 3.46 | 3.75 | 3.78 | 3.62 | 3.42 | 3.46 |
| Arginine | 1.73 | 1.44 | 1.66 | 2.54 | 2.62 | 2.65 | 2.74 | 2.59 | 2.56 | 2.73 |
| Glutamic acid | 3.74 | 1.65 | 5.10 | 5.83 | 5.89 | 5.76 | 5.93 | 5.94 | 5.70 | 5.55 |
| Glycine | 1.68 | 1.00 | 1.20 | 1.60 | 1.65 | 1.63 | 1.73 | 1.65 | 1.63 | 1.65 |
| Histidine | 0.22 | 0.03 | 0.56 | 0.85 | 0.83 | 0.81 | 0.81 | 0.84 | 0.83 | 0.85 |
| Isoleucine | 1.11 | 0.63 | 1.03 | 1.46 | 1.48 | 1.40 | 1.41 | 1.50 | 1.47 | 1.47 |
| Leucine | 1.99 | 0.53 | 1.95 | 2.51 | 2.53 | 2.58 | 2.57 | 2.60 | 2.51 | 2.53 |
| Lysine | 1.50 | 1.19 | 1.80 | 1.97 | 1.99 | 2.09 | 2.19 | 2.18 | 1.99 | 1.89 |
| Methionine | 0.46 | 0.34 | 0.37 | 0.55 | 0.63 | 0.46 | 0.49 | 0.44 | 0.56 | 0.46 |
| Phenylalanine | 1.56 | 0.76 | 1.28 | 1.57 | 1.58 | 1.66 | 1.67 | 1.71 | 1.57 | 1.70 |
| Proline | 1.38 | 0.30 | 2.43 | 2.35 | 2.35 | 2.02 | 1.93 | 1.98 | 2.25 | 1.91 |
| Serine | 1.57 | 0.85 | 1.14 | 1.63 | 1.64 | 1.53 | 1.57 | 1.53 | 1.67 | 1.65 |
| Threonine | 1.43 | 0.81 | 0.90 | 1.36 | 1.39 | 1.24 | 1.30 | 1.25 | 1.39 | 1.33 |
| Tyrosine | 0.75 | 0.54 | 0.85 | 1.10 | 1.29 | 1.15 | 1.13 | 1.20 | 1.09 | 1.23 |
| Valine | 2.03 | 0.79 | 1.36 | 1.65 | 1.68 | 1.85 | 1.89 | 1.84 | 1.68 | 1.68 |
| *Fatty acids (mg 100g$^{-1}$ diet as fed)* | | | | | | | | | | |
| 14:0 | 95 | 57 | 60 | 40 | 47 | 53 | 57 | 52 | 58 | 72 |
| 16:0 | 960 | 370 | 810 | 840 | 930 | 1020 | 970 | 970 | 920 | 960 |
| 18:0 | 480 | <10 | 190 | 280 | 290 | 300 | 240 | 280 | 290 | 290 |
| 10:1 | 56 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 |
| 14:1 | 10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 |
| 15:1 | 70 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 |
| 16:1 | 58 | <10 | 120 | 75 | 86 | 84 | 99 | 92 | 98 | 110 |
| 17:1 | 23 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 |
| 18:1n-7 | 280 | 30 | 87 | 85 | 100 | 110 | 130 | 110 | 100 | 110 |
| 18:1n-9 | 24 | 46 | 1040 | 1440 | 1490 | 1340 | 1260 | 1440 | 1580 | 1620 |
| 18:2n-6 | 210 | <10 | 1240 | 1710 | 1720 | 1430 | 1430 | 1660 | 1640 | 1520 |
| 20:4n-6 | 29 | 420 | 20 | 11 | 13 | 11 | 18 | 15 | 62 | 110 |
| 18:3n-3 | 680 | <10 | 140 | 200 | 210 | 190 | 250 | 230 | 200 | 190 |
| 18:4n-3 | 550 | <10 | 14 | <10 | 17 | 16 | 37 | 26 | <10 | <10 |
| 20:4n-3 | 39 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | 10 |
| 20:5n-3 | 85 | <10 | 66 | 30 | 33 | 27 | 38 | 35 | 36 | 39 |
| 22:5n-3 | 79 | <10 | 24 | 30 | 12 | 10 | 16 | 14 | 14 | 16 |
| 22:6n-3 | <10 | <10 | 140 | 98 | 98 | 68 | 97 | 98 | 110 | 110 |
| Diet stability (%)[2] | - | - | 80.65 | 79.75 | 78.75 | 77.56 | 75.62 | 55.03 | 46.34 | 33.61 |

EPA, Eyre Peninsula Aquafeed Pty Ltd; NA, no algae, "-" variables not analysed

[1] Calculated by difference, carbohydrate% = 100% - (moisture% + protein % + lipid % + ash %);

[2] Diet stability calculated by immersing diets in water at 22 °C in experimental tanks for 16.5 h, and sieved through a fine mesh net (500 μm), and dried to constant weight.

**Table 7.1 cont.** Nutrient composition of test ingredients and experimental diets.

| | Ingredients (meals) | | Diet | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Macroalgal species | *Ulva* sp. | *Gracilaria* sp. | NA | | *Ulva* | | | *Gracilaria* | | |
| Inclusion level (%) | | | EPA | 0 | 5 | 10 | 20 | 5 | 10 | 20 |
| *Minerals (mg kg$^{-1}$as fed)* | | | | | | | | | | |
| Calcium | 17000 | 9300 | 4700 | 6100 | 6900 | 6900 | 8700 | 6200 | 7100 | 7700 |
| Chromium | 3.5 | 2.1 | - | - | - | - | - | - | - | - |
| Cobalt | 0.29 | 1.40 | - | - | - | - | - | - | - | - |
| Copper | 110.0 | 6.3 | - | - | - | - | - | - | - | - |
| Iodine | 59 | 200 | - | - | - | - | - | - | - | - |
| Iron | 360 | 1300 | - | - | - | - | - | - | - | - |
| Magnesium | 18000 | 7300 | - | - | - | - | - | - | - | - |
| Manganese | 290 | 390 | - | - | - | - | - | - | - | - |
| Molybdenum | 0.60 | 0.48 | - | - | - | - | - | - | - | - |
| Nickle | 4.5 | 6.4 | - | - | - | - | - | - | - | - |
| Phosphorus | 14000 | 4500 | 6200 | 8200 | 8600 | 8700 | 9800 | 8200 | 8500 | 8400 |
| Potassium | 16000 | 72000 | - | - | - | - | - | - | - | - |
| Selenium | 0.110 | 0.089 | - | - | - | - | - | - | - | - |
| Sodium | 29000 | 44000 | - | - | - | - | - | - | - | - |
| Zinc | 280 | 51 | - | - | - | - | - | - | - | - |

EPA, Eyre Peninsula Aquafeed Pty Ltd; NA, no algae, "-" variables not analysed

### *7.3.4 Biochemical and water quality analysis*

At the commencement of the experiment, the soft tissue of 20 animals ($n$ = 4 replicates) were collected, shucked and stored at -20 °C to analyse the initial soft tissue proximate composition. At the conclusion of the experiment, five abalone from each tank were collected, shucked and stored at -20 °C. Abalone soft tissue was pooled for each tank, and proximate composition was analysed. Proximate composition analyses of diets, and soft tissue composition were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (2000). The gastrointestinal region (combined tissue and mucus) from four abalone per tank were also collected at the conclusion of the experiment. Gastrointestinal samples were snap-frozen in liquid nitrogen and stored at -80 °C prior to the analysis of digestive enzyme activity.

All data reported for animal performance were based on the pooled data from each tank. All calculations using abalone weight were based on wet values, while feed use values were based on dry values:

Biomass gain (g $tank^{-1}$) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)

Specific growth rate (SGR, % $day^{-1}$) = ([ln final weight - ln initial weight] / days) × 100

Shell growth rate (μm $day^{-1}$) = (final shell length - initial shell length) / days

Apparent feed consumption = (feed offered - uneaten feed collected - (uneaten feed collected / % retained without animals × % leaching loss without animals) / tank biomass

Apparent feed conversion ratio (FCR) = feed consumed / abalone weight gain

Apparent protein efficiency ratio (PER) = abalone weight gain / protein consumed

Apparent energy efficiency ratio (EER) = abalone weight gain / energy consumed

Apparent protein deposition = ([final soft body protein - initial soft body protein] / protein intake) × 100

Apparent energy deposition = ([final soft body energy - initial soft body energy] / energy intake) × 100

Water quality parameters were monitored daily. Water temperature was measured using a thermometer. Dissolved oxygen (mg $L^{-1}$ and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, IL, USA). Salinity (g $L^{-1}$) was measured using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, NH, USA).

### *7.3.5 Preparation of gut extracts and digestive enzymatic assays*

Gastrointestinal samples from four abalone ($n$ = 4) per tank replicate were partially thawed, weighed, pooled and homogenised in four volumes of distilled water (W/V) using a Dounce homogeniser. As each kit had set pH levels, the homogenate was resuspended in four volumes of the buffer supplied with each kit (V/V). The suspensions were centrifuged at 17530 *g* for 20 min at 4 °C. The resulting supernatants were analysed in triplicate for α-amylase, β-glucosidase, β-galactosidase, trypsin and lipase activities at 22 °C using spectrophotometric techniques and commercial enzyme test kits.

Colourimetric analyses were used to determine α-amylase (E.C 3.2.1.1) activity by reading the absorbance of samples at a wavelength of 405 nm at 10 and 20 mins (Catalogue No. K711-100; Biovision, Inc., California, USA). Colourimetric analyses were used to determine β-glucosidase (E.C 3.2.1.21) activity by reading the absorbance of samples at a wavelength of 405 nm at 0 and 20 mins (Catalogue No. MAK129; Sigma-Aldrich Co., Missouri, USA). Colourimetric analyses were used to determine β-galactosidase (E.C 3.2.1.23) activity by reading the absorbance of samples at a wavelength of 405 nm at 0 and 30 mins (Catalogue No. 75707; Thermo Scientific Inc., Massachusetts, USA). Colourimetric

analyses were used to determine trypsin (E.C 3.4.21.4) activity by reading the absorbance of samples at a wavelength of 405 nm at 0 and 1 h (Catalogue No. K771-100; Biovision). Fluorometric analyses were used to determine lipase (E.C 3.1.1.) activity by reading Ex/Em=529/600 nm at 0 and 40 mins (Catalogue No. K724-100; Biovision). Total protein was determined using a bicinchoninic acid (BCA) protein assay kit with bovine serum albumin solution as the standard (Biovision, Catalogue No. K813-2500). Aside from specific β-galactosidase activity, which was defined as the ΔA405 nm $h^{-1}$ mg soluble $protein^{-1}$, specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of one μ mole of substrate per minute per mg of protein (i.e. U mg soluble $protein^{-1}$) at 22 °C.

### *7.3.6 Statistical analyses*

IBM SPSS (Version 22 for Windows; IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro–Wilk test, respectively. All percentage data were arcsine transformed before analyses. Two-tailed T-tests were used to determine differences between abalone fed the 0% diet and commercial diet. Data were analysed using two-factor ANOVA to determine interactive effects between macroalgae meal species (*Ulva* sp. and *Gracilaria* sp.) and inclusion level (0, 5, 10 and 20%). When significant interactions were observed, pairwise comparisons were used to determine significant differences between treatment combinations (Fisher's least significant difference). A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

## 7.4 Results

### *7.4.1 General observations*

There were no significant differences between diets for abalone initial weight and shell length ($P > 0.05$). The average initial weight and shell length was 2.89 ± 0.00 g and 22.41 ± 0.07 mm, respectively (Table 7.2). Water quality parameters were monitored daily and maintained at levels appropriate for greenlip abalone (mean ± standard deviation, range): water temperature (21.9 ± 0.3, 21.0 - 22.8 °C), dissolved oxygen (94 ± 4, 85 - 104% saturation; 6.8 ± 0.3, 6.0 - 7.5 mg $L^{-1}$), pH (8.15 ± 0.05, 8.03 - 8.31) and salinity (36 ± 1, 34 - 37). Throughout the study, abalone exhibited normal signs of feeding and fed actively on all diets. No visual signs of disease were observed in experimental animals and abalone mortality during the study was low (5.63%). Mortalities primarily occurred during the first two weeks due to handling, and were not affected by diet ($P > 0.05$; Table 7.2).

### *7.4.2 Growth performance*

Abalone fed the commercial diet and 0% diet had similar final weight (12.45 and 11.49 g), biomass gain (143.29 and 129.16 g), SGR (1.58 and 1.49% $day^{-1}$), final shell length (45.89 and 44.51 mm) and shell growth rate (256.55 and 239.47 μm $day^{-1}$), respectively ($P > 0.05$; two-tailed T-test).

Final weight, biomass gain, SGR, final shell length and shell growth rate were significantly influenced by macroalgae meal species (*Ulva* sp. and *Gracilaria* sp.), inclusion level (0, 5, 10 and 20%) and the interaction between these two factors ($P < 0.05$; two-factor ANOVA; Table 7.2). The interaction between macroalgae species and inclusion level affected growth parameters similarly. Abalone fed 5% *Ulva* sp. meal or 5% *Gracilaria* sp. meal grew significantly more rapidly than abalone fed 0%, and did not depend on macroalgae species meal. Abalone fed 10 and 20% *Gracilaria* sp. exhibited significantly superior growth,

compared to those fed 0 and 5% *Gracilaria* sp. (Table 7.2). In contrast, the growth performance of abalone fed 10 and 20% *Ulva* sp. were similar to abalone fed 0 and 5% *Ulva* sp.

### *7.4.3 Feed use*

Feed consumption rate for abalone fed the commercial diet (10.21 g as fed kg abalone $^{-1}$ day$^{-1}$) was significantly higher than those fed the 0% diet (9.81 g as fed kg abalone $^{-1}$ day$^{-1}$; $P = 0.004$; T-test). Feed conversion ratio was not significantly different between abalone fed the commercial diet (0.76) and the 0% diet (0.73; $P = 0.107$; T-test).

Feed consumption rate (g as fed kg abalone$^{-1}$ day$^{-1}$) of abalone was significantly influenced by macroalgae meal species, inclusion level and the interaction between macroalgae meal species and inclusion level ($P < 0.001$; two-factor ANOVA; Table 7.2). This interaction was due to a significant increase in feed consumption for abalone fed 5 and 10% *Gracilaria* sp. compared to 0%, while the feed consumption of abalone fed 5 and 10% *Ulva* sp. inclusions were similar to those fed 0% ($P > 0.05$). There was also a significantly greater increase in feed consumption for abalone fed 20% *Gracilaria* sp. than 20% *Ulva* sp., compared to abalone fed 0%.

Macroalgae meal species, inclusion level and the interaction between these two factors had a significant effect on the apparent FCR of abalone ($P < 0.001$; two-factor ANOVA; Table 7.2). The significant interaction was due to significantly higher apparent FCR for abalone fed 5 and 10% *Gracilaria* sp. than abalone fed 0%. In contrast, abalone fed 5 and 10% *Ulva* sp. had similar apparent FCRs to those fed 0%. As the dietary *Gracilaria* sp. inclusion level increased from 5% to 20%, apparent FCR for abalone significantly decreased, while abalone fed 20% *Ulva* sp. had significantly higher apparent FCR than abalone fed 0, 5 and 10% *Ulva* sp.

### *7.4.4 Soft tissue composition*

Abalone fed the 0% diet had significantly higher soft tissue moisture content (75.02%), compared to the commercial diet (73.33%; $P < 0.001$; T-test). Soft tissue protein, lipid and energy contents were similar for abalone fed the 0% diet (65.4%, 6.1% and 19.72 MJ $kg^{-1}$, respectively) and the commercial diet (64.2 and 5.6% and 20.03 MJ $kg^{-1}$, respectively; $P > 0.05$).

Soft tissue moisture, protein, energy or ash content of abalone was not influenced by macroalgae meal species, inclusion level or the interaction between these two factors ($P > 0.05$; two-factor ANOVA; Table 7.2). Soft tissue lipid content of abalone was not influenced by macroalgae meal species ($P = 0.085$) or inclusion level ($P = 0.186$), but was significantly affected by the interaction between these two factors ($P = 0.030$; Table 7.2). The interaction was due to significantly higher lipid levels for abalone fed 20% *Gracilaria* sp. than 20% *Ulva* sp. meal, relative to abalone fed 5% *Gracilaria* sp. and *Ulva* sp., respectively. Soft tissue lipid content was not influenced by other interactions between macroalgae meal species and inclusion level.

### *7.4.5 Nutrient use*

Abalone fed the commercial diet had significantly higher apparent PER and apparent protein deposition (3.92 and 48.31, respectively) than abalone fed the 0% diet (3.48 and 40.98, respectively) ($P = 0.002$ and $P < 0.001$, respectively; T-test). Energy efficiency ratio was similar for abalone fed the commercial diet (6.88) and the 0% diet (6.87; $P = 0.976$; T-test). Apparent energy deposition for abalone fed the commercial diet was significantly higher than those fed the 0% diet (27.58 and 25.38, respectively; $P = 0.017$; T-test).

Macroalgae meal species, inclusion level and the interaction between macroalgae meal species and inclusion level significantly affected the apparent PER and EER of abalone ($P < 0.001$; two-factor ANOVA; Table 7.2). The interaction effects between macroalgae meal species and inclusion level was similar for both the apparent PER and EER of abalone. The PER and EER for abalone fed 5 and 10% *Gracilaria* sp. meal was significantly lower than abalone fed 0%. In contrast, PER and EER of abalone fed 5 and 10% *Ulva* sp. were similar to those fed 0%. In addition, PER and EER for abalone fed 20% *Ulva* sp. and 20% *Gracilaria* sp. were significantly lower and higher than animals fed 5% *Ulva* sp. and 5% *Gracilaria* sp., respectively.

Apparent protein deposition and apparent energy deposition of abalone was significantly affected by macroalgae meal species, inclusion level and the interaction between these two factors ($P < 0.001$; two-factor ANOVA; Table 7.2). The significant interaction was due to the significant lower apparent protein and energy depositions for abalone fed 5 and 10% *Gracilaria* sp. meal relative to abalone fed 0%, while protein and energy depositions for abalone fed 5 and 10% *Ulva* sp. meal were similar to abalone fed 0%. Additionally, abalone fed 20% *Gracilaria* sp. or *Ulva* sp. meal diets had significantly reduced protein and energy depositions compared to abalone fed 0%, but this response did not depend on macroalgae meal species.

### *7.4.6 Digestive enzymes*

The α-amylase activity for abalone fed the 0% diet was significantly higher than abalone fed the commercial diet (92.33 and 44.08 U mg soluble protein$^{-1}$, respectively; $P = 0.016$; T-test). Abalone fed the 0% diet and commercial diet had similar trypsin (0.38 and 0.30 U mg soluble protein$^{-1}$, respectively; $P = 0.435$), lipase (19.98 and 18.67 U mg soluble protein$^{-1}$, respectively; $P = 0.751$), β-glucosidase (3.18 and 3.42 U mg soluble protein$^{-1}$,

respectively; $P = 0.875$) and β-galactosidase activities (0.35 and 0.33 ΔA405nm $h^{-1}$ mg soluble $protein^{-1}$, respectively; $P = 0.875$).

Trypsin activity of abalone was significantly affected by macroalgae species ($P = 0.003$), inclusion level ($P = 0.041$), and the interaction between these two factors ($P = 0.020$; two-factor ANOVA; Table 7.3). Abalone fed *Ulva* sp. had significantly higher trypsin activity than abalone fed corresponding *Gracilaria* sp. The significant interaction was primarily due to a more pronounced trypsin up-regulation for abalone fed 5% *Ulva* sp. than 5% *Gracilaria* sp., compared to abalone fed 0% ($P < 0.05$; two-factor ANOVA; Table 7.3).

Abalone fed *Gracilaria* sp. had significantly higher β-galactosidase activity than those fed *Ulva* sp. ($P = 0.037$; two-factor ANOVA; Table 7.3). Inclusion level and the interaction between macroalgae species and inclusion level did not influence β-galactosidase activity ($P > 0.05$; Table 7.3). Lipase, α-amylase, β-glucosidase activities were not significantly influenced by macroalgae meal species, inclusion level, and the interaction between macroalgae meal species and inclusion level ($P > 0.05$; two-factor ANOVA; Table 7.3).

**Table 7.2** Growth performance, feed efficiency and nutrient retention of greenlip abalone fed dried macroalgae meal inclusions ($n$ = 4)[1].

| Macroalgal species | | *Ulva* sp. meal | | | *Gracilaria* sp. meal | | | Pooled SE | ANOVA ($P$ =) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macroalgal inclusion level (%) | 0 | 5 | 10 | 20 | 5 | 10 | 20 | | Species (A) | Inclusion level (%) (B) | A × B |
| *Growth performance and mortality* | | | | | | | | | | | |
| Initial weight (g) | 2.90 | 2.88 | 2.89 | 2.89 | 2.89 | 2.90 | 2.88 | 0.00 | 0.821 | 0.443 | 0.746 |
| Final weight (g) | 11.49 | 11.84 | 11.63 | 11.73 | 12.38 | 13.48 | 13.33 | 0.17 | < 0.001 | 0.002 | 0.006 |
| Biomass gain (g tank$^{-1}$) | 129.16 | 134.25 | 131.00 | 132.08 | 142.01 | 158.77 | 156.35 | 2.53 | < 0.001 | 0.002 | 0.006 |
| SGR (% day$^{-1}$) | 1.49 | 1.53 | 1.51 | 1.52 | 1.58 | 1.67 | 1.66 | 0.01 | < 0.001 | < 0.001 | 0.003 |
| Mortality (%) | 5.00 | 3.33 | 8.33 | 5.00 | 13.33 | 0.00 | 5.00 | 1.52 | 0.891 | 0.763 | 0.204 |
| *Somatic growth parameters* | | | | | | | | | | | |
| Initial shell length (mm) | 22.47 | 22.28 | 22.65 | 22.27 | 22.56 | 22.47 | 22.28 | 0.07 | 0.832 | 0.444 | 0.668 |
| Final shell length (mm) | 44.51 | 45.33 | 45.25 | 45.82 | 46.27 | 47.49 | 47.76 | 0.24 | < 0.001 | < 0.001 | 0.020 |
| Shell growth rate (µm day$^{-1}$) | 239.47 | 250.58 | 245.63 | 256.00 | 257.76 | 271.98 | 276.99 | 2.70 | < 0.001 | < 0.001 | 0.006 |
| *Feed utilisation* | | | | | | | | | | | |
| Feed consumption rate (g as fed kg abalone$^{-1}$ day$^{-1}$) | 9.42 | 10.11 | 9.84 | 11.50 | 13.91 | 13.91 | 13.03 | 0.35 | < 0.001 | < 0.001 | < 0.001 |
| Apparent FCR | 0.73 | 0.77 | 0.75 | 0.88 | 1.03 | 0.99 | 0.93 | 0.02 | < 0.001 | < 0.001 | < 0.001 |
| *Nutrient retention* | | | | | | | | | | | |
| Apparent PER | 3.48 | 3.30 | 3.36 | 2.90 | 2.43 | 2.55 | 2.70 | 0.08 | < 0.001 | < 0.001 | < 0.001 |
| Apparent PD | 40.98 | 40.24 | 37.11 | 34.72 | 28.88 | 29.84 | 33.55 | 0.98 | < 0.001 | < 0.001 | 0.001 |
| Apparent EER | 6.87 | 6.57 | 6.75 | 5.91 | 4.88 | 5.17 | 5.64 | 0.15 | < 0.001 | < 0.001 | < 0.001 |
| Apparent ED | 25.38 | 26.09 | 23.81 | 22.38 | 18.25 | 19.10 | 21.92 | 0.60 | < 0.001 | < 0.001 | < 0.001 |
| *Proximate composition* | | | | | | | | | | | |
| Moisture (%) | 75.02 | 73.76 | 75.85 | 74.45 | 75.28 | 75.42 | 74.71 | 0.21 | 0.316 | 0.090 | 0.190 |
| Protein (% dry) | 65.4 | 64.7 | 64.4 | 64.7 | 66.1 | 65.7 | 66.5 | 0.3 | 0.066 | 0.943 | 0.767 |
| Lipid (% dry) | 6.1 | 6.1 | 6.1 | 5.7 | 5.8 | 6.6 | 6.5 | 0.1 | 0.085 | 0.186 | 0.030 |
| Ash (% dry) | 11.06 | 9.19 | 11.38 | 11.36 | 10.86 | 10.74 | 10.32 | 0.23 | 0.993 | 0.255 | 0.101 |
| Energy (MJ kg$^{-1}$ dry) | 19.72 | 20.17 | 19.67 | 19.60 | 20.00 | 20.01 | 20.19 | 0.07 | 0.128 | 0.198 | 0.088 |

SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; PD, protein deposition; EER, energy efficiency ratio; ED, energy deposition; Standard errors, SE. Initial soft tissue composition of greenlip abalone (dry): moisture (80.06%), protein (73.8%), lipid (4.3%), ash (13.2%) and energy (18.93 MJ kg$^{-1}$).

[1] A significance level of $P < 0.05$ was used for all statistical tests. For variables with a significant interaction, pairwise comparisons were used to determine significant differences between treatment combinations (Fisher's least significant difference).

**Table 7.3** Trypsin, lipase, α-amylase, β-glucosidase and β-galactosidase activities in the gastrointestinal region of greenlip abalone fed dried macroalgae meal inclusions ($n$ = 4, four pooled abalone per replicate)[1].

| Macroalgal species | | *Ulva* | | | *Gracilaria* | | | Pooled SE | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macroalgal inclusion level (%) | 0 | 5 | 10 | 20 | 5 | 10 | 20 | | Species (A) | Inclusion level (%) (B) | A × B |
| Trypsin activity[2] | 0.38 | 1.08 | 0.51 | 0.66 | 0.35 | 0.39 | 0.44 | 0.06 | 0.003 | 0.041 | 0.020 |
| Lipase activity[2] | 19.98 | 20.15 | 18.61 | 17.00 | 21.62 | 21.50 | 18.65 | 1.05 | 0.449 | 0.739 | 0.972 |
| α-amylase activity[2] | 92.33 | 101.85 | 103.06 | 82.02 | 99.44 | 104.54 | 112.42 | 4.92 | 0.422 | 0.844 | 0.592 |
| β-glucosidase activity[2] | 3.18 | 4.39 | 4.72 | 4.29 | 4.40 | 4.46 | 3.46 | 0.31 | 0.664 | 0.366 | 0.962 |
| β-galactosidase activity[3] | 0.35 | 0.33 | 0.31 | 0.35 | 0.37 | 0.38 | 0.36 | 0.01 | 0.037 (U < G) | 0.976 | 0.298 |

Standard errors, SE; U, *Ulva* sp. meal; G, *Gracilaria* sp. meal.

[1] A significance level of $P < 0.05$ was used for all statistical tests. For variables with a significant interaction, pairwise comparisons were used to determine significant differences between treatment combinations (Fisher's least significant difference).

[2] U mg soluble protein$^{-1}$

[3] ΔA405nm h$^{-1}$ mg soluble protein$^{-1}$

## 7.5 Discussion

Further development of a macroalgae industry in Australia is limited by cost effective "farm to market" value chains. Therefore, our goal in the current study was to utilise macroalgae meal to improve abalone growth, and also build linkage between the macroalgae and abalone industry. To achieve this goal, the effects of dietary inclusion of dried macroalgae meal (*Ulva* sp. and *Gracilaria* sp.) on the growth performance and feed utilisation of greenlip abalone was investigated in the current study. Specific growth grate of abalone ranged from 1.49 to 1.67 % $day^{-1}$, which compares favourably to other laboratory and commercial growth studies on greenlip abalone (Vandepeer, 2005; Stone et al., 2013; Bansemer et al., 2015b). The growth and feed conversion ratio of abalone fed the commercial diet and 0% diet were similar, which gives confidence in interpreting results for experimental diets in the current study.

Abalone fed *Gracilaria* sp. or *Ulva* sp. meal inclusions immediately displayed active feeding behaviours when feed was added to the tank during the light phase. This behavioural response was not observed in abalone fed diets without macroalgae meal (0% diet or commercial diet). Feeding stimulation by supplying macroalgae was reported in greenlip abalone fed live *Ulva* sp. (Bansemer et al., 2015c) and *Gracilaria* sp. (Buss et al., 2015). However, abalone fed live macroalgae exhibited inferior growth to those fed formulated diets (Bansemer et al., 2014b). In the current study, increased feeding stimulation for abalone fed 5% *Gracilaria* sp. may have resulted in significantly higher feed intake, and in turn improved growth, compared to those fed the 0% diet. These results are consistent with a previous study by Allen et al. (2006), which reported numerically higher feed intake and significantly higher shell growth rates (15%) for *Haliotis iris* fed a formulated diet with dried, mulched *Gracilaria* spp. particles (300 - 500 μm) suspended in the system, compared to a formulated diet alone (Allen et al. 2006). Feed attractants, supplied in the form of dried macroalgae

inclusions (5% wet weight [0.88% dry weight] *Ecklonia maxima*) in formulated diets, are currently utilised by a South African feed company for *Haliotis midae* (Personal communication, Kurt Mätschke, Marifeed, Western Cape, South Africa). Results from the current study indicate that it would also be beneficial to formulate greenlip abalone diets with macroalgae meal (*Gracilaria* sp.) to stimulate feeding and also improve growth.

In the current study, abalone fed 10 and 20% *Gracilaria* sp. inclusions exhibited superior growth performance to those fed other diets. Dietary protein is the first limiting factor for abalone growth. In the current study, experimental diets were formulated to contain optimal dietary protein levels (~35% crude protein) for greenlip abalone at 22 °C (Stone et al., 2013). Diets were also formulated using highly palatable and digestible ingredients at realistic inclusion levels (Fleming et al., 1998; Vandepeer, 2005; Stone et al., 2013). However, ingredients can be highly digested, but may be poorly utilised (Stone et al., 2003). The reserve carbohydrates in terrestrial plants and *Gracilaria* sp. are primarily glucose polymers with α-(1,4) glycosidic linkages that are hydrolysed by α-amylase (Viola et al., 2001; Yu et al., 2002). In contrast, floridean starch, the primarily reserve carbohydrate in *Gracilaria* spp., lacks amylose, has a shorter glucose polymer chain length and a higher branching frequency than starch from terrestrial plants and reserve carbohydrate in *Ulva* sp. (Viola et al., 2001; Yu et al., 2002). Structural carbohydrates of *Gracilaria* spp. also differ from other ingredients used in the current study. The primary structural carbohydrates in terrestrial plants and *Ulva* sp. are cellulose, xylans, ulvan and mannans, while the most abundant structural carbohydrate in *Gracilaria* spp. is agar, which is composed of galactose and glucose repeating units with β-glycosidic linkages (McCandless, 1981; Evan, 1989; Lahaye and Robic, 2007). As the digestive system of abalone is adapted to digest and utilise macroalgae, composition and structural carbohydrate differences may affect carbohydrate digestion and utilisation.

The digestive capacity of abalone is dependent on the type and activities of digestive enzymes. Digestive enzyme activities in abalone are influenced by diet (Knauer et al., 1996; Erasmus et al., 1997; García-Carreño et al., 2003). For example, higher alginate lyase, carboxymethylcellulase and laminarinase activities were reported in abalone (*H. midae*) fed *Ecklonia maxima* than those fed *Gracilaria verrucosa* (Erasmus et al., 1997). In contrast, higher agarase and carrageenase activities were reported in abalone fed *G. uerrucosa* than those fed *E. maxima*. The authors suggested that the regulation of carbohydrase activity in abalone was associated with carbohydrate differences between macroalgae species (Erasmus et al., 1997). In the current study, diet also affected abalone digestive enzyme activities. Agar is hydrolysed by agarose degrading enzymes, including β-galactosidase (Michel et al., 2006; Lee et al., 2014). Abalone significantly up-regulated β-galactosidase activities when fed *Gracilaria* sp. meal compared to those fed *Ulva* sp. meal. There was also tendency for α-amylase activities to increase with increased dietary inclusions of *Gracilaria* sp. The β-galactosidase activities and α-amylase activity up-regulation may increase carbohydrate utilisation for energy, and spare protein for growth. However, protein utilisation (apparent PER and protein deposition) of abalone fed a 10 and 20% *Gracilaria* sp. meal inclusions was significantly lower than those fed the 0% diet. Although abalone may have been supplied with carbohydrates they efficiently digest and utilise, due to their increased energy requirements during periods of fast growth (Duong et al., 2014), abalone may have also deaminated protein for energy metabolism. This hypothesis is supported by a significantly lower energy deposition in abalone fed *Gracilaria* sp. meal, indicating that during periods of rapid growth abalone fed *Gracilaria* sp. meal may have a different energy budget to those fed diets without *Gracilaria* sp. meal. It would be beneficial in future studies to investigate greenlip abalone energy budgets when fed dried macroalgae meal inclusions to further improve the nutritional knowledge on greenlip abalone.

Superior growth performance for abalone fed *Gracilaria* sp. meal inclusions may also be related to fatty acid profile differences between *Gracilaria* sp. and *Ulva* sp. Abalone have low lipid requirements, but some fatty acids are essential for abalone growth (Nelson et al., 2002; Dunstan, 2010). The C20 long chain polyunsaturated fatty acids (LC PUFA) levels and higher arachidonic acid (20:4n-6, ARA) to eicosapentaenoic acid (20:5n-3, EPA) ratios can promote superior growth for abalone (*Haliotis fulgens*; Nelson et al., 2002). Eicosapentaenoic acid, a LC n-3 PUFA, is important for cellular membrane structure and function, and controlling and regulating cellular metabolism (Dunstan, 2010; Bautista-Teruel et al., 2011). Additionally, ARA, a LC n-6 PUFA, is required for cell membrane function, combating infection, blood coagulation and as an anti-inflammatory (Nelson et al., 2002). In the current study, the EPA levels of experimental diets were relatively similar, but ARA levels in *Gracilaria* sp. diets increased due to high ARA levels in *Gracilaria* sp. meal. As a result, as dietary inclusions of *Gracilaria* sp. increased, diets contained higher C20 LC PUFA levels and ARA to EPA ratio, which may have also influenced abalone growth in the currents study. Recent research by Viera et al. (2015), suggested that abalone (*Haliotis tuberculata coccinea*) have the capacity to desaturate and chain elongate linoleic acid (18:2n-6, LA) to ARA. In the current study, *Ulva* sp. was low in ARA, but contained high levels of LA, which may have also supplemented low ARA levels. While the LC-PUFA biosynthesis in fish is well understood, few studies have focused on this area for abalone. Further research into fatty acid metabolism and gene expression of greenlip abalone, with particular focus on the desaturation and chain elongation of LA to ARA, is required before further conclusions can be made.

In addition to dietary macronutrients, micronutrients in *Gracilaria* sp. meal may also improve abalone growth. Dietary minerals are required for normal metabolic function. The optimal inclusion level for some minerals, including calcium, phosphorus, copper, iron,

selenium, and zinc, are established for abalone (Coote et al., 1996; Tan and Mai, 2001; Wang et al., 2009; Wang et al., 2012).Other dietary vitamin and mineral requirements for abalone are typically based on fish requirements (Sales and Janssens, 2004). In the current study, *Gracilaria* sp. meal contained some minerals, including cobalt, iodine, iron and manganese, at levels considerably higher than *Ulva* sp. meal. These minerals may be required for optimal abalone growth. This area has not been thoroughly explored in previous studies, and further research to understand the mineral requirements and optimal level for abalone diets is required. The mineral composition of *Gracilaria* sp. meal may provide a useful benchmark to explore this area.

Abalone fed 5% *Ulva* sp. meal inclusions also exhibited superior growth to those fed 0%. In contrast to abalone fed 5% *Gracilaria* sp., the apparent feed consumption and FCR of animals fed 5% *Ulva* sp. were similar to those fed 0%. Superior growth of abalone fed 5% *Ulva* sp. may be related to digestive enzyme activity regulation. Trypsin activity was significantly up-regulated (184%) in abalone fed 5% *Ulva* sp. compared to those fed 0%. Trypsin is important in protein digestion and is a useful indicator for fish growth (Lemieux et al., 1999; Rungruangsak-Torrissen et al., 2006). In the current study, the significant trypsin up-regulation by feeding 5% *Ulva* sp. likely increased dietary protein utilisation and subsequent growth. Trypsin and other protease activities are typically influenced by dietary protein levels (Knauer et al., 1996). Diets in the current study however, were isonitrogenous (~35% crude protein). Resident bacteria in the gastrointestinal tract of abalone are associated with nutrient digestion (Erasmus et al., 1997; Harris et al., 1998b). Dietary inclusions of *Ulva* sp. may alter the luminal environment and influence microbiota composition, which may have resulted in a proliferation of trypsin-secreting bacteria in the current study. This would ultimately increase the protein digestion and growth of abalone. Further research on the

complex interaction between dietary macroalgae meal inclusions, luminal environment and microbiota composition is required to support this hypothesis.

In Australia, there are currently no commercial producers of *Gracilaria* sp. Results from the current study are positive and provide considerable scope to develop and grow a *Gracilaria* sp. industry in Australia, which will be capable of supplying *Gracilaria* sp. meal for abalone formulated diets to improve abalone production. In contrast, Venus Shell Systems in Australia already produce a high quality *Ulva* sp. product that was used in diets in the current trial. At present, *Ulva* sp. meal produced by Venus Shell Systems is primarily for human applications, and is sold for >\$20 $kg^{-1}$, which is based on a three tonne per annum production capacity. At this price, dietary inclusions of *Ulva* sp. meal are not economically viable. In the near future, Venus Shell Systems envisage a short term future price of \$10 $kg^{-1}$, and an order of magnitude lower again once production exceeds 100 tonnes per annum (Personal communication, Dr. Pia Winberg, Venus Shell Systems Pty. Ltd., Bomaderry, NSW, Australia). In addition to the two macroalgae species investigated in the current study, Australia has a diverse and endemic macroalgae species and there are likely numerous other macroalgae species that may be beneficial to incorporate into formulated diets for greenlip abalone. For example, two red alga species, *Gelidium australe* and *Solieria robusta*, were identified as the best candidates for aquaculture in integrated multi-trophic aquaculture (IMTA), which have additional ecological benefits of removing nutrient wastes from other aquaculture species (Lorbeer et al., 2013; Wiltshire et al., 2014). Further research focused on incorporating different dried macroalgae meal species into formulated diet for greenlip abalone would also likely benefit both the Australian abalone and macroalgae industries.

In conclusion, when considering the growth and feed utilisation of greenlip abalone fed *Gracilaria* sp. meal inclusions, we recommend 10% dietary inclusions of *Gracilaria* sp. meal for abalone diets. Although there are currently no commercial producers, results from the

current study suggest the need to develop and grow a *Gracilaria* sp. industry to supply a high quality ingredient for abalone diets. With regard to *Ulva* sp. meal, once production is economically viable for inclusion into abalone feeds, we recommend a dietary inclusion of 5% *Ulva* sp. meal to stimulate digestive enzyme activity and improve abalone growth. Furthermore, up to 20% inclusion of *Ulva* sp. meal did not compromise growth indicating *Ulva* sp. meal may be successfully used to replace solvent extracted soybean meal, de-hulled lupin meal and wheat meal in abalone formulated diets. Results from the current study will contribute to further improvements of formulated diets for abalone, which may ultimately lead to abalone growth improvements. Furthermore, this study built a linkage between the macroalgae and abalone industries, and also provides momentum to grow and diversify an Australian macroalgae industry.

# Chapter 8: General Discussion

## 8.1 Introduction

The main thrust of this PhD research focused on improving the nutritional knowledge for greenlip abalone. The two main research objectives were to: (i) improve the nutritional knowledge of greenlip abalone on the interaction between age, water temperature and dietary protein; (ii) and to provide a fundamental understanding, and practical means to use macroalgae as feed for Australian abalone on-farm. The specific research aims were to: (i) identify the optimal protein level for 6-month greenlip abalone at different water temperatures; (ii) identify changes to the digestive enzyme activities in greenlip abalone throughout the production cycle; (iii) understand feeding behaviour, growth and feed utilisation of abalone fed fresh macroalgae and formulated diets; and (iv) to identify the type and amount of dried macroalgae meal required to achieve the "algal effect" in formulated abalone feeds. Major findings and outcomes are outlined below, and future research directions are recommended.

## 8.2 Summary of Major Findings

1. Superior growth rates were observed in 6-month old abalone at 20 °C, while a 3 °C reduction in water temperature resulted in significantly depressed growth rates. Lipase and α-amylase activity of 1- and 2-year old greenlip abalone were significantly higher at warmer water temperatures, while trypsin activities were not affected by water temperature.
2. Dietary protein did not influence abalone growth in the protein range tested (27 - 36% crude protein). Faster growing abalone at 20 °C up-regulated feed intake when fed low protein diets to increase protein intake to maintain optimal growth potential. In contrast, abalone at 14 and 17 °C had significantly higher feed consumption when fed high protein diets, which was detrimental to the animal's FCR.

3. Trypsin and lipase activities of 2-year old abalone were significantly down-regulated by 53% and non-significantly up-regulated by 8%, respectively, compared to 1-year old abalone. In addition, α-amylase activity differed between 1- and 2-year old greenlip abalone, but was dependent on dietary protein levels. While 1-year old abalone significantly up-regulated α-amylase activities as dietary protein level increased, 2-year old abalone down-regulated α-amylase activities by 55% when fed 33% CP, compared to abalone fed 30% CP.
4. Feeding fresh *Ulva* sp. to abalone stimulated animals to feed. However, abalone fed fresh macroalgae exhibited depressed growth compared to animals fed commercial formulated diets.
5. Abalone fed 5% *Gracilaria* sp. or *Ulva* sp. meal exhibited superior growth to abalone fed 0%. Increasing *Gracilaria* sp. inclusions (> 10%) led to further growth improvements, but impaired protein and energy retentions. In contrast, abalone fed > 10% *Ulva* sp. exhibited similar growth to abalone fed 0 and 5% *Ulva* sp.

## 8.3 Recommendations for management

1. Heating land-based nursery systems before abalone are transferred to grow-out tanks will take advantage of accelerated growth of juvenile greenlip abalone at warmer water temperature. This will likely reduce the grow-out period of abalone and production costs associated with the reduction of production time. The construction and implementing temperature controlled nursery systems are costly. Therefore, a cost-benefit analysis should be undertaken before temperature controlled nursery systems are implemented on-farm (Fig. 8.1).

2. For 6-month old abalone, switching the dietary protein level from 29% crude protein < 17 °C, to ~35% CP at 20 °C will improve the feed efficiency and growth of abalone (Fig. 8.1).
3. The reduced trypsin activity in 2-year old abalone, compared to 1-year old greenlip abalone, indicate that the optimal dietary protein level for 2-year old abalone is lower than 1-year old (Fig. 8.1).
4. Based on the superior growth of abalone fed commercial formulated diets compared to abalone fed fresh macroalgae, it is recommended that fresh macroalgae are not fed to cultured abalone in Australia when growth is the parameter of interest. When commercial diets are not suited as feed for abalone, such in offshore systems, it may be beneficial to feed abalone enriched and mixed macroalgae diets (Fig. 8.1). In addition, the carbohydrates in *G. cliftonii* (including agar and floridean starch), which are unique to red algae species, are important for abalone growth.
5. An inclusion of 10% *G. cliftonii* meal or 5% *Ulva* sp. meal for greenlip abalone diets leads to improved growth. Furthermore, up to 20% inclusion of *Ulva* sp. meal can be used to replace other common dietary ingredients (solvent extracted soybean meal, de-hulled lupin meal and wheat meal), without compromising growth (Fig. 8.1).

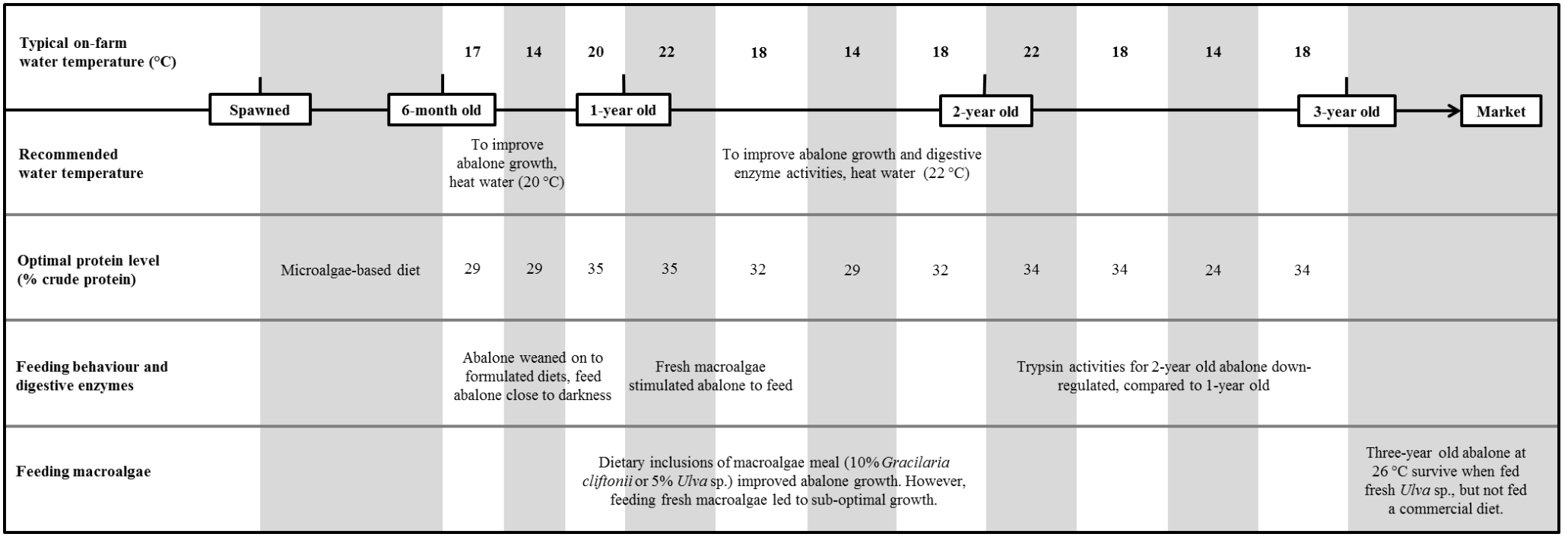


**Figure 8.1** Summary of recommendations for the production of cultured greenlip abalone (*Haliotis laevigata*), based on my PhD research and also co-authored publications throughout my PhD candidature.

## 8.4 Recommended future research

1. While optimising the dietary protein level for greenlip abalone throughout their production was the primary focus of this research, optimising other macro- and micro-nutrient levels may also improve abalone growth. Further research to optimise the protein to energy ratio for different age classes of greenlip abalone, especially when fed high dietary protein levels is recommended. In addition, optimising dietary lipid level, and fatty acid composition, for different age classes of greenlip abalone may also improve greenlip abalone production. Future research should also focus on understanding the mineral requirements and optimal levels for abalone diets.
2. The main thrust of this thesis was focused on improving the nutritional knowledge for greenlip abalone. The second major issue identified that impedes the development of Australian abalone aquaculture was health, which was not investigated in this thesis. Summer mortality is one of the major health concerns to greenlip abalone farmers. During periods of high summer water temperatures (> 22 °C), mortality rates for larger, 3-year old, abalone can be up to 50% (Vandepeer, 2006). Although novel dietary intervention can improve the survival of greenlip abalone at high water temperatures (Lange et al., 2014; Stone et al., 2014), further research focused on utilising cost-effective dietary ingredients and extracts is needed to improve the survival of these highly valuable, larger animals.
3. Based on this research, there is considerable scope to further enhance abalone growth and also an Australian macroalgae aquaculture industry by investigating dietary inclusions of other macroalgae meal species for greenlip abalone diets. Although only two macroalgae species were investigated in this study, Australia has a diverse range of endemic macroalgae species. There are likely numerous other macroalgae species that may be beneficial to incorporate into formulated diets for greenlip abalone. Two red

alga species in particular, *Gelidium australe* and *Solieria robusta* are excellent candidates and have the additional ecological benefits of removing nutrient wastes from other aquaculture species (Lorbeer et al., 2013; Wiltshire et al., 2014).

4. Further research focused on dietary inclusions of sustainable ingredients is recommended. In particularly, dietary ingredients from industry waste streams, including the wine, peanut or beer industry, may be of great potential to improve abalone growth or decreased feed costs by partial or complete substitution of other bulk dietary ingredients.
5. Bacteria have previously been suggested to play a major role in the digestion of macroalgae (Erasmus et al., 1997). Further research to understand gastrointestinal bacteria composition and dynamics of abalone, in particular how macroalgae meal inclusion may promote a 'favourable' gut is recommended.

## 8.5 Conclusion

In conclusion, this research contributes to improve dietary formulations and effective abalone production in Australia. This research provides new knowledge of the protein requirements in juvenile greenlip abalone at seasonally relevant water temperatures, and also changes to digestive enzyme activities depend on age, water temperature and dietary protein. Furthermore, this study indicates that greenlip abalone fed fresh macroalgae had slower growth compared with those fed formulated diets, but inclusions of dried macroalgae improve abalone growth. Currently, there is more known about the nutritional requirements of greenlip abalone than other international abalone species, which has helped transform the greenlip abalone aquaculture industry. The abalone industry is still relatively new compared to other aquaculture industries, such as Atlantic salmon, and further improvements to dietary formulations and abalone production thought dietary manipulation are likely. Looking to the

future, this research will also serve as a stepping-stone to further enhance the productivity and sustainability of the greenlip abalone aquaculture industry.

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