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Gastrointestinal evacuation time, but not nutrient digestibility, of greenlip abalone, *Haliotis laevigata* Donovan, is affected by water temperature and age

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ABSTRACT

The effects of water temperature (14, 18, 22 and 26 °C) and age (2-y-old, 6.7 g; 3-y-old, 25.7 g) on the gastrointestinal evacuation (GIE) time and apparent nutrient digestibility coefficients (ADC) for greenlip abalone (*Haliotis laevigata*). GIE time of greenlip abalone was evaluated using a marker diet containing chromic oxide and the total faecal collection technique. Additionally, GIE values were compared from samples collected from abalone that were sampled once only or repeatedly to compare the rigour of each method. GIE time was also assessed using radiography. GIE times were significantly affected by water temperature ($P < 0.001$) and age ($P < 0.05$). GIE times decreased for both age classes with increasing temperatures, and the GIE time of 2-y-olds was significantly faster than for 3-y-olds. GIE times never exceeded 60 h. There was no significant effect of either factor on the duration that the marker diet was voided, suggesting that differences in GIE times are due to factors other than the transit of feed through the intestine. Dry matter ADCs were not affected by temperature ($P > 0.05$) but were affected by age ($P < 0.001$; 2-y-old <3-y-old). Protein and energy ADCs were not affected by temperature or age. Results may be used to predict purging times and also indicate that further investigations into feeding frequencies are recommended.

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1. Introduction

Gastrointestinal evacuation (GIE) time is important, because the rate at which food passes all the way through the intestinal tract is likely to influence how effectively nutrients are digested and absorbed. Additionally, GIE is an important parameter for modelling feed intake (Jobling, 1987) and is partially responsible for the return of appetite (Riche et al., 2004). As GIE is partially linked to the return of appetite, GIE times may be used to tailor specific feeding frequency regimes. Additionally, information relating to GIE time in abalone can be used to assess purging times for a range of applications including in-feed chemical withholding periods, and the voiding of feed and sand prior to harvesting, live transport and consumption.

A number of methods for determining GIE, or alternatively gastric evacuation time or rate methods which can be applied to assessing GIE time have been used in fish. These methods include dissection (Miegel et al., 2010), stripping (Storebakken, 1985), inert markers (Boyce et al., 2000; Richter et al., 2008; Storebakken et al., 1999), radioactive isotopes (Storebakken et al., 1981), radiography (Hossain et al., 2000; Jobling, 1987; Jobling et al., 1977; Tablot and Higgins, 1983) and faecal collection by settlement (Sales and Britz, 2001; Shipton and Britz, 2001). Due to handling logistics, only faecal collection by settlement and radiography may be suitable methods for the determination of GIE time in abalone. Digestibility studies may also be carried out using faecal collection by settlement (Sales and Britz, 2001; Shipton and Britz, 2001; Vandepeer et al., 2002); therefore, using methods in which both studies can be employed simultaneously would be beneficial in order to refine future studies for this species.

To date, limited research has been done in order to determine the GIE time in abalone. South African abalone (*Haliotis midae* L) have been shown to have a gastric evacuation time of 18 to 24 h when fed a formulated diet (Britz et al., 1996), and produced faeces up to 96 h after being fed a formulated feed with 0.5 % chromic oxide as a marker (Shipton and Britz, 2001). When *H. midae* are fed algae, less digestible algal species remain identifiable in the stomach for more than 48 h, whereas preferred algal species are mostly digested within 24 h (Day and Cook, 1995). A problem with these studies is that the animals were fasted for three days (Britz et al., 1996), four days (Sales and Britz, 2001; Shipton and Britz, 2001) or four weeks (Day and

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Cook, 1995) prior to feeding the test diet. Additionally, after receiving their test ration, the abalone in these studies were also fasted for the remainder of the experiment. The impact of fasting, or starvation, on the GIE time is currently not known for abalone. However, starvation periods are known to alter the intestinal structure of greenlip abalone (Harris et al., 1998). Consequently, starving abalone prior to sampling may influence the GIE time and potentially nutrient digestibility. Therefore, sampling methods should not employ starvation periods that may interfere with normal feeding patterns.

The effect of water temperature and animal age on GIE time in greenlip abalone have not been reported. Both temperature (Jobling et al., 1977; Miegel et al., 2010; Santulli et al., 1993) and animal size (Bassompierre et al., 1998; Heyraud, 1979; Jobling et al., 1977) influence the GIE time and the gastric evacuation time and rate of other aquatic animals. The trend common to these studies is that decreasing water temperatures and increasing animal sizes leads to an increased GIE times. Based on these findings it is reasonable to suggest that the same trend may be observed in greenlip abalone.

A dependable measure of the digestibility of various nutrients is an essential component required for effective animal nutrition research and diet formulation (De la Noue and Choubert, 1986). Feed digestibility coefficients also provide essential information to correctly formulate diets to reduce nutrient discharge into the environment (Montaño-Vargas et al., 2002). Little reported research has been conducted on the influence of water temperature and animal size or age on nutrient digestibility in abalone. Dixon (1992) reported that peak digestibility occurred at 18 °C compared to 14 °C or 22 °C in *H. midae*. The influence of animal size in abalone digestibility studies has been neglected as often larger abalone (>50 mm) are used in order to obtain larger amounts of readily identifiable faecal material in the shortest period of time (Fleming, 1995).

The aims of this study were to: 1) investigate the effects of water temperature and age on the GIE time and dietary nutrient digestibility in greenlip abalone; 2) investigate the effects of single versus repeated faecal collection methods on the GIE time of greenlip abalone; and 3) compare the GIE times in single sampled greenlip abalone using Ballotini balls and radiography versus repeated faecal collection methods.

2. Materials and methods

2.1. Experimental animals

Greenlip abalone were purchased from South Australian Mariculture (Boston Point, Port Lincoln, SA, Australia) and were then held at the South Australian Research and Development Institute (SARDI) Aquatic Sciences Centre (ASC) in 5000 L fibre glass tanks with a flow-through, UV-treated, seawater system, fed a commercial abalone pellet diet (Eyre Peninsula Aquafeed Pty Ltd (EPA), Lonsdale, SA, Australia) ad libitum, and were not used in any other studies prior to these experiments. Upon commencement of the experiments a sub-set of abalone were randomly selected from a larger population and transferred into 250 L holding tanks with flow-through UV-treated seawater. Two age classes of greenlip abalone (2-y-old: initial weight 6.70 ± 0.47 g, initial shell length (SL) 37.03 ± 0.90 mm, n = 192; 3-y-old: initial weight 25.71 ± 1.28 g, initial SL 56.90 ± 0.97 mm, n = 192) were used.

2.2. Experimental system

The experiments were conducted in a temperature (20 ± 1 °C) and photoperiod (12 h low intensity, fluorescent lighting at 3.4 Lx: 12 h dark) controlled room. Thirty two blue plastic tanks (Nally IH305, Viscount Plastics Pty Ltd; length, 39.2 cm; width, 28.8 cm; depth, 11.0 cm) were used in this experiment. Water level was set at 2.5 cm in each tank using a standpipe providing a water volume of 2.8 L in each tank. Flow-through UV-treated water was supplied to tanks from a saltwater system described in Stone et al. (2013) at a rate of 300 mL min^{-1}. Water temperature was controlled by using either a chiller (240 V, 2.2 kW, 50 Hz; Daeil Cooler Co., Ltd., Busan, Korea) or an immersion heater (240 V, 3 kW; JQ20, Austin & Cridland., Carlton, NSW, Australia).

2.3. Experimental diets

One commercial diet formulation was used for the experiments. Eyre Peninsula Aquafeeds provided the diet dry mash, and two separate diets were made using the same mash: 1) a control diet; and 2) a marker diet which contained chromic oxide (20 g kg^{-1} dietary inclusion) and 0.5 mm diameter Ballotini balls (7.5 g kg^{-1} dietary inclusion, dry basis). The diets were mixed and cold pressed into flat chips (~5 mm × 5 mm × 2 mm thick) using a commercial pasta machine (La Prestigiosa medium, IPA, Vicenza, Italy). Biochemical analyses on moisture, ash, crude lipid, gross energy, crude protein and amino acid composition of the commercial diet were analysed according to the methods of the AOAC (1995). The commercial control diet was oven dried to a constant weight at 105 °C for 16 h to determine moisture content. 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. Ash was determined by a muffle furnace at 550 °C for 16 h. Gross energy content was determined using a bomb calorimeter calibrated with benzoic acid. Carbohydrate was evaluated using the Molisch test (Lampman et al., 2010) and a glucose standard curve. Amino acids were analysed using the methods of Bosch et al. (2006). The analysed biochemical composition of the experimental diets is presented in Table 1. For the marker diet four replicate groups of ten Ballotini balls were weighed to 0.1 mg to determine the individual weight of balls in order to establish the relationship between feed weight and the number of Ballotini balls present.

2.4. Temperature acclimation and experimental stocking and feeding procedures

Prior to the stocking of each experiment, the animals were pre-acclimatised in the holding system to their respective water temperature treatment by adjusting the water temperature of the holding tank by 1 °C per day until the desired water temperature was reached. The abalone were then held in the holding tank for a period of at least 7 days at the selected temperature and fed the control diet to excess (4% body weight (bw) d^{-1}) prior to stocking. At stocking, each abalone was individually weighed (wet whole weight, ±0.01 g), measured (shell length, ±0.01 mm) and transferred systemically to result in three 2-y-old (n = 16 tanks) or three 3-y-old abalone (n = 16 tanks) per tank. The water temperature was then maintained at the desired treatment temperature (±1 °C) for each experiment.

Table 1
The analysed and calculated biochemical composition of the experimental diets.[1]

Item (dry basis)	Control diet
Moisture content (g kg^{-1})	100.0
Crude protein (g kg^{-1})	306.0
Lipid (g kg^{-1})	45.0
Crude carbohydrates (g kg^{-1})	518.7
Ash (g kg^{-1})	62.0
Gross energy (MJ kg^{-1})	15.2
NFE (g kg^{-1})[2]	587.0
Chromic oxide (g kg^{-1})	0.0
Ballotini balls (g kg^{-1})	0.0

[1] Diet mash was provided by Eyre Peninsula Aquafeeds (Lonsdale, South Australia, Australia).
[2] NFE = nitrogen-free extract was calculated by difference = 100% − (crude protein % + total fat % + ash %).

Following stocking into the experimental tanks and prior to commencement of the GIE experiments abalone were fed the control diet at 4% bw d^{-1} for a period of 5 days. Feeding was at 7 pm daily (beginning of dark period) and uneaten food was collected and tanks were cleaned at 7 am the following day. Uneaten feed was collected by sieving the entire tank contents through a fine mesh as per Stone et al. (2013). The wet, uneaten feed was weighed daily and stored frozen at −20 °C. The wet uneaten feed was then dried in an oven at 105 °C for 16 h to obtain the dry weights. The proportion of uneaten feed that was lost to leaching and through the collection net without animals in the tank was also determined over 12 h, and used to calculate the corrected apparent feed intake per tank. Apparent feed intake was calculated by subtracting the uneaten feed (dry weight) and the amount lost due to leaching (dry weight) from the total amount of feed delivered to each tank and then we converted these values to an as fed basis using the original moisture content of the diets.

2.5. Experimental series 1: determination of gastrointestinal evacuation time using the repeated sampling faecal collection method

In brief, the GIE time in this study was initiated when the control diet was replaced by the marker diet. Faeces were then collected at set time intervals until the marker diet was no longer excreted, the times at which the green coloured faeces appeared, which corresponded to the marker diet, were then used to assess GIE times. In this study tanks were repeatedly sampled so that all tanks were sampled at each collection time.

On the day of the commencement of each repeated sampling GIE experiment abalone were fed one meal (4% bw d^{-1}) of the marker diet at 7 pm. Feeding on the days following the marker diet returned to as previously described with the control diet. Faeces were then collected at set collection times until the green marker diet (green faeces) was no longer voided and only white faecal material, originating from the control diet was collected. This resulted in collection times, post marker diet feeding, of: 3, 6, 9, 12, 18, 24, 30, 36, 42 and 48 h at 26 °C; 3, 6, 9, 12, 18, 24, 30, 36, 42, 48 and 54 h at 22 °C; and 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, 60 and 66 h at both 18 and 14 °C. Each of the 16 replicate tanks for each animal size were sampled at each collection time. At each sampling time faecal pellets were collected using a clear 3 mL pipette and transferred, depending on colour, into one of three pre-weighed Eppendorf tubes (tube 1, white; tube 2, a mixture of white and green; and tube 3, green). During the day (light) period, faecal colour was assessed under white light. During the night (dark) periods, faecal material was collected under red lighting and colour was assessed in the pipette over a white light source contained in a light-proof housing.

In order to obtain a standardised wet weight of the faecal material, immediately following collection and classification, tubes were centrifuged for 30 s at 1000 rpm (G = 59) at 21 °C (model Hettich Zentrifugen Universal 32 R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Baden-Württemberg, Germany) to concentrate the faecal material at the bottom of the tube, without over compacting it. The supernatant water in each tube was then removed using a pipette, so that only a thin film of water covering the faeces remained. Tubes containing the remaining faecal material were then immediately weighed to four decimal places (model Mettler AE 240, Mettler-Toledo Ltd., Melbourne, Victoria, Australia). Each tube was then stored at −20 °C until it was freeze dried (Labconco FreeZone ® 18 L dry system model 7755030, Labcaonco, Kansas, MO, USA) for 2 days at −80 °C, and reweighed for the determination of dry weight and moisture content. The dried faecal material was also later assessed using radiography analysis.

After the faecal collection period for each temperature experiment the average start and end GIE times were then determined. The start time (hours) was defined as the first time the marker diet was excreted. The end GIE time (hours) was defined as the last time the marker diet was excreted per tank prior to only the control diet being excreted. The duration of faecal excretion was determined by subtracting the start time from the end time of the excretion of the marker diet. The percent evacuated was then modelled over time, assessed by the following calculation;

Percent evacuated (at time x)
= sum of faeces at time x (hours)/total amount of faeces collected per tank.

2.6. Experimental series 2: determination of gastrointestinal evacuation time using the single sample faecal collection method

In brief, this study employed similar methods to those previously used. However, as opposed to the previous study, the replicate tanks this study were only sampled once so that all replicate tanks were not sampled at each sample time point.

Immediately following the conclusion of each repeated sampling experiment (Section 2.5), abalone were fed the control diet for a further 3 days and then fed the marker diet at 7 pm and sampled at the following predetermined intervals, which were established after examining data from each of the previous experiments, resulting in collection times of: 6, 9, 12, 18, 24, 30, 36 and 48 h at 26 and 22 °C; and 9, 12, 18, 24, 30, 42, 48 and 60 h at 18 and 14 °C. Subsequent feeding and faecal material collection, classification, drying and storage used the same methods as described for Experiment 1 (Section 2.5). However, faeces were collected once only from two tanks per animal size at each sampling time as opposed to the repeated sampling methods, in which all tanks were sampled at each collection time. In addition, at each sampling time the abalone from the respective tanks were harvested, weighed and measured and snap frozen in liquid nitrogen. The abalone were then stored at −20 °C until undergoing radiography analysis.

2.6.1. Assessment of gastrointestinal evacuation time and feed intake from abalone using Ballotini balls and radiography

Frozen, shucked abalone were pinned, foot side down, on foam boards and X-rayed using a portable diagnostic X-ray unit (model PMX-20BT, United Radiology Systems, Inc., Deerfield, Illinois, USA) suspended 455 mm directly above. Exposure time was 1 s at 40 kV and 0.4 mAs. Images were attained using a portable Empower Wireless 1417 DR system (Vetel Diagnostics, San Luis Obispo, CA, USA) and Metron software (Metron-DVM Version 6.06). The Ballotini balls in the X-ray images were readily identifiable as white dots. Images were examined in order to determine: 1) if the abalone ingested the Ballotini balls in the diet; 2) if the Ballotini balls could be counted in the digestive tract and related back to the feed intake of the animals; and 3) if the Ballotini balls were absent from the animals after the marker diet had been fully excreted (based on visual observations).

In order to relate the number of Ballotini balls to feed intake the average number of balls counted for each abalone age class and each temperature at 12 h post marker feed in X-radiographs were used. As the average weight of each ball was 0.4 mg and the inclusion of the Ballotini balls in the marker diet was 0.75 g 100 g $diet^{-1}$ (as fed), the predicted number of Ballotini balls consumed for each age class at each temperature was calculated by the following equation:

$$\text{Predicted number of Ballotini balls} = \text{Average feed intake(as fed)} \times (\text{grams per abalone}) \times 0.75\ \text{g}\ 100\ \text{g}\ \text{diet}^{-1}/0.4\ \text{mg}.$$

2.6.2. Assessment of gastrointestinal evacuation using faecal samples, Ballotini balls and radiography

Dried faecal samples were X-rayed using the same methods as described for whole abalone in Section 2.6.1, with the exception that samples were X-rayed while in the Eppendorf tubes. Images were examined

in order to determine if the Ballotini balls were excreted at the same rate as the marker in the faecal material (based on visual observations).

2.7. Digestibility analysis

In order to obtain a sufficient quantity of green faeces for the direct determination of apparent digestibility coefficient (ADC), duplicate samples were prepared from Experiment 1 by pooling samples from 8 replicate tanks at each temperature for each animal size. There was sufficient faecal material to create duplicate samples for 26, 22 and 18 °C experiments for both age classes. However, due to insufficient quantities of faecal material only one pooled sample was obtained from 16 tanks for each age class at 14 °C. Dietary ADC for dry matter, protein and energy were estimated using the direct method, and the following equations modified from Montaño-Vargas et al. (2002) and De la Noue and Choubert (1986):

$$\text{Dietary dry matter ADC}(\%) = (\text{feed intake–faeces})/\text{feed intake} \times 100$$

where dry feed intake is in units of mg abalone^{-1} d^{-1} and faeces corresponds to the total dry weight of green faeces recovered (mg abalone^{-1}).

Dietary apparent protein or energy ADC (%) = (nutrient in feed − nutrient in faeces) / nutrient in feed x 100.

2.8. Water quality

Water temperature and dissolved oxygen (mg L^{-1} and % saturation) were measured daily using a Handygamma dissolved oxygen meter (Oxyguard Handy Polaris 2 oxygen probe and meter, Oxyguard International A/S., Birkerod, Denmark), calibrated for oxygen daily in 100% water saturated air. The pH was measured daily using a pH meter (Oakton pHtestr 20; Oakton Instruments., Vernon Hills, IL, USA). Salinity (g L^{-1}) was determined every three days using a portable salinity refractometer (model RF20; Extech Instruments., Nashua, NH, USA).

Water quality was measured and maintained at levels appropriate for optimal health of abalone throughout the experimental period (Hutchinson and Vandepeer, 2006). The average water temperatures were 13.8 ± 0.56 (13.0–14.9), 18.3 ± 0.17 (18.0–18.6), 21.6 ± 0.35 (21.0–22.4), 25.7 ± 0.46 (25.0–26.6) for the 14, 18, 22 and 26 °C experiments respectively. The dissolved oxygen ranged from 85–105% and 6.9–7.4 mg L^{-1}, pH ranged from 8.1–8.3 and the salinity ranged from 35–37 g L^{-1}.

2.9. Statistical analysis

All data was log transformed to conform to Levene's test for equality of variance and Shapiro–Wilk test of normality. An analysis of covariance (ANCOVA) was used to assess if the weight of the abalone between experiments confounded the effects of temperature for results examined. This was assessed by using the temperature was as a fixed factor and initial weight of the abalone as a covariate. The initial weight of the abalone was not found to have a significant effect on results ($P > 0.05$). Using age as a variable was therefore deemed appropriate. Data was analysed using two-factor analysis of variance (ANOVA), with age class as the first factor and water temperature as the second factor. When significant interactions were observed, the data for all temperatures within each age class were analysed using one-factor ANOVA. The Student Newman–Keuls (SNK) test was used to identify significant differences among multiple treatment means. Where samples sizes were unequal a Games Howell post hoc test was used in conjunction with an SNK test to assess to account for the unequal samples sizes. In all cases where a significant difference was recorded using the SNK case a significant difference was also reported for the Games Howell test. Presenting results from the SNK test was therefore deemed appropriate. Linear regression analysis was conducted on GIE end time of each age class in order to create a model to estimate GIE time. A significance level of $P < 0.05$ was used for all statistical tests. All statistical analyses were done using IBM SPSS, version 21 for Windows (IBM SPSS Inc., Chicago, IL, USA). All linear analyses and graphical data were prepared using Sigmaplot, version 12 (Systat Software Inc., San Jose, CA, USA). All values are presented as mean ± standard error (SE) of the mean unless otherwise stated.

3. Results

3.1. General observations

Animals exhibited normal signs of feeding behaviour throughout the study and no gross symptoms of disease or mortalities were observed. Faecal material from the control diet was readily identifiable as a white colour and the faecal material from the marker diet was a medium green colouration. Mixing between the two diets was evident through the appearance of white/green faecal pellets. Two variations of white/green pellets were observed: 1) half and half i.e. one half of pellet was white and the other green; or 2) pellets were coloured either green with white horizontal stripe through the middle of the faecal pellet or white with a green horizontal stripe. Generally, with the second type of faecal pellets, the green with a white stripe was initially observed as the marker diet was initially voided and the white with a green stripe appeared as marker diet began reaching the end of evacuation. Only two out of three types of faeces described for *Haliotis rubra* by Wee et al. (1992) and *H. midae* by Shipton and Britz (2001) were observed. Two types observed were discrete whole pellets and long, thin strings.

3.2. Feed and nutrient intake

There was a significant effect of water temperature ($P < 0.001$; 26 > 22 > 18 > 14 °C) and age class ($P < 0.001$, 2-y-old < 3-y-old) on the feed intake (as fed) of abalone (g kg abalone^{-1}), and there was no significant interaction ($P > 0.05$) between water temperature and age class (Table 2). On a gram per kg of abalone basis, 2-y-old abalone consumed 20% more feed than 3-y-old abalone, and as temperature decreased from 26 °C to 14 °C, feed consumption decreased.

3.3. Experimental series 1: determination of gastrointestinal transit time using the repeated sampling method

Gastrointestinal evacuation rates of the marker diet were evaluated for 2-y-old and 3-y-old abalone at 14, 18, 22 and 26 °C and are presented in Figs. 1A and 2A. There were significant effects of water temperature and age class on the GIE start time ($P < 0.001$; $P < 0.001$) and end time ($P < 0.001$; $P < 0.05$) and there were no significant interactions between the two factors for either variable ($P > 0.05$; Table 3). The start and end times were equal for 22 °C and 26 °C and increased as temperature decreased, thereafter (Fig. 1A and 2A; Table 3). Additionally, the start and end times were also significantly later in the 3-y-old than 2-y-old abalone (Fig. 1A and 2A; Table 3). In contrast, there was no significant effect of water temperature or age on the duration of GIE time ($P > 0.05$), and there was no significant interaction between the two factors ($P > 0.05$; Table 3). The duration of GIE time ranged from 22.8 to 27.0 h (Table 3). Regression analysis of GIE end time gave the following equations; $y = 53.0000 - 0.7591x$ ($R^2 = 0.9300$) and $y = 65.1026 - 1.2174x$ ($R^2 = 0.9054$) [where y = end GIE time (h) and x = water temperature (°C)] for 2-y-old and 3-y-old abalone, respectively.

3.3.1. Experimental series 1: observations of feeding behaviour

Throughout sampling periods the behaviour of the abalone was examined in order to determine if the faecal collection methods interfered with the normal feeding behaviour of the animals or caused

Table 2
Feed and nutrient intake of 2-y-old and 3-y-old greenlip abalones.

Temperature (°C)	14		18		22		26		ANOVA[1]					
Age (years)	2	3	2	3	2	3	2	3	Age (a)	Temperature (b)				Interaction
									2 vs 3	14	18	22	26	(a × b)
Feed consumption rate (*as fed*)														
g kg abalone^{-1} d^{-1}	3.6 ± 0.27	3.2 ± 0.18	5.5 ± 0.28	4.2 ± 0.25	10.5 ± 0.36	7.9 ± 0.27	11.8 ± 0.64	9.8 ± 0.34	***>	D	C	B	A	NS

Values are expressed as means ± SE, n = 16.
A, B, C, and D for variables with a significant effect of temperature and no interaction, values without a common uppercase letter are significantly different (A indicates the highest value; $P < 0.05$; two-factor ANOVA; SNK test).
For variables with a significant effect of age ($P < 0.05$), < or > indicates whether the values measured at 2-y-old were less than or greater than that measured for 3-y-old.
[1] NS, non significant; *denotes a significant difference: *** denotes a signifincant difference, $P < 0.001$.

any obvious signs of distress. Extreme care was taken not to touch or disturb the animals during this process. Feeding behaviour carried on as normal and abalone were not observed to suddenly withdraw tentacles or cease movement or feeding activity when the faeces were removed with the pipette. Additionally, behaviour of serial sampled abalone was observed to be comparable to the feeding behaviour of abalone in tanks that were not sampled.

3.4. Experimental series 2: determination of gastrointestinal transit using the single sampling method

Abalone of each age class and at each temperature sampled once only (Table 4; Figs. 1B and 2B) showed similar GIE rate trends to abalone of each age class at each respective temperature that were repeatedly sampled in Experimental series 1 (Figs. 1A and 2A; Table 3).

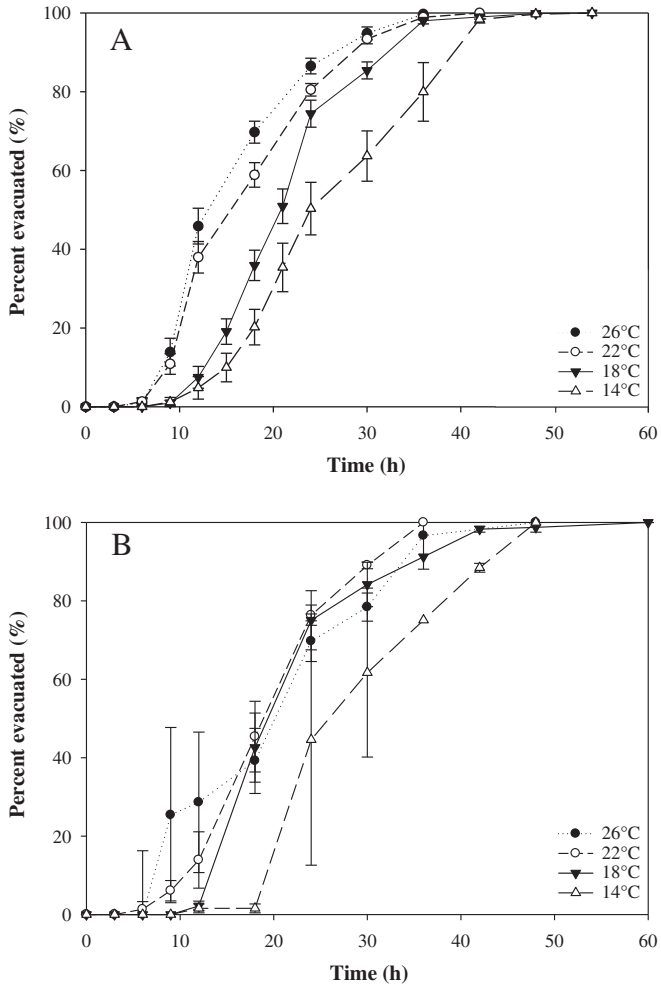

Fig. 1. The gastric evacuation rate in 2-y-old (6.7 g; 37 mm SL) greenlip abalone, *Haliotis laevigata*, based on the cumulative proportion of green faeces collected following the feeding of the marker diet using (A) the repeated sampling method in Experiment 1 (mean ± SE; n = 16 for each sampling time) and (B) the single sampling method in Experiment 2 (mean ± SE, n = 2 for each sampling time).

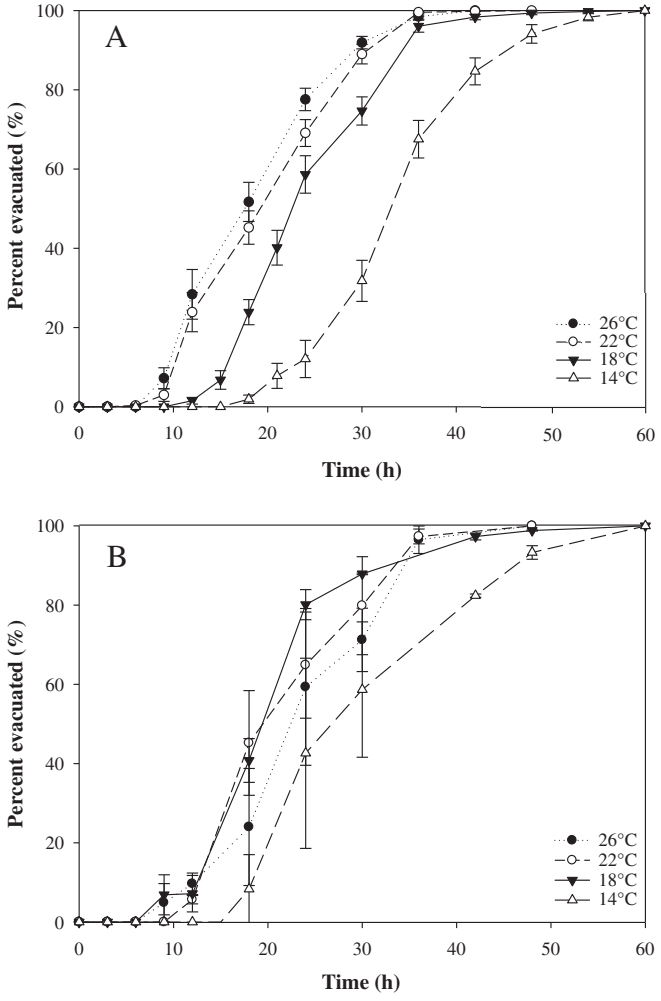

Fig. 2. The gastric evacuation rate in 3-y-old (25.7 g; 56.9 mm SL) greenlip abalone, *Haliotis laevigata*, based on the cumulative proportion of green faeces collected following the feeding of the marker diet using (A) the repeated sampling method in Experiment 1 (mean ± SE; n = 16 for each sampling time) and (B) the single sampling method in Experiment 2 (mean ± SE, n = 2 for each sampling time).

Table 3
Gastrointestinal evacuation time and faecal output from 2-y-old and 3-y-old greenlip abalone repeatedly sampled in Experiment 1.

Temperature (°C)	14		18		22		26		ANOVA[1]					
Age (years)	2	3	2	3	2	3	2	3	Age (a)	Temperature (b)				Interaction
									2 vs 3	14	18	22	26	(a × b)
Gastrointestinal evacuation time (h)[2]														
Start (h)	19.1 ± 2.28	27.0 ± 1.52	13.9 ± 0.85	17.2 ± 1.08	9.9 ± 0.71	12.0 ± 1.02	9.6 ± 0.43	12.6 ± 1.00	***<	A	B	C	C	NS
Duration (h)	24.2 ± 2.38	22.8 ± 2.68	27.0 ± 1.79	24.0 ± 2.25	25.7 ± 1.09	24.7 ± 1.63	23.6 ± 0.96	22.6 ± 2.10	NS	NS				NS
End (h)	41.5 ± 1.58	49.9 ± 1.95	40.9 ± 1.47	41.3 ± 1.96	35.6 ± 0.86	36.7 ± 1.08	33.2 ± 0.80	35.1 ± 1.65	*<	A	B	C	C	NS
Total green faecal output over whole collection period (dry basis)[2]														
g kg abalone^{-1}	0.9 ± 0.13	0.4 ± 0.05	1.8 ± 0.37	0.8 ± 0.12	2.7 ± 0.27	1.5 ± 0.16	2.6 ± 0.17	1.1 ± 0.13	***>	C	B	A	A	NS
Total white faecal output over whole collection period (dry basis)														
g kg abalone^{-1}	2.4 ± 0.27	1.0 ± 0.10	4.2 ± 0.28	2.3 ± 0.23	6.3 ± 0.41	3.8 ± 0.17	4.2 ± 0.42	1.6 ± 0.23	***>	D	B	A	C	NS
Total white/green faecal output over whole collection period (dry basis)														
g kg abalone^{-1}	0.2 ± 0.03	0.1 ± 0.01	0.4 ± 0.08	0.2 ± 0.05	0.4 ± 0.04	0.3 ± 0.03	0.6 ± 0.11	0.2 ± 0.05	***>	B	A	A	A	NS

Values are expressed as means ± SE, n = 16 (unless otherwise indicated).
A, B, C, and D for variables with a significant effect of temperature and no interaction, values without a common uppercase letter are significantly different (A indicates the highest value; $P < 0.05$, two-factor ANOVA; SNK test).
For variables with a significant effect of age ($P < 0.05$), < or > indicates whether the values measured at 2-y-old was less than or greater than that measured for 3-y-old.
[1] NS, non significant; *denotes a significant difference; *, $P < 0.05$; ***, $P < 0.001$.
[2] n < 16 as green faecal material was not produced in all 16 replicate tanks, n = 14 for 2-y-old at 14 °C, n = 15 for 2-y-old at 26 °C, n = 14 for 3-y-old at 26 °C.

Additionally, the variation at each time point was consistently large for singly sampled abalone for each GIE variable compared to that of repeatedly sampled abalone in Experimental series 1 (Figs. 1A and 2A; Table 3). There was no statistical effect of age class ($P > 0.05$) on start time, duration or end time (Table 4) for singly sampled abalone. There were, however, statistically significant effects of temperature on start time, duration and end time of singly sampled abalone ($P < 0.05$) (Table 4), while there were no significant interactions between either factor for start time, duration or end time of singly sampled abalone ($P > 0.05$).

3.5. Experimental series 2: radiography analysis

3.5.1. Radiography analysis of whole abalone

Ballotini balls were readily identifiable as small white dots throughout the digestive tract of abalone in the X-radiograph images (Fig. 3) and could easily be counted. X-radiographs of whole abalone showed that Ballotini balls were present in the digestive tract of the animals longer than the end GIE time, compared to the end GIE time determined in abalone for both age classes and at all temperatures in the repeated sampling (Table 3) and single sampling (Table 4) methods in Experimental series 1.

Based on the actual feed intake rates (Table 2), the predicted number of Ballotini balls versus the observed number of balls obtained in X-radiographs from the abalone at 12 h post marker feed (representative of the entire feeding period) were only similar to expected value for 2-y-old abalone at 22 °C and 18 °C, and 3-y-old abalone at 18 °C. In addition, based off animals collected at 12 h, which is representative of the entire feeding period (n = 2 tank; n = 6 animals), the proportion of animals that ate varied from 0% at 14 °C for 2-y-old abalone to 100% for 3-y-old abalone held at 18 and 22 °C.

3.5.2. Radiography analysis of faecal samples

The Ballotini balls were not excreted by abalone at the same rate as the chromic oxide in the marker diet. Ballotini balls were present in white, white/green and green faeces, and were still present in white faeces after the marker diet had been fully excreted. Additionally, at 14 °C, Ballotini balls were observed in white faecal material before the marker diet started to be excreted for both 2-y-old and 3-y-old abalone.

3.6. Apparent digestibility of diets

There was no significant effect of temperature ($P > 0.05$; Table 5) on dietary dry matter ADC for abalone, but there was a significant effect of age (3-y-old > 2-y-old; $P < 0.05$). There was no significant interaction between the two factors ($P > 0.05$). There was also no significant effect of temperature ($P > 0.05$) or age ($P > 0.05$) on dietary protein ADC for abalone (ranged from 88.7–90.1%), and there was also no significant interaction between the two factors ($P > 0.05$; Table 5). There was also no significant effect of temperature ($P > 0.05$) or age ($P > 0.05$) on dietary

Table 4
Gastrointestinal evacuation time and faecal output from 2-y-old and 3-y-old abalones sampled once only in Experiment 2.

Temperature (°C)	14		18		22		26		ANOVA[1]					
Age (years)	2	3	2	3	2	3	2	3	Age (a)	Temperature (b)				Interaction
									2 vs 3	14	18	22	26	(a × b)
Gastrointestinal evacuation time (h)														
Start (h)	16.5 ± 3.00	19.5 ± 1.50	12.0 ± 3.00	10.5 ± 1.50	7.5 ± 1.50	12.0 ± 0.00	7.5 ± 3.00	10.5 ± 1.50	NS	A	B	B	B	NS
Duration (h)	31.5 ± 4.50	34.5 ± 1.50	39.0 ± 3.00	43.5 ± 1.50	28.5 ± 1.50	27.0 ± 3.00	31.5 ± 1.50	28.5 ± 4.50	NS	B	A	B	B	NS
End (h)	48.0 ± 0.00	54.0 ± 0.00	51.0 ± 3.00	54.0 ± 0.00	36.0 ± 0.00	39.0 ± 3.00	39.0 ± 3.00	39.0 ± 3.00	NS	A	A	B	B	NS

Values are expressed as means ± SE, n = 2.
A, B, C, and D for variables with a significant effect of temperature and no interaction, values without a common uppercase letter are significantly different (A indicates the highest value; $P < 0.05$, two-factor ANOVA; SNK test).
For variables with a significant effect of age ($P < 0.05$), < or > indicates whether the values measured at 2-y-old was less than or greater than that measured for 3-y-old.
[1] NS, non significant.

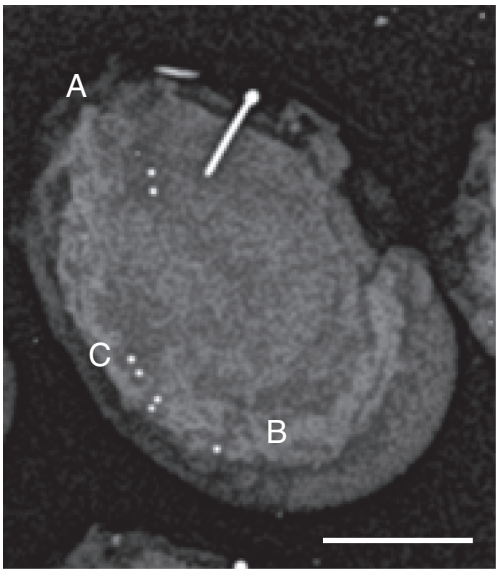

Fig. 3. X-radiograph image displaying x-ray opaque Ballotini balls in the gastrointestinal tract of a 3-y-old abalone at 18 °C, 60 h post-marker feeding at 4% body weight per day (dorsal view). Ballotini balls are identifiable as white dots. The white line in the top right of the abalone is a pin through the lower cephalic region of the abalone. A = cephalic region; B = stomach region; C = anus. Scale bar represents 1 cm.

energy ADC (ranged from 91.4–92.8%) and there was no significant interaction between the two factors ($P > 0.05$; Table 5).

There were significant effects of temperature ($P < 0.001$) and age ($P < 0.001$) on the amount of faecal material produced by abalone [g kg abalone^{-1} day^{-1} (dry)] over a 24 h period (Table 5). There was also a significant interaction between temperature and age for these variables ($P < 0.05$). These interactions may be explained by the relatively reduced production of faecal material at 26 °C compared to 22 °C for 3-y-old abalone. Relative faecal production was highest at 22 °C then declined as temperature either decreased or increased thereafter. More 2-y-old greenlip abalone produced relatively more faeces (g kg abalone^{-1}) in 24 h than 3-y-old greenlip abalone (Table 5).

4. Discussion

A number of studies have previously focussed on the gastric (stomach) evacuation time in different fish species (Andersen, 2001; Andersen and Beyer, 2005, 2007; Riche et al., 2004) and on the abalone species *H. midae* (Britz et al., 1996; Day and Cook, 1995). However, little consideration has been given to the GIE time. The present study, therefore, aimed to examine GIE time in greenlip abalone in relation to water temperature and animal age utilising multiple methods.

Based on the present study, the repeated sampling method used in Experiment 1 was the most effective method to determine GIE time and rate in greenlip abalone. The trends in GIE times and rates were similar between the repeated versus single sampled abalone. Based on visual observations, there was minimal impact on the feeding behaviour and health of the abalone in response to repeated sampling and no mortalities were observed. This method also allowed for a direct estimation of dietary ADCs. In addition, compared to previous total collection methods used in abalone (Montaño-Vargas et al., 2002; Sales, 2001; Sales and Britz, 2001; Shipton and Britz, 2001), this study did not include a fasting period prior to faecal collection. Fasting periods have previously been included in studies in order to be certain that the faeces collected pertained to the last meal eaten. By using a coloured marker diet, the method employed in this study negates the need for fasting, and hence, may be more reliable as the extended fasting period may alter the gastric evacuation rate, and also include a contribution of nutrients from the catabolism of digestive tract tissue, which combined would lead to an underestimate of digestibility (Sales, 2001). The use of single sample sampling as a method for the determination of the GIE time, and rate, was ineffective due, in part, to the low level of replication used. This was apparent by the large variability between standard errors of treatment means over time (Figs. 1B and 2B). The single sampling approach may have potential application beyond that trialled in this study; however, a larger sample size is recommended for determining GIE time and rate in abalone species if this method is to be used, and the lack of results using this method should not be viewed as a limitation of this method. The lower level of resources required for direct sampling shows that this method is the more practical method for analysing GIE time.

In contrast to radiography studies used effectively on fish to determine gut transit times or GIE times (Jobling, 1987; Jobling et al., 1977; Tablot and Higgins, 1983) and feed intake (Azaza et al., 2010; Hossain et al., 1998; Palson et al., 1992), the use of radiography as a method to determine both GIE time and feed intake in greenlip abalone in this study was not effective. The reason radiography was not effective in greenlip abalone was most likely due to differential rate of movement of Ballotini balls in comparison to feed through the gastrointestinal tract. Ballotini balls used in the current study were 0.5 mm, which were larger than pre-ingested feed particles, which were approximately

Table 5
Total faecal output and apparent dietary digestibility coefficients (ADC) from 2-y-old and 3-y-old abalones from Experiment 1.

Temperature (°C)	14		18		22		26		ANOVA[1]					
Age (years)	2	3	2	3	2	3	2	3	Age (a)	Temperature (b)				Interaction
									2 vs 3	14	18	22	26	(a × b)
Total faecal output over a 24 h period (dry basis) g^{-1} kg abalone^{-1} day^{-1}	1.0 ± 0.15^{c}	0.4 ± 0.04^{w}	1.8 ± 0.35^{b}	0.9 ± 0.09^{x}	3.6 ± 0.17^{a}	2.4 ± 0.16^{z}	4.0 ± 0.33^{a}	1.4 ± 0.17^{y}	***>	***				**
Apparent diet digestibility														
Dry matter (%)[2,3]	72.7 ± 3.95	88.6 ± 1.73	73.1 ± 2.43	82.6 ± 2.05	74.9 ± 2.24	80.9 ± 1.91	72.4 ± 2.29	89.4 ± 1.17	***<	NS				NS
Protein (%)[4,5]	88.7	90.1	89.2 ± 0.69	89.4 ± 0.97	90.0 ± 0.19	89.0 ± 1.00	89.2 ± 0.31	89.1 ± 0.57	***<	NS				NS
Energy (%)[4,5]	92.8	92.7	92.0 ± 0.00	91.5 ± 0.18	91.7 ± 0.44	91.4 ± 0.01	91.7 ± 0.05	91.9 ± 0.31	NS		NS			NS

Values are expressed as means ± SE, n = 16 (unless otherwise indicated).
For variables with a significant effect of age ($P < 0.05$), < or > indicates whether the values measured at 2-y-old was less than or greater than that measured for 3-y-old.
For variables with a significant interaction, differences in temperature are compared within age class ($P < 0.05$) (one-way ANOVA; SNK test), $^{a, b, c, d}$ for 2-y-old and $^{w, x, y, z}$ for 3-y-old values without a common superscript are different (^{a} and ^{z} indicate the highest value for 2-y-old and 3-y-old respectively; $P < 0.05$).
[1] NS, non significant; *denotes a significant difference; **, $P < 0.01$; ***, $P < 0.001$.
[2] n < 16 as green faecal material was not produced in all 16 replicate tanks, n = 14 for 2-y-old at 14 °C, n = 15 for 2-y-old at 26 °C, n = 14 for 3-y-old at 26 °C.
[3] Apparent dry matter digestibility was calculated using the direct method. Refer to methods for calculation.
[4] n = 1 for 14 °C treatments and n = 2 for 18, 22 and 26 °C treatments.
[5] As only one sample was obtained for 14 °C treatments, values were not included in the two-factor ANOVA analysis.

≤0.3 mm, and larger than endogenous particles (fragmentation spherules) in the digestive tract which have been observed to be approximately 5–10 μm, or 100–200 times smaller than the Ballotini balls used (Harris et al., 1998). In regard to feed intake, radiography may have not been useful in greenlip abalone due to the nature in which abalone feed. In contrast to fish, abalone do not ingest pellets whole, instead they graze on their feed using their radula, and as a consequence, all the Ballotini balls in a ration of feed may not have been ingested. Nevertheless, radiography may have its uses in terms of qualitative, rather than quantitative data, in order to determine the proportion of animals that eat per day.

Feed intake in this study increased as temperature increased from 14 to 26 °C, and was higher in 2-y-old abalone than 3-y-old abalone on a grams per kg basis. The increase in feed intake from 22 °C to 26 °C in the present study is unexpected, as the animals are near their thermal maximum of 27.5 °C (Gilroy and Edwards, 1998), hence generally it would be expected that the feed intake at 26 °C would be lower than at 22 °C. For example, both Lange et al. (2014) and Stone et al. (2014) reported an approximate 50% reduction in feed intake by 2 and 3-year-old greenlip abalone between 22 to 26 °C when fed the same diet as used in the current study. Abalone in the present study were likely able to cope at 26 °C better than those in the Lange et al. (2014) and Stone et al. (2014) studies, due to lower stocking densities in combination with better water quality.

The GIE end time of a single meal by greenlip abalone decreased with increasing water temperature, and was faster in 2-y-olds than 3-y-olds (Table 3). The maximum GIE time of 60 h, at 18 °C for 3-y-old greenlip abalone, was shorter than that observed in *H. midae* at 18 °C (Shipton and Britz, 2001). Faeces were voided by *H. midae* up to 96 h post feeding, while the majority of faeces were voided between 12 and 60 h after feeding (Shipton and Britz, 2001). Differences in maximum GIE time are likely due to differences between species, diet and animal size. The *H. midae* in the study of Shipton and Britz (2001) weighed 72.4 g and were larger than 3-y-old greenlip abalone (25.7 g) used in this study, which fits with the pattern shown in this study of increasing GIE time with increasing age. Most interestingly, while the initial appearance of faecal material from greenlip abalone in the present study was affected by temperature and age class, the duration of the faecal voiding period was not. This suggests that the differences in GIE times are not caused by a difference in feed passage rate through the intestinal tract, but are due to other factors. Enzymatic activity has been demonstrated in other poikilothermic animals to be lower with decreasing water temperatures (Bowyer et al., 2012; Miegel et al., 2010). Similarly, age or animal size has also been demonstrated to affect enzyme activity. Generally, enzyme activity decreases with increasing age or animal size (Kuz'mina, 1996; Wang et al., 2006). It is likely that the abalone are compensating for the lower enzyme activity by reducing food intake and possibly retaining feed in the body for longer, as observed in this study. Increasing meal size, or increasing the amount of meals an animal ingests daily, has been demonstrated to increase GIE times in other poikilothermic animals (Flowerdew and Grove, 1979; Lee et al., 2000; McGaw and Curtis, 2013; Riche et al., 2004), however, in the present study, increasing feed intake that occurred with increasing temperatures had no significant effect on the duration on faecal excretion. Hence, these animals appear to adapt to lower enzymatic activity by consuming less food, commencing their faecal excretion later and completing their voiding of faeces later, resulting in no change to GIE duration. This is an unusual response that has not been observed in any literature that we can find.

GIE time data from this study may be used on-farm for greenlip abalone of comparable sizes and at similar water temperatures for the establishment of purging times, for the voiding of faeces and sand, which are necessary procedures prior to transportation and consumption. Few purging times are provided in literature for abalone species, although those that include purging times prior to experimentation, involving transport, report a purging period of 2–3 days (Bubner et al., 2009; Sales, 2001). Based on this study the recommended purging times would be any time after the maximum GIE time was recorded in relation to both temperature and age (Figs. 1A and 2A).

The increased GIE time at lower water temperatures in 3-y-old greenlip abalone suggest that an investigation into feeding frequency would be worthwhile, as GIE time is partially linked to the return of appetite (Riche et al., 2004). Little research has been conducted on feeding frequency in abalone, particularly in relation to water temperature and animal age. Greenlip abalone (1 g), held at 13.9–18.8 °C over 2 months, fed every 2 or 4 days had SGRs 41 and 52% higher, respectively, compared to daily feeding (Maguire, 1995). Conversely, Britz et al. (1996) recommended a feeding frequency of once per day for *H. midae* as the gastric evacuation time of *H. midae* was 18–24 h. Feeding frequency has been extensively studied in other cultured aquatic species, with research mainly focused on increasing growth and feed efficiency (Andrews and Page, 1975; Biswas et al., 2010; Booth et al., 2008; Riche et al., 2004). From the perspective of an abalone farmer the ability to feed on alternate days, particularly during periods of suboptimal water temperatures, without compromising growth rates and health would be a significant outcome, as feeds and feed delivery represent major economic costs in inland abalone aquaculture.

Using the direct method for determination of dietary ADC was possible during this study. Three year old greenlip abalone exhibited higher dry matter ADC (81–89%) than 2-year-old abalone (72–75%), whereas temperature was not found to have a significant effect on dry matter ADC by greenlip abalone (Table 5). In contrast, the dry matter ADC of a semi-purified diet by 70 mm *H. midae* was affected by water temperature; dry matter ADC values were reduced as water temperatures decreased from of 22 °C to 15 °C (Dixon, 1992). In regards to protein and energy ADC, there was no significant effect of temperature or age observed in greenlip abalone in the present study. However, due to low replication the 14 °C treatment was excluded from statistical analysis (Table 5). Protein ADC ranged from 88.7–90.1% while energy ADC ranged from 91.4–92.8% (Table 5).

The variation in age affecting dry matter ADC but not protein and energy ADC is unusual considering the protein and energy ADCs do not differ due to temperature or age and therefore dry matter ADC should not either. The causal effect for the differences in the present study may have been in relation to the rate of breakdown of the faecal pellets by 2-y-old abalone. Two year old abalone produced smaller faecal pellets than the 3-y-old abalone, hence, as the surface area to volume ratio would have been higher, faeces produced by the younger abalone would have been more subject to breakdown and the pellet mass may have decreased at a greater rate that faecal material produced by 3-y-old abalone.

In order to further validate the use of the direct methods in determining dry matter ADC and nutrient ADCs in greenlip abalone, comparisons are required with the indirect methods. Montaño-Vargas et al. (2002) compared the direct method using total collection to the indirect method using acid insoluble ash and chromic oxide as markers in determining nutrient digestibility in *Haliotis fulgens*. The results showed that dry matter ADC obtained via total collection, and with the acid insoluble ash marker, were similar (80.5 ± 2.73% vs. 84.4 ± 5.21%, respectively); whereas result obtained using the chromic oxide marker were significantly lower (61.7 ± 2.0%). To improve the direct method used in this study, ideally the feed intake that the faecal output is contrasted to should be compared only to the feed intake specific to the day the marker diet was fed. This would ensure that any variation in the average fed intake, i.e. eating less or more on a given day, would not affect results. The breakdown of faecal material over time should also be assessed to use as a correction factor.

In conclusion, the use of a coloured inert marker, chromic oxide, in combination with repeated sampling and the total faecal collection technique in a time course study is an appropriate method in determining the GIE time for greenlip abalone. The GIE time was significantly affected by both water temperature and age class. Gastrointestinal

evacuation time was longer as age increased and water temperature decreased. As there was no significant effect on the duration in which faeces was voided due to either temperature or age, it is suggested that the difference in GIE time is due to factors other than the transit of feed through the intestine of the animal. Using the direct methods, dry matter ADC was higher in 3-y-old greenlip abalone in comparison to 2-y-old abalone, and there was no significant effect of water temperature. However, there was no significant effect was recorded in protein or energy ADCs in relation to age or water temperature. The variation in age affecting dry matter ADC but not protein or energy ADCs suggest that refinement to using the direct methods of assessing ADCs is necessary. Results suggest that due to the extended GIE time, examining feed frequency would be a beneficial endeavour to improve feed efficiency and growth. Examining feeding frequency is an area of research which has been neglected in abalone species, even though it has the potential to significantly reduce feed and feed associated cost. The results of this study can be applied on-farm to purging feed and sand from abalone prior to harvesting.

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