Comparative Histological Changes in the Greenlip Abalone *Haliotis laevigata* Gastrointestinal Tract in Response to Water Temperature, Different Dietary Protein Levels, and Animal Age

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COMPARATIVE HISTOLOGICAL CHANGES IN THE GREENLIP ABALONE *HALIOTIS LAEVIGATA* GASTROINTESTINAL TRACT IN RESPONSE TO WATER TEMPERATURE, DIFFERENT DIETARY PROTEIN LEVELS, AND ANIMAL AGE

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ABSTRACT The land-based culture of greenlip abalone *Haliotis laevigata* in southeastern Australia is carried out using seawater that is prone to seasonal temperature fluctuations and is done almost exclusively using artificial feeds. Although some nutrition research has been done to identify the optimum dietary protein level, typically farms use only 1 diet for grow-out after the juveniles are weaned. Little consideration has been given to the effects of fluctuating water temperatures and dietary protein levels on the morphology of the gastrointestinal tract (GIT) of abalone. Because these factors are known to impact growth in other aquatic species, it is important that they are investigated further to improve growth in abalone species. In this study, the histological changes of the GIT of greenlip abalone in response to 2 water temperatures and 4 dietary levels of crude protein (juvenile, 27% and 36%; subadult, 24% and 33%) for juveniles (1.75 g) and subadults (22.93 g) were investigated. The epithelial thickness of the stomach and crop; intestinal villus height, width, and area; lamina propria height; and stomach, crop, and intestinal neutral and acidic goblet cell numbers were measured. The stomach epithelium was significantly thicker at 14°C than 22°C in both juveniles and subadults, whereas the crop epithelium was significantly thicker at 22°C than 14°C in juveniles. The crop epithelial thickness of subadults was reduced by increasing dietary protein; however, juveniles did not show the same response. Juvenile abalone were more sensitive to temperature fluctuations than subadults, whereas significant effects of dietary protein levels were only observed in subadults. The alterations in the morphology of the GIT did not appear to be detrimental to the health and growth of the abalone. Further research is required to investigate the interactive effects of water temperature and dietary ingredients, particularly in regard to antinutritional factors, on the morphology and function of the GIT to improve our understanding of abalone physiology.

KEY WORDS: greenlip abalone, *Haliotis laevigata*, temperature, dietary protein, nutrition, histology

INTRODUCTION

Abalone are marine gastropods belonging to the genus *Haliotis,* with approximately 100 species found worldwide (Hahn 1989). In Australia, the greenlip abalone *Haliotis laevigata* Donovan, the blacklip abalone *Haliotis rubra* Leach, and the hybrid of these 2 species, the tiger abalone, are exploited commercially, mostly from land-based systems (Shepherd & Turner 1985, Coote et al. 2000). Wild abalone are herbivorous, with greenlip abalone feeding primarily on red macroalgae (Shepherd & Turner 1985); however, this food preference is a limiting factor for aquaculture because of its expense, availability, and low growth rates of abalone fed macroalgae diets (Viana et al. 1993, Coote et al. 2000, Hernandez et al. 2009). Dry pelleted feeds are currently being used for abalone culture because they are easier to handle and store, reduce labor costs, and can be formulated to meet nutritional requirements of abalone (Bautista-Teruel & Millamena 1999). Cultured abalone have a slow and variable growth rate, typically taking 2–5 y to reach market size (Hahn 1989). This slow growth rate is related to seasonal fluctuations in water temperature (Gilroy & Edwards 1998), and potentially limiting levels of dietary protein provided during shifts in ontogenic development (Britz & Hecht 1997).

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Temperatures experienced during the grow-out of greenlip abalone oscillate seasonally between 10°C and 25°C, and optimum temperature for growth is 18.3°C (Gilroy & Edwards 1998). Because abalone are also limited in their capacity to move to different geographical locations when suboptimal environmental conditions prevail, they must adapt to changing environments to survive, which may be achieved in several ways. Phenotypic plasticity and phenotypic flexibility are mechanisms used by animals to respond to changing environmental and nutritional conditions. Phenotypic plasticity, previously defined as the ability of an organism to change its phenotype in response to changes in the environment (Hazel & Prosser 1974), has been described more recently as variation that is irreversible, and affects more than 1 individual (Piersma & Drent 2003). Phenotypic flexibility is used to describe changes that are plastic in nature, but are reversible within-individual changes that occur over shorter time periods (Piersma & Drent 2003). Both these mechanisms can be used by animals to adapt to their environmental conditions. When the external temperature changes in poikilothermic animals, adaptation occurs by compensatory adjustments to physiology and biochemistry. The digestive tract is the primary organ for the digestion and absorption of nutrients, and is in contact with the external environment (Starck 2003); therefore, a high level of plasticity is required such that metabolic rate can be controlled during times of change. Morphological changes in gastrointestinal tract (GIT) enterocytes, changes in intestinal mucosal layer thickness, and

shifts in nutrient transport systems have been observed in fish exposed to different water temperatures (Lee & Cossins 1988, Houpe et al. 1996), assisting in controlling metabolism and, therefore, growth, reproduction, and survival. There have been several studies that have investigated the structure and function of the abalone GIT. Most notable are those conducted by Crofts (1929) on *Haliotis tuberculata* L., Campbell (1965) and Bevelander (1988) on *Haliotis cracherodii* Leach, McLean (1970) on *Haliotis rufescens* Swainson, and Harris et al. (1998) on *Haliotis laevigata*. Although these studies have focused on morphology and histology of the digestive tract, the environmental and dietary effects have yet to be covered extensively in the available literature.

There is considerable variation in the reported optimal dietary protein levels within and among abalone species. Coote et al. (2000) observed an optimum crude protein (CP) level of 27% for juvenile greenlip abalone (0.6–2.5 g) cultured at 20°C. Similarly, Bautista-Teruel and Millamena (1999) reported an optimum level of 27% for juvenile *Haliotis asinina* (0.6–3.0 g) at 27–31°C. In contrast, the optimal protein levels for a range of other abalone species appear to be higher, but variable. There is also evidence to suggest that abalone of different life stages have different protein requirements. Britz and Hecht (1997) reported that maximum growth of *Haliotis midae* L. at 18°C occurred in large (7.8 ± 0.25 g) abalone fed a CP level of 44%, whereas the maximum growth occurred in small (0.2 ± 0.01 g) abalone at a CP level of 34%. From these previous studies, greenlip abalone may also require different protein levels at different age classes and at different water temperatures.

Although morphology of the abalone digestive tract has been studied over many years, there is a dearth of published information from studies investigating the effects of dietary protein level, or protein source, on alterations to GIT morphology in abalone. It is important to consider the source of protein on digestive tract health. In an attempt to reduce costs, plant ingredients are being used more frequently as protein sources to replace fishmeal in aquaculture diets (Tacon & Metian 2009). Inclusions of yellow lupins, wheat gluten, soybean meal (SBM), cottonseed meal, peas, faba beans, and vetch into experimental diets for abalone have been investigated previously (Bautista-Teruel et al. 2003, Vandepeer 2005, Cho 2010). Some plant ingredients, such as SBM, contain antinutritional factors (ANFs) such as protease inhibitors, lectins, and saponins (Francis et al. 2001), which has been observed to induce histopathological damage in the GIT (subacute enteritis) and inhibit nutrient digestibility and growth performance in sensitive fish species such as Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) (van den Ingh et al. 1991, Baeverfjord & Krogdahl 1996, Bakke-McKellep et al. 2000, Carter & Hauler 2000, Storebakken et al. 2000, Krogdahl et al. 2003).

In response to the lack of available literature investigating the interactive effects of environmental and dietary factors in relation to differences in abalone age, the aim of this study was to investigate the effects of water temperature, dietary protein level, and animal age on GIT morphology of juvenile and subadult greenlip abalone by histological examination, and to investigate the processes that may be contributing to suboptimal growth in greenlip abalone.

MATERIALS AND METHODS

Abalone

The study was conducted at the South Australian Research and Development Institute (SARDI) Aquatic Sciences Center (ASC) at West Beach, South Australia. Greenlip abalone were sourced from SAM Abalone Pty Ltd (Boston Pt, Port Lincoln, South Australia). Animals were shipped by air to SARDI ASC and were acclimated in 5,000-L flow-through holding tanks at 15°C for 24 h prior to the commencement of the study.

Experimental design

Two age classes (juvenile, year 1: initial weight, 1.75 ± 0.1 g; initial shell length (SL), 23.31 ± 0.1 mm; subadult, year 2: initial weight, 22.93 ± 0.1 g; initial SL, 56.64 ± 0.1 mm) of greenlip abalone were held at either 14°C or 22°C and fed 2 separate series of diets of varying nominal protein levels. Juvenile abalone were fed the diet series containing 27% and 36% CP, and subadults were fed the diet series containing 24% and 33% CP.

Experimental Diets

The 4 test diets were formulated using practical ingredients at commercially acceptable inclusion levels to contain nominal CP levels of 24%, 27%, and 33%; 36% and 4.9% crude lipid; and 12.4 MJ/kg digestible energy (Table 1). The diets were manufactured into flat pellets using cold pellet pressing technology at the SARDI Australasian Experimental Stockfeed Extruded Center at Roseworthy, South Australia.

Experimental Procedure

The experiment was conducted in a temperature (14 ± 1°C and 22 ± 1°C) and photoperiod-controlled (12 h low intensity light (3.2 lux): 12 h dark) flow-through seawater system over a 12-wk period beginning September 2011. Prior to initial weighing and measuring, abalone were anesthetized using 1 mL/L (juvenile) or 2 mL/L (subadult) ethyl p-aminobenzoate (Sigma Chemicals, Balcatta, WA, Australia) in 30 L oxygenated seawater. All abalone were weighed individually (±0.1 g) then measured using digital vernier calipers (±0.1 mm). Each individual abalone was then transferred systemically into 32 2-L flow-through tanks. Each tank was stocked with either 20 juvenile or 10 subadult abalone. There were 4 replicate tanks provided for each treatment combination. All tanks were fed to excess, and feed rates were maintained in the range of 4.00%–5.25% of tank biomass for juvenile abalone and 1.00%–1.75% of tank biomass for subadult abalone. Abalone were fed at 4 PM daily, and uneaten food was collected at 9 AM the following day. Water temperature was maintained to ±1.0°C of the treatment temperatures for the duration of the experiment (Table 2). Because of the low ambient water temperature at the start of the experiment (15°C), the water temperature was adjusted at 1°C/day until the desired water temperature was achieved. Water quality was measured and recorded throughout the experiment (Table 2). Water temperature and dissolved oxygen (milligrams per liter and percent saturation) was measured daily using the OxyGuard Handy Gamma dissolved oxygen meter (OxyGuard International A/S,

TABLE 1.

Ingredient and chemical composition for abalone diets.

	24% CP	27% CP	33% CP	36% CP
Ingredient composition (g/100 g, as fed)				
Salmon fishmeal	4	4	4	4
Solvent-extracted soybean meal	16.4	18.9	23.9	26.47
Wheat gluten meal	5	5	5	5
Fish oil	1.61	1.22	0.46	0.1
EPA vitamin/mineral premix	0.2	0.2	0.2	0.2
Waxy maize starch (Mazaca 3401X)	30.4	30.67	27.59	19.96
Sodium alginate	0.3	0.3	0.3	0.3
Vitamin E	0.01	0.01	0.01	0.01
Lupins (gungaru, dehulled)	17.95	20.8	26.4	29.14
Diatomaceous earth	2.61	1.76	1.77	4.6
Calcium sulfate	0.5	0.43	0.3	0.22
Casein	4.43	5.48	7.59	8.63
Pregelatinized waxy maize starch	15.4	10	1.15	0
Monosodium phosphate	0.75	0.72	0.65	0.61
Arginine	0.27	0.31	0.41	0.46
Threonine	0.17	0.2	0.27	0.3
Sum	100	100	100	100
Chemical composition (g/100g, as fed)				
Moisture	10.1	10.35	10.61	10.3
Crude protein	23.93	26.93	32.93	35.93
Lipid	4.94	4.94	4.94	4.94
Gross energy (MJ/kg)	17.01	17.21	17.39	17.08
Digestible energy (MJ/kg)	12.39	12.39	12.39	12.39
Ash	5.78	4.97	5.07	7.93

Yttrium oxide was added at 0.02% to all diets. CP, crude protein.

Birkerød, Denmark), pH was measured daily using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, IL), and salinity (grams per liter) was measured weekly using a portable salinity refractometer (model RF20; Extech Instruments, Nashua, NH). Light intensity was measured using a LI-COR 1400 Quantum light meter (LI-COR Environmental, Lincoln, NE).

Additional abalone were held at the 2 water temperatures, fed a commercial diet, and were used to replace any mortalities that occurred throughout the experiment. Mortalities were recorded, measured, weighed, and replaced with abalone of a similar weight and size to reduce variability in tank biomass. Replacement animals were not tagged and were sampled, which could be a potential source of variation. However, because the number of mortalities was extremely low, any variation resulting from the use of these animals would be minimal. Ammonia (NH_4^+/NH_3, milligrams per liter) was not recorded because water exchange rate was maintained by the fresh seawater flow-though system, and tanks were cleaned daily to ensure the levels were kept low.

Sample Collection

At the conclusion of the study, 6 juvenile and 3 subadult abalone from each tank were sampled for histological investigation. The abalone were anesthetized in 2 mL/L ethyl p-aminobenzoate (Sigma Chemicals) in seawater and weighed and measured as described previously. They were then killed with an incision by scalpel to the cephalic region to sever the cerebral ganglia (Upatham et al. 1998). Abalone were fixed whole in 10% neutral buffered formalin and, after 4 days, were removed and shucked. An incision to the midsection of the animal was made to obtain a cross-section of the crop, stomach, and midsection of the intestine (Fig. 1). This cross-section was placed in a histology cassette and stored in 70% ethanol solution at room temperature.

Histology

Fixed tissue samples were dehydrated through an ethanol series to chloroform, embedded in paraffin wax, and sectioned at 5 μm using a Leica RM 2135 rotary microtome (Leica Microsystems GmbH, Wetzlar, Germany). Sections were then floated onto Objektträger microscope slides (ground edge, 90-deg frosted end; HDS Scientific Supplies, Wetherill Park, NSW, Australia) and dried for more than 48 h before staining.

To show GIT morphology, and to differentiate between neutral and acidic mucins present within goblet cells, slides were stained using pH 2.5 Alcian Blue (Sigma Chemicals) and periodic acid–Schiff stain (Sigma Chemicals). Periodic acid–Schiff stain stains neutral goblet cells magenta; Alcian Blue stains acidic mucins blue.

All sections were mounted in DPX (Ajax Finechem Pty Ltd, Taron Point, NSW, Australia) and examined under a light microscope (Olympus WH B10X\20 microscope; Olympus, Tokyo, Japan) and a Colorview Soft Imaging System CX41 camera (pixel resolution, 2,048 × 1,538) (Soft Imaging System; Brook-Anco Corporation, Rochester, NY) at 40× magnification. Slides were analyzed using Motic Image Plus version 2.0 (Motic Group, Wetzlar, Germany). Epithelial thickness measurements of the stomach, crop, and villi width, height, and area; and lamina propria height of the intestine were taken.

TABLE 2.

Water-quality parameters for each temperature-controlled system.

Treatment system	Temperature* (°C)	Dissolved oxygen† (% saturation)	Dissolved oxygen† (mg/L)	pH†	Salinity† (ppt)
14°C	14 + 0.2 (13.2–14.7)	99.1 ± 1.6 (88.4–104.2)	8.0 ± 0.2 (6.8–8.7)	8.3 ± 0.1 (8.1–8.4)	35.0 ± 0.6 (34–36)
22°C	22.0 ± 0.4 (21–23)	92.5 ± 4.5 (73.6–101.6)	6.7 ± 0.4 (5.3–8.1)	8.3 ± 0.1 (8.2–8.4)	35.0 ± 0.6 (34 –36)

All values are mean ± SE. Values in parentheses indicate parameter range. * Data for water temperature are recorded after the completion of the temperature acclimation period ($n = 74$). † $n = 79$.

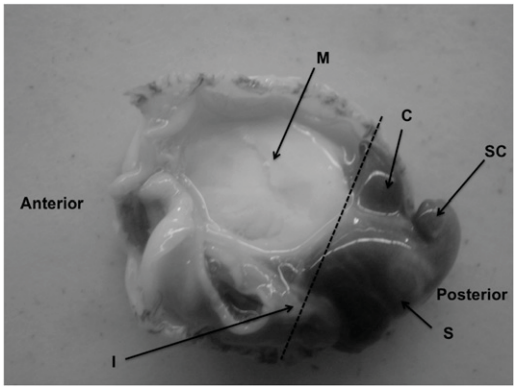

Figure 1. Dorsal view of shell-less greenlip abalone. The dotted line represents where the tissue cross-section was taken. Scale bar = 1 cm. C, crop; I, intestine; M, muscle, S, stomach; SC, spiral cecum.

Goblet cells containing neutral and acidic mucins were also counted in the epithelial layer of the stomach and crop.

Gastrointestinal Measurements

To reduce bias and variability, measurements were taken from the same histological section in each slide. A 1,000-μm length was measured along the basal lamina of the stomach or crop epithelium. Twenty measurements of epithelial thickness were taken approximately every 50 μm from the basal lamina to the apical membrane of the cell, which has been defined as epithelial thickness in this study. Goblet cells were counted within the same measurement. All measurements were taken from the stomach and crop areas adjacent to the intestine.

For the intestine, a 1,000-μm length was measured along the basal lamina of the intestinal epithelium adjacent to the stomach. Villus height and lamina propria height were taken from the basal lamina, and 2 measurements of villi width (basal and apical measurements) were also taken. Villus area was calculated using villus width × villus height.

Statistical Analysis

Homogeneity of variances among mean values was tested using Levene's test of equality of variance errors, and normality was assessed with standardized residuals against the predicted mean plot. Data for mean crop or stomach epithelial thickness; villus width, height, and area (square micrometers); and goblet cell numbers were analyzed using 2-way analysis of variance, with the fixed factors temperature and CP level. When necessary, a Student-Newman-Keuls test was used to evaluate significant differences among the means. Bivariate Pearson correlation analyses were used to investigate whether there were correlations between crop or stomach epithelial thickness in relation to the whole weight or SL of juvenile or subadult abalone. For all statistical tests, the significance level was set at $\alpha = 0.05$, and all statistics were done using IBM SPSS version 19.0 for Mac OSX (IBM SPSS Inc., Chicago, IL). All values shown are mean ± SE.

RESULTS

Mortality throughout the experiment was low at 2.92%, and normal signs of feeding and no gross symptoms of disease were observed.

Stomach Epithelium

The stomach epithelium of both juvenile and subadult greenlip abalone (Fig. 2) was characterized by a single layer of simple, uniform columnar cells of approximately 60–70 μm in thickness, interspersed sporadically with large goblet cells containing neutral and acidic mucins.

There was a significant effect of temperature on stomach epithelial thickness for juvenile abalone ($P = 0.027$; Fig. 3A). However, there was no significant effect of dietary protein level ($P = 0.557$; 27% CP, 61.08 ± 3.16 μm; 36% CP, 59.38 ± 3.31 μm), and there was no significant interaction ($P = 0.657$). A significant effect of temperature on the stomach epithelial thickness was also observed in subadult abalone ($P = 0.017$; Fig. 3B). The effect of dietary protein level ($P = 0.365$; 24% CP, 75.56 ± 4.68; 33% CP, 71.27 ± 7.37) and the interaction between the 2 factors ($P = 0.103$) were not significant. Stomach epithelial thickness was less for both age classes of abalone at the higher temperature.

Significant moderate negative correlations for juvenile stomach epithelial thickness and whole animal weight ($P = 0.006$, $r = -0.652$, $n = 16$; Fig. 4A) and SL ($P = 0.009$, $r = -0.62$, $n = 15$; Fig. 4B) were observed. There were also significant moderate negative correlations for subadult stomach epithelial thickness and whole animal weight ($P = 0.045$, $r = -0.52$, $n = 15$; Fig. 4C) and SL ($P = 0.020$; $r = -0.59$, $n = 16$; Fig. 4D).

Crop Epithelium

In both juvenile (Fig. 5b) and subadult abalone, the crop epithelium had tall, simple columnar cells of approximately 100–110 μm in thickness, although cells were less uniform in thickness and were taller than the stomach epithelial cells.

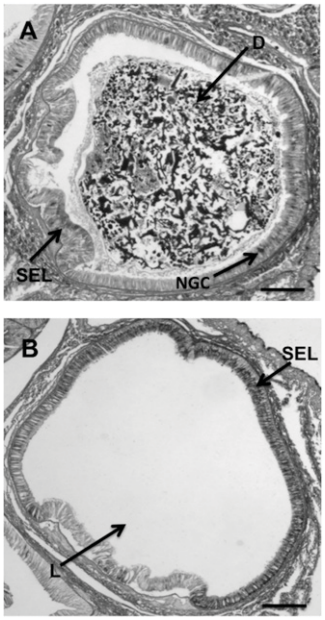

Figure 2. (A, B) Juvenile stomach epithelium at 14°C (A) and 22°C (B). Tissue was fixed in 10% formalin and stained with periodic acid–Schiff stain. Scale bar = 100 μm, 40× magnification. D, digesta; L, lumen; NGC, neutral goblet cells; SEL, stomach epithelial layer.

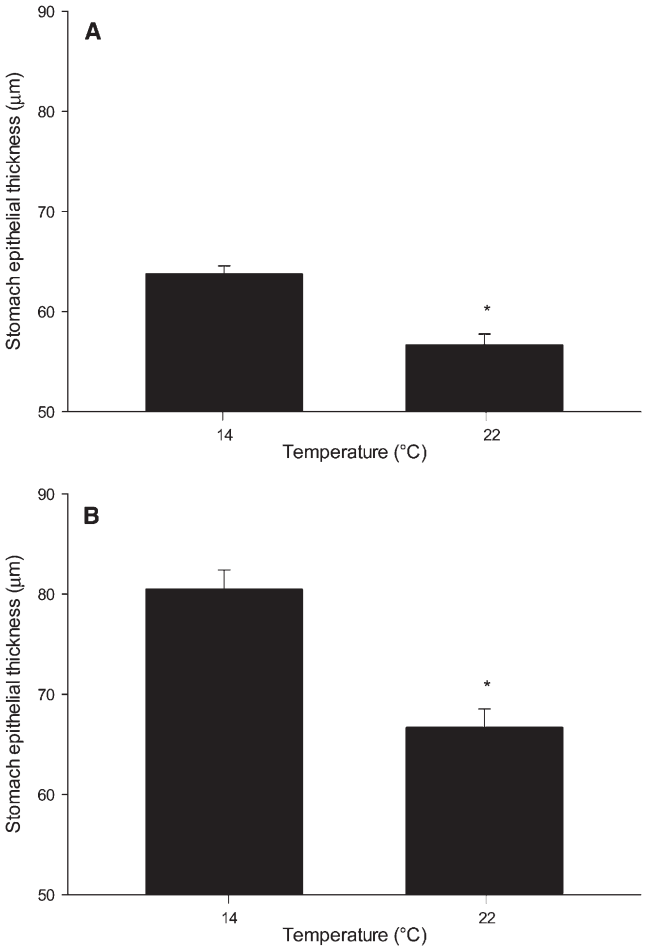

Figure 3. (A, B) Mean juvenile ($P = 0.027$, $n = 16$) stomach epithelial thickness at 14°C and 22°C (A), and subadult ($P = 0.017$, $n = 15$) abalone stomach epithelial thickness at 14°C and 22°C. *Significant difference at $P < 0.05$. Values are mean ± SE.

Neutral and acidic goblet cells were interspersed throughout the crop epithelium and were more numerous than found in the stomach epithelium.

In juvenile abalone, temperature had a significant effect on epithelial thickness, being significantly thicker at 22°C compared with 14°C ($P = 0.044$; Fig. 6A). Dietary protein had no significant effect on epithelial thickness ($P = 0.456$; 27% CP, 106.38 ± 8.19 µm; 36% CP, 99.43 ± 8.14 µm) and there was no significant interaction between the 2 factors ($P = 0.801$). In subadult abalone, there was no significant effect of temperature on crop epithelial thickness ($P = 0.490$; 14°C, 80.79 ± 5.06 µm; 22°C, 66.69 ± 5.20 µm). However, there was a significant effect of protein level on this variable, with 33% CP having a significant decrease in epithelium compared with 24% CP ($P = 0.011$; Figs. 6B and 7). There was no significant interaction ($P = 0.590$).

There were significant moderate positive correlations for juvenile crop epithelial thickness and whole animal weight ($P = 0.023$, $r = 0.58$, $n = 16$; Fig. 8A) and SL ($P = 0.020$, $r = 0.57$, $n = 16$; Fig. 8B). There was no significance or correlations for subadult crop epithelial thickness and whole weight ($P = 0.183$, $n = 16$; Fig. 8C) or SL ($P = 0.118$, $n = 16$; Fig. 8D).

Intestinal Epithelium: Villus Height, Width, and Area; and Lamina Propria Height

Villi height from the midsection of the intestine in juvenile and subadult abalone was not significantly affected by water temperature ($P = 0.512$ and $P = 0.091$, respectively) or protein level ($P = 0.359$ and $P = 0.527$, respectively), and there was no significant interaction between the 2 factors ($P = 0.736$ and $P = 0.137$, respectively). The mean villi height was 285.59 ± 18.77 µm and 558.15 ± 41.18 µm for juvenile and subadult abalone, respectively.

There was also no significant effect of water temperature ($P = 0.558$, $P = 0.387$) or protein level ($P = 0.686$, $P = 0.172$) on the villi width from the midsection of the intestines of juvenile or subadult abalone, respectively, and there was no significant interaction between the 2 factors ($P = 0.562$, $P = 0.394$). The mean villi widths were 166.03 ± 7.73 µm and 208.14 ± 9.98 µm for juveniles and subadults, respectively.

There was no significant effect of water temperature ($P = 0.367$, $P = 0.081$) or protein level ($P = 0.427$, $P = 0.149$) on the villi area from the midsection of the intestines of juvenile or subadult abalone, respectively, and there was no significant interaction between the 2 factors ($P = 0.636$, $P = 0.110$). The mean villi area was 48,236.75 ± 4,358.32 µm^2 and 119,700.48 ± 12,695.58 µm^2 for juveniles and subadults, respectively.

There was also no significant effect of water temperature ($P = 0.399$, $P = 0.919$) or protein level ($P = 0.399$, $P = 0.484$) on the lamina propria height from the midsection of the intestines of juvenile or subadult abalone, respectively, and there was no significant interaction between the 2 factors ($P = 0.919$, $P = 0.067$). The mean lamina propria height was 217.93 ± 20.45 µm and 406.01 ± 29.22 µm for juvenile and subadult abalone, respectively.

Goblet Cells

There was no significant effect of water temperature on the number of goblet cells containing acidic mucins in the stomach ($P = 0.082$, $P = 0.494$) or crop ($P = 0.604$, $P = 0.570$) and no significant interaction in juvenile and subadult abalone, respectively. There was no significant effect of dietary protein on acidic goblet cells in the stomach ($P = 0.301$, $P = 0.086$) or crop ($P = 0.607$, $P = 0.248$) in juvenile or subadult abalone, respectively. There were also no significant interactions between water temperature and dietary protein level for acidic mucins in the stomach ($P = 0.707$, $P = 0.337$) or crop ($P = 0.445$, $P = 0.582$) for juvenile or subadult abalone, respectively. The number of goblet cells containing acidic mucins in the stomach was highly variable and the mean was 4.43 ± 0.44 cells/mm tissue and 6.06 ± 1.51 cells/mm tissue for juvenile and subadult abalone, respectively. The number of goblet cells containing acidic mucins in the crop was also highly variable and the mean was 15.92 ± 2.11 cells/mm tissue and 7.86 ± 2.97 cells/mm tissue for juveniles and subadults, respectively.

There was no significant effect of water temperature on the number of goblet cells containing neutral mucins in the stomach ($P = 0.842$, $P = 0.782$) or crop ($P = 0.422$, $P = 0.482$) in juvenile or subadult abalone, respectively. There were also no significant effects of dietary protein on the number of goblet cells containing neutral mucins in the stomach ($P = 0.758$, $P = 0.235$) or crop ($P = 0.840$, $P = 0.227$) in juvenile or subadult abalone, respectively. There were no significant interactions between

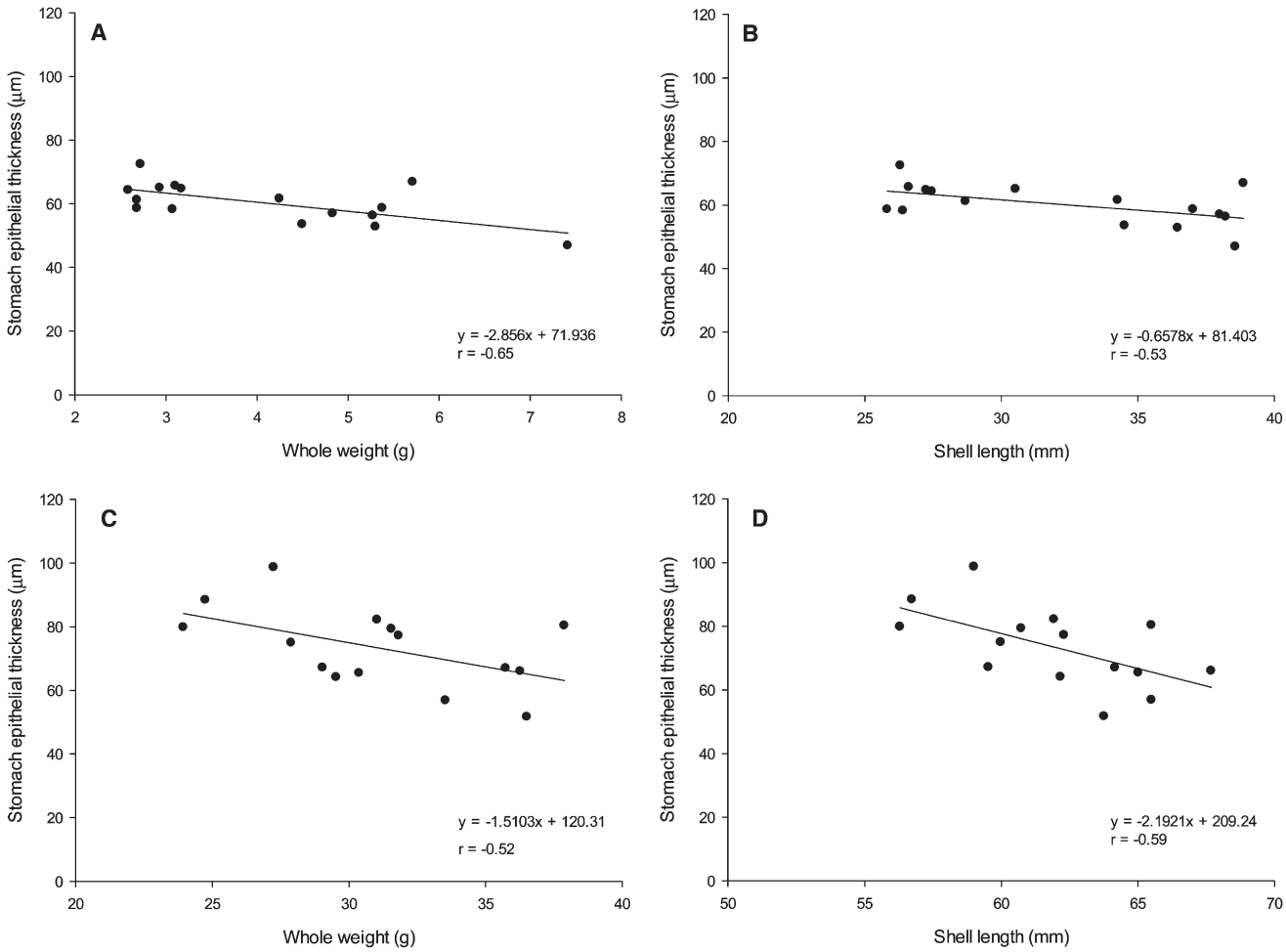

Figure 4. (A) Significant moderate negative correlation for juvenile stomach epithelial thickness and whole animal weight ($P = 0.006$, $r = -0.65$, $n = 16$). (B) Significant moderate negative correlation for juvenile stomach epithelial thickness and shell length ($P = 0.009$, $r = -0.53$, $n = 15$). (C) Significant moderate negative correlation for subadult stomach epithelial thickness and whole weight ($P = 0.045$, $r = -0.52$, $n = 15$). (D) Significant moderate negative correlation between subadult stomach epithelial thickness and shell length ($P = 0.020$, $r = -0.59$,; $n = 16$).

water temperature and dietary protein level for neutral mucins in the stomach ($P = 0.816$, $P = 0.092$) or crop ($P = 0.523$, $P = 0.214$) for juveniles or subadults, respectively. The number of goblet cells containing neutral mucins in the stomach was highly variable and the mean was 3.11 ± 0.39 cells/mm tissue and 6.01 ± 1.33 cells/mm tissue for juveniles and subadults, respectively. The number of goblet cells containing neutral mucins in the crop was also highly variable, with 15.09 ± 2.58 cells/mm tissue and 2.33 ± 0.95 cells/mm tissue for juvenile and subadult abalone, respectively.

The goblet cell counts for the mid intestine were discounted as a result of the lack of goblet cells present in this section of tissue.

DISCUSSION

In this study, we set out to investigate the effects of water temperature, dietary protein level, and age class on the morphology and function of the GIT of greenlip abalone. There were marked differences in the response of juvenile and subadult abalone to environmental and dietary alterations. Juvenile and subadult abalone showed moderate negative correlations in the stomach epithelial thickness as whole weight and length increased. In the crop epithelium, juvenile abalone showed a moderate positive correlation of thickness to whole weight and SL. Conversely, subadults showed a weak negative correlation in crop epithelial thickness as whole weight and SL increased. Thus, it appears that alterations in epithelial thickness in response to animal age for both the stomach and the crop are independent processes, and that both need to be assessed together. The correlations of stomach and crop epithelial layers to animal age are moderate, so it is likely that other factors are affecting the epithelial layers in juvenile and subadult abalone apart from age alone.

Abalone are affected in many ways by temperature because it influences all aspects of molecular and cellular processes. For example, membrane stability and enzymatic activity are affected by water temperature, hence metabolism and physiological activity of the organism are also affected (Guderley 2004). This fact is reflected in growth rates; as temperature increases, growth rates of abalone also increase until the thermal threshold is reached. This growth response has been demonstrated in numerous abalone species (Britz et al. 1997, Lopez et al. 1998, Cho & Kim 2012) and was also observed with greenlip abalone in the parent study to this work (Stone et al. 2013).

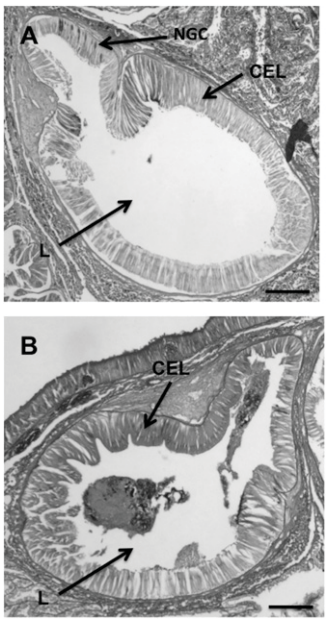

Figure 5. (A, B) Juvenile crop epithelium at 14°C (A) and 22°C (B). Tissue was fixed in 10% formalin and stained with periodic acid–Schiff stain. Scale bar = 100 μm, 40× magnification. CEL, crop epithelial layer; L, lumen; NGC, neutral goblet cells.

Water temperature had an effect on the stomach epithelial thickness in both age classes of abalone. Those kept at 14°C had a significantly thicker stomach epithelial layer than those at 22°C, and this finding is consistent with studies conducted on fish species. Lee and Cossins (1988) investigated the effects of water temperature on the common carp *Cyprinus carpio* L. and found that as water temperature decreased from 30–15°C, intestinal diameter increased, and mucosal surface area increased by almost 50%. The increased surface area was reported to be a result of the enterocytes of the intestinal villi undergoing considerable hypertrophy (Lee & Cossins 1988). Carp are a warm-water fish, and their optimum temperature for growth is reported to be 27°C (Goolish & Adelman 1984), indicating that the GIT hypertrophy reported in the study of Lee and Cossins (1988) was in response to colder water temperatures. Similar results were also reported by Kitchin and Morris (1971) in the goldfish *Carassius auratus* L., who suggested that the observed hypertrophy was to increase the affinity for valine absorption. Similarly, Lee et al. (1991), while investigating transepithelial transport of amino acids in the villi of carp, observed that amino acid uptake was almost double in cold-acclimated fish compared with warm-acclimated fish. Houpe et al. (1996) reported similar phenotypic responses to cold acclimation in the channel catfish *Ictalurus punctatus* Rafinesque. Intestinal lengths of fish reared in 15°C water were 20% longer than those in 30°C. Houpe et al. (1996) hypothesized that even without accounting for increased surface area of villi and microvilli, intestinal surface area was likely to increase in fish kept in cooler water.

In mammals, studies of cold acclimation show similar results. Gut length and mass increased as the temperature decreased in the rodents *Microtus ochrogaster* Wagner, and *Dicrostonyx groenlandicus* Traill (Hammond & Wunder 1995). A similar result was also observed in the small intestine of the omnivorous rodent *Akodon azarae* Fischer (del Valle et al. 2004). However, the stomach and large intestine showed no changes in length or mass (del Valle et al. 2004). The increase in the stomach epithelial thickness for all abalone kept at 14°C in this study is consistent with the results from the aforementioned studies. Greenlip abalone in this study appeared to demonstrate a compensatory effect by increasing the surface area in the stomach, even if this process is energetically costly (Houpe et al. 1996). This tissue hypertrophy is generally not maintained in warmer water, as demonstrated in by the study of Lee and Cossins (1988). They hypothesized that extensive hypertrophy is not required to maintain the absorptive capacity when enzymatic activity increases, or is within its optimal range, in response to warmer water temperatures. The findings of this study concur with their study (Lee & Cossins 1988). Analysis of the growth of the abalone in this experiment showed that animals kept at 14°C had a depressed growth rate compared with those kept at 18°C and 22°C (Stone et al. 2013). It could be suggested that even with a decrease in the stomach epithelial layer at 22°C, the absorptive capacity was maintained and was adequate to provide the nutrients required for reasonable levels of growth at the warmer temperature.

As observed in other animals, greenlip abalone may demonstrate phenotypic plasticity or flexibility in response to cold acclimation, which may have been achieved by increasing stomach epithelial surface area in response to depressed enzymatic activity and absorptive capacity at the colder temperature in an attempt to increase nutrient absorption and/or enzyme secretion. The question arises as to why the increase in absorptive surface area was not observed in the crop of either age class of greenlip abalone at 14°C. As mentioned previously, rodents do not increase the size and mass of all organs in the GIT during cold acclimation (del Valle et al. 2004). del Valle et al. (2004) reported changes to the small intestine only, which is the main site of nutrient absorption and digestion in mammals. In abalone, nutrient absorption was originally thought to only occur in the intestine, whereas the crop and stomach were regarded as areas for food collection, and in the stomach, the addition of digestive enzymes from the hepatopancreas (Crofts 1929). However, after subsequent investigations, Campbell (1965) and McLean (1970) disagreed with Crofts (1929), and suggested that the esophagus, crop, and stomach are also sites for nutrient absorption in *Haliotis* species. The stomach contains a spiral cecum, and the hepatopancreas is connected to the stomach and spiral cecum through a series of ducts, where protease, diastase, and lipase are secreted (McLean 1970) and nutrients are absorbed (Campbell 1965). The stomach of abalone retains digesta for a greater period of time than the crop, seemingly to allow ample time for both extracellular and intracellular digestion (Crofts 1929, Campbell 1965). In addition, the hepatopancreas is known to be an important site for nutrient absorption in molluscs (Ahearn et al. 1992), and because it is connected to the stomach, this suggests an increased capacity for nutrient absorption compared with other areas of the digestive tract, such as the crop. McLean (1970) suggests that the crop is the first site of absorption in abalone, because phenylalanine permeability was observed in this region. Currently, there is limited new information on the digestive and absorptive capacity of regions other than the hepatopancreas in molluscs (Martin et al. 2011). More research in this area is warranted for greenlip abalone.

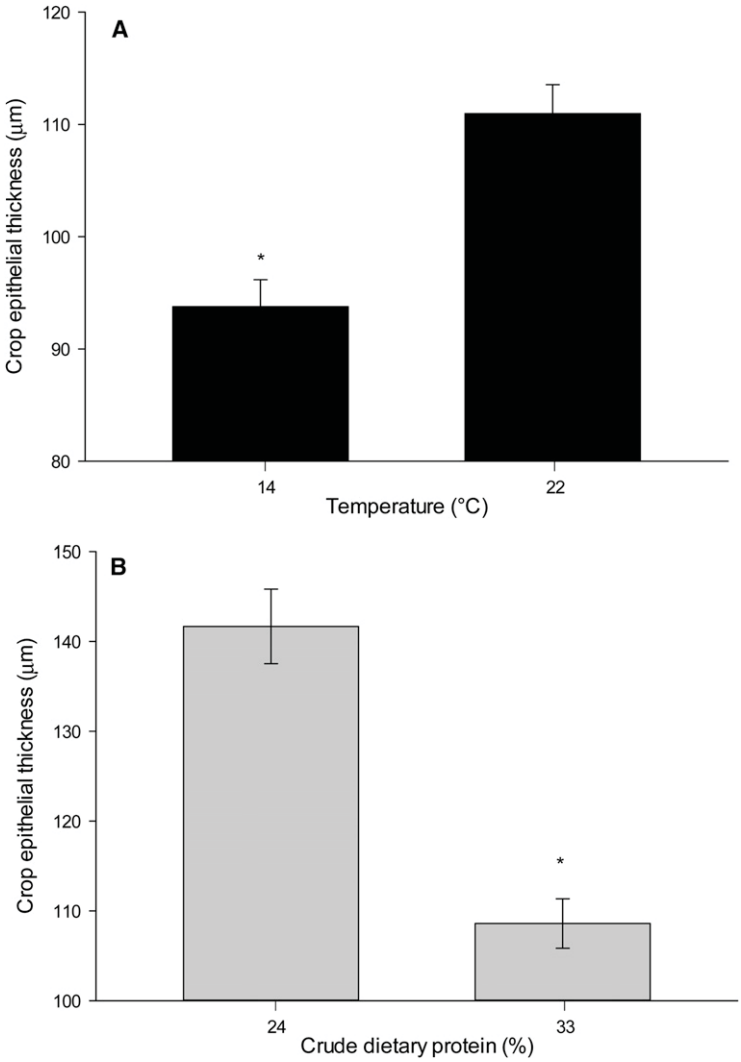

Figure 6. (A, B) Mean juvenile ($P = 0.044$, $n = 16$) crop epithelial thickness at 14°C and 22°C (A), and (b) subadult ($P = 0.011$, $n = 15$) crop epithelial thickness at 24% crude protein and 33% crude protein. *Significance at $P < 0.05$. Values are mean ± SE.

The information from these previous studies suggests it is possible that the crop is responsible mainly for digestion, mixing, and secreting and/or receiving enzymes, and its absorptive capacity is limited, which could account for the increase in stomach epithelial thickness in cold water, whereas the crop epithelium in both age classes showed a limited response. As mentioned previously, it may be energetically costly to build and maintain an extensive digestive epithelium, therefore increasing the surface area of the organ with the highest absorptive capacity would be advantageous in colder water temperatures.

Although the stomach had a thinner epithelium at 22°C, it cannot be assumed that this thinning was entirely in response to warmer water temperature, as hypothesized by Lee and Cossins (1988). In addition, the crop epithelium was thicker in juveniles at 22°C compared with those kept at 14°C, indicating that tissue hypertrophy or hyperplasia is not only restricted to cold water. Growth at 22°C was significantly greater than at 14°C (Stone et al. in press), and thus it is evident that the capacity of the GIT has been maintained at warmer water temperatures; however, other factors that could be inducing these responses cannot be discounted.

In this study, solvent-extracted SBM was used as the primary protein source. The diets were formulated to contain ~19% and 27% solvent-extracted SBM for juveniles, and ~16% and 24% solvent-extracted SBM for subadults. Solvent-extracted SBM is known to induce damage in the intestinal mucosa of Atlantic salmon, referred to as subacute enteritis, with a concurrent reduction in growth performance (van den Ingh et al. 1991, Baeverfjord & Krogdahl 1996, Uran et al. 2008). There was no significant reduction in the growth performance of abalone in response to increasing protein content (see Stone et al. in press). However, it is possible that the stomach epithelium for both juveniles and subadults is demonstrating a thinning at warmer water temperatures because of the presence of solvent-extracted SBM in the diets. More research is needed to investigate the effects of alternative protein sources, and their constituent ANFs, on the GIT of greenlip abalone.

It is possible that, in juveniles, the crop epithelium is compensating for the stomach epithelial thinning by increasing its cell size, therefore increasing surface area and digestive capacity. Compensatory growth that leads to an increase in

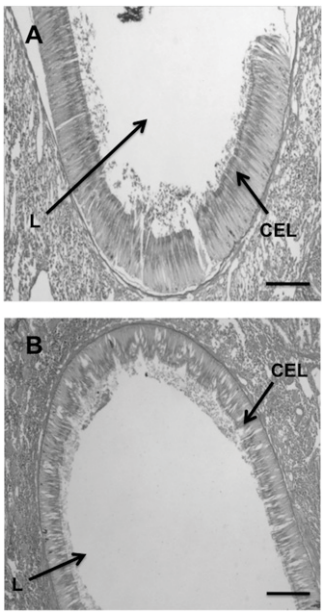

Figure 7. (A, B) Subadult crop epithelium at 24% crude protein (A) and 33% crude protein (B). Tissue was fixed in 10% formalin and stained with periodic acid–Schiff stain. Scale bar = 100 μm, 40× magnification. CEL, crop epithelial layer; L, lumen.

the surface area of the GIT has been observed in humans and other animals that have had significant surgery or damage to the intestine. Adaptive responses in rats and pigs that increase surface area include mucosal hypertrophy (Whang et al. 1996) and increases in villus height and crypt depth (Digalakis et al. 2011). A similar adaptive response could be occurring in the crop of the juvenile abalone because they have a greater growth rate compared with subadult abalone (Stone et al. in press) and, therefore, a concurrent increased need for nutrients at warmer water temperatures. If the stomach is indeed demonstrating an ANF-induced change, then the abalone may be attempting to compensate by increasing crop surface area and, therefore, enzymatic activity and digestive capacity. Subadult abalone did not demonstrate the same response in the crop epithelium as juvenile abalone, and this could be a result of the enterocyte turnover rate, which could be masking the effects of epithelial hypertrophy in subadults compared with juveniles. This hypothesis requires further research.

An alternative hypothesis is the crop epithelium in juveniles is responding to gastric transit time. Water temperature is known to affect digestive efficiency and gastric evacuation rates of several fish species. European sea bass (*Dicentrarchus labrax* L.) showed increased gastric evacuation rates with warmer water temperatures (Santulli et al. 1993), whereas the yellowtail kingfish (*Seriola lalandi* Valenciennes) had the same response (Miegel et al. 2010). Animal size also affects gastric evacuation rate. In brown trout (*Salmo trutta* L.) and rainbow trout, the amount of food processed per gram per hour decreased with increasing body weight, and gastric emptying was found to be almost twice as fast in smaller trout compared with larger trout (Bassompierre et al. 1998, Kristiansen 1998). This situation was also corroborated in turbot (*Scophthalmus maximus* L.); in larger fish, the gastric evacuation rate was slower (Bromley 1987).

Because smaller animals have a faster gastric transit rate compared with larger animals, then it could be likely that an upregulation of crop epithelium is occurring to increase enzyme secretion and mixing of digesta. If the gastric transit rate is increased, then use of food may be reduced because there is a decrease in digesta contact time with the GIT in juvenile abalone. Because gastric transit rate is reduced in larger animals, then digesta is in contact with the GIT for greater lengths of time in subadult abalone. This increased time of digesta with the crop epithelium in subadults could account for why crop epithelial hypertrophy was not observed in this age class, but was observed in juveniles. The decreased transit time in subadult abalone may indicate that the crop epithelium does not need to expand its surface area because digestive capacity is maintained as a result of the increased contact time of digesta to the GIT. Alternatively, a solvent-extracted SBM-induced response is hampering the ability of the subadult crop epithelium to increase surface area, and so the dietary protein effects are masking crop hypertrophy. In support of this theory, a dietary protein effect was seen in subadult abalone, with a significant thinning of the crop epithelium at 33% CP compared with 24%. The same trend, although insignificant, was observed in the juvenile crop epithelium. The assumed decrease in gastric transit rate in subadults compared with juveniles, and increased contact time with digesta containing ANFs could account for crop epithelial thinning in response to dietary protein, and may explain why this was only seen in subadults and not in juvenile greenlip abalone. More research is needed to investigate gastric transit rate in relation to temperature and ANFs for greenlip abalone.

There were no significant results obtained from the villi or goblet cell data because of a very high amount of variability within and between individuals. Villi were interspersed somewhat intermittently throughout the intestinal region sections, and goblet cell numbers had a high amount of variability. There were more goblet cells containing neutral and acidic mucins in the crop than in the stomach, perhaps suggesting that movement of digesta through the crop is greater than in the stomach region, as discussed earlier. Villi were not uniform in height or width, resulting in the high amounts of variability.

In conclusion, it is apparent that both water temperature and dietary protein level are affecting the morphology of the GIT of greenlip abalone. The alterations in the morphology of the GIT did not appear to be detrimental to the health and growth of the abalone during this study. A more pronounced influence of water temperature, rather than dietary protein level, was observed. In addition, there were more pronounced changes in the morphology of the GIT in response to water temperature for juvenile abalone compared with subadult abalone. This observation, in turn, suggests a greater sensitivity to temperature fluctuations for juvenile abalone, whereas subadults were more prone to the effects of changes in dietary protein levels. Abalone appear to be able to compensate for temperature and dietary changes adequately by phenotypic plasticity or flexibility, enhancing digestive and absorptive capacity at the temperatures tested in this study. With regard to plant ingredients and ANFs, further research is required to determine whether these ingredients, in combination with fluctuating water temperature, are the causative factors behind crop hypertrophy/stomach hypotrophy in abalone. In addition, further investigations are required to determine whether the presence of ANFs in combination with extended gastric transit times is also having an effect on the GIT morphology and, in turn, the enzymatic secretion and activity of each region of the GIT.

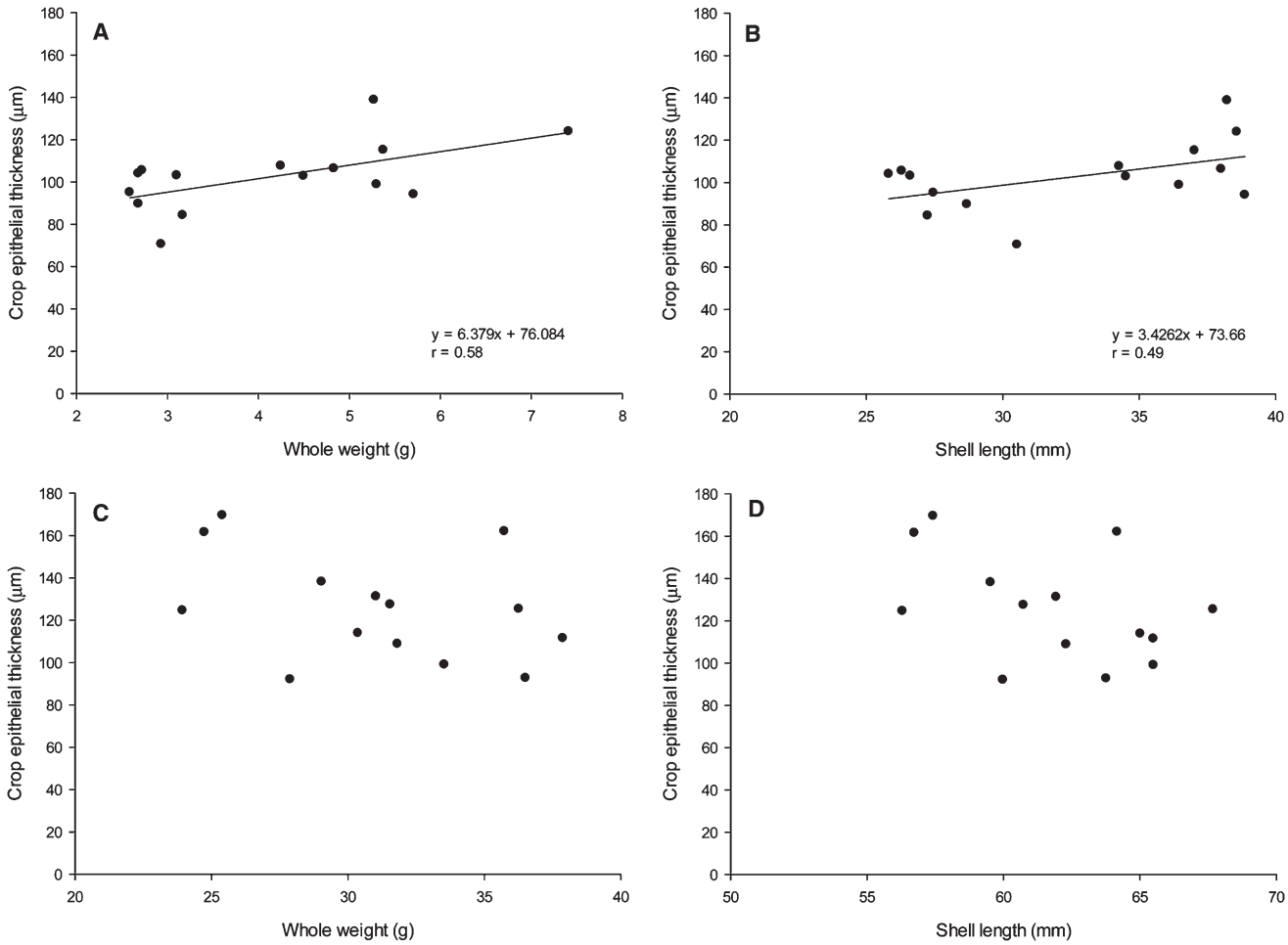

Figure 8. (A) Significant moderate positive correlation for juvenile crop epithelial thickness and whole weight ($P = 0.023$, $r = 0.58$, $n = 16$). (B) Significant moderate positive correlation for juvenile crop epithelial thickness and shell length ($P = 0.020$, $r = 0.49$, $n = 16$). (C) Nonsignificant correlation for subadult crop epithelial thickness and whole weight ($P = 0.183$, $n = 16$). (D) Nonsignificant correlation between subadult crop epithelial thickness and shell length ($P = 0.118$, $n = 16$).

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