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

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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 was significantly lower (53%) than that of 1-year old abalone. The α-amylase activities of 1-year old abalone were significantly up-regulated as dietary protein levels 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 to 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.
Statement of relevance: Results may contribute to further diet development research


## 1. 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

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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). However, 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., 2015). 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 that 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.

## 2. Methods

### 2.1. 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 days. 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). 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). 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 (Stone et al., 2013).

### 2.2. 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 min (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 min (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 μmol 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).

### 2.3. 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

**Table 1**
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[1] | 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 2**
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) *P* | Water temperature (°C; B) *P* | Protein level (%; C) *P* | A × B *P* | A × C *P* | B × C *P* | A × B × C *P* |
|---|---|---|---|---|---|---|---|
| Trypsin activity | <0.001 (Y1 > Y2) | 0.910 | 0.172[1] | 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 |

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

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.

## 3. Results

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

### 3.2. 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 1). The α-amylase activity of abalone significantly increased as water temperature increased from 14 to 18 to 22 °C (Table 1). 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 1). Lipase activity was significantly higher in abalone at 22 °C, compared to abalone at 14 or 18 °C (Table 1). 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 1).

### 3.3. 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 2). 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 2).

The α-amylase activity of abalone significantly increased as water temperature increased from 14 to 18 to 22 °C (Table 2). 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 2). 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 2).

Lipase activity was significantly influenced by water temperature (14 = 18 < 22 °C; Table 2), 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 2).

## 4. 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*; García-Carreño et al., 2003; 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 rasping on 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 cultured 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 that 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. However, 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 that 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 *H. 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.

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