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Dietary intervention improves the survival of cultured greenlip abalone (*Haliotis laevigata* Donovan) at high water temperature

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ABSTRACT

Summer mortality (SM), a disease caused by an interaction between biotic and abiotic environmental factors at high water temperatures (>22 °C), impacts health, growth and mortality (up to 50%) of larger (≥60 mm) cultured abalone in southern Australia. We aimed to determine if dietary intervention could alleviate mortality demonstrated by abalone at high water temperatures. As this issue is most relevant to farm production, we aimed to demonstrate that different mortality patterns are evident for 2- and 3-year-old greenlip abalone (*Haliotis laevigata* Donovan), irrespective of reproductive state, and to provide potential, practical solutions to this issue. Growth rate, feed intake and haemolymph variables were measured. To test if dietary intervention could minimise mortality at high water temperatures, we selected a commercial diet routinely fed on-farm when SM occurred, as a negative survival control, and live macroalgae (*Ulva lactuca* Linnaeus) as a positive survival control. In Experiment 1, 30 mm 2-year-old and 70 mm 3-year-old abalone were subjected to water temperatures of 18, 22 and 26 °C for 36 days. In Experiment 2, 60 mm 3-year-olds were subjected to 22 and 26 °C for 38 days. In Experiment 1, survival was >95% for all treatments at 18 and 22 °C ($P > 0.05$); whereas, survival was significantly reduced by 35% in 3-year-olds fed the commercial diet at 26 °C compared to all other treatments ($P < 0.05$). In Experiment 2, there was no mortality at 22 °C. At 26 °C, survival was significantly reduced by 50% ($P < 0.05$) for the commercial diet, whereas, survival was >97% for the *U. lactuca* diet. We demonstrated that dietary intervention reduced mortality in larger abalone at 26 °C. We also demonstrated a pattern of mortality in response to high water temperatures that differed for age classes. This information is invaluable for further systematic research to alleviate on-farm abalone mortality associated with high summer water temperatures, particularly in the areas of nutritional or therapeutic intervention.

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1. Introduction

Greenlip abalone (*Haliotis laevigata* Donovan) in southern Australia are predominantly cultured in land-based systems in water temperatures that can fluctuate from 10 °C in winter to 25 °C in summer (Stone et al., 2013). Abalone cultured in southern Australia spend a large period of the summer months in water temperatures above their preferred optimum. Optimal water temperatures for growth in this species are size- and strain-dependent and have been recently reported to be 22 °C for 25 mm shell length (SL) animals originating from South Australian waters (Stone et al., 2013), whereas 18.9 °C has been reported to be the preferred temperature for 82 mm SL animals originating from the cooler waters of Tasmania (Gilroy and Edwards, 1998).

When water temperatures exceed 22 °C, increased health problems and significant levels of mortality occur on abalone farms in southern Australia. Small (<60 mm SL) abalone typically show little mortality during summer water temperatures. In contrast, mortality patterns with larger (>60 mm SL) 3-year-old stock during these events are typically sporadic between culture units and have been reported to range from 15 to 50% (Dang et al., 2011a; Vandepeer, 2006), although there is no peer-reviewed published information quantifying these levels. Discussions with abalone farm managers indicate that these figures are an accurate representation of the mortality patterns each summer (Australian Abalone Growers' Association, pers. comm.). In Australia, this mortality event observed is referred to as "summer mortality" (SM) (Dang et al., 2011a; Handlinger et al., 2005; Vandepeer, 2006). A similar condition, "summer immune depression", associated with high summer water temperatures, spawning processes, low immune status,

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and an increased susceptibility to *Vibrio* spp. infections (Nicolas et al., 2002; Travers et al., 2008, 2009a) has been reported in cultured and wild abalone (*H. tuberculata*) in France.

Currently the aetiology of SM in abalone is hypothesised to be related to a complex interaction between the physiology and metabolism of abalone and a range of abiotic and biotic environmental factors at elevated summer water temperatures (Dang et al., 2011a,b; Handlinger et al., 2005; Vandepeer, 2006), contributing to mortalities caused by bacterial infection (Travers et al., 2009a,b). Additionally, as the condition is prevalent in larger 3-year-old stock, maturation and spawning processes may also be involved (Travers et al., 2009a), however, handling stresses are also implicated (Cardinaud et al., 2014). Nutritional factors have also been implicated in the aetiology of SM (Dang et al., 2011b; Vandepeer, 2006).

In Australia, abalone cultured in land-based systems are fed formulated compounded diets composed predominantly of terrestrial plant, and to a much lesser extent, marine ingredients. In contrast, in their natural environment, larger abalone have a distinct dietary preference for macroalgae, particularly species of the red (*Gracilaria* spp.) and green (*Ulva* spp.) taxa (Shepherd, 1973). Macroalgae possess a range of attributes that are beneficial to the environment, and potentially to the organisms consuming them. Benefits include the removal of nitrogenous wastes and carbon dioxide from the environment to produce oxygen, they also contain a range of bioactive compounds that exhibit antimicrobial and antioxidant properties (Abirami and Kowsalya, 2011; Silva et al., 2013; Tierney et al., 2010), that when consumed may enhance the immune response of the abalone (Dang et al., 2011b).

The aim of this study was to determine if dietary intervention could alleviate mortality demonstrated by abalone at high water temperatures. As this issue is most relevant to farm production, we aimed to demonstrate that different mortality patterns are evident for 2- and 3-year-old abalone, irrespective of reproductive state, and to provide potential, practical solutions to this issue.

2. Methods

2.1. Experimental animals

Greenlip abalone, not previously used for any other experiments, were purchased from South Australian Mariculture at Boston Point, Port Lincoln, South Australia. Upon arrival at the SARDI SAASC the abalone were transferred to flow through 5000-L cylindro-conical tank seawater systems, held at ambient photoperiod and water temperatures, and fed a 5 mm commercial abalone diet [Eyre Peninsula Aquafeed Pty Ltd (EPA), Lonsdale, South Australia, Australia] prior to stocking into the experiments. Experiment 1 used 2-year-old [spawned September 2010; initial weight, 5.95 ± 0.01 g abalone^{-1}; shell length (SL), 38.54 ± 0.01 mm; condition factor (CF), 0.60 ± 0.01] and 3-year-old abalone (spawned September 2009; initial weight, 36.27 ± 0.02 g abalone^{-1}; SL, 63.23 ± 0.03 mm; CF, 0.83 ± 0.01). Experiment 2 used 3-year-old abalone (spawned September 2010; initial weight, 26.83 ± 0.63 g abalone^{-1}; SL, 57.07 ± 0.42 mm; CF, 0.81 ± 0.01).

2.2. Experimental design

In this study the interactive effects of water temperatures, abalone sizes, and different diet types on the survival of greenlip abalone were investigated. Both 2-year-old and 3-year-old abalone at 18, 22 and 26 °C fed the EPA 5 mm commercial abalone diet (negative control diet) or the live *Ulva lactuca* Linnaeus (positive control diet) were trialled for 36 days in Experiment 1. To confirm our findings and to provide more information about feed intake and water quality changes, Experiment 2 was run at 22 and 26 °C with only 3-year-old abalone fed the commercial abalone diet (negative control diet) or the *U. lactuca* (positive control diet) for 38 days.

2.3. Experimental system

The temperature (20 ± 1 °C) and photoperiod [12 h low intensity fluorescent lighting at 3.4 lx (equates to dark limit of civil twilight under a clear sky): 12 h dark] controlled experimental facility consisted of identical temperature controlled salt water systems (Exp. 1 used three systems; Exp. 2 used two systems) described in Stone et al. (2013) supplied with 30 μm sand-filtered, UV treated seawater. Each system supplied sixteen (Exp. 1) or six (Exp. 2) 12.5-L blue plastic culture units (Nally IH305, Viscount Plastics Pty Ltd.; length, 39.2 cm; width, 28.8 cm; 11.0 cm depth; bottom surface area of 1129 cm^2). The culture units were each provided with temperature controlled flow-through water from the reservoir by gravity feed at a rate of 300 mL min^{-1}. Water level was set at 5 cm in each culture unit using a standpipe with a mesh screen (0.8 mm nominal mesh size) on the outlet to retain uneaten food.

2.4. Experimental stocking

For Experiment 1, ten 2-year-old, or ten 3-year-old abalone were weighed, measured and stocked, using systematic interspersion, into each of the four replicate culture units per treatment combination. For Experiment 2, twelve 3-year-old abalone were stocked into each of the three replicate culture units per treatment. A one week acclimation period was used to slowly raise the water temperature (~1 °C day^{-1}) to the desired temperatures. For each experiment, dead abalone were recorded, measured, weighed and in an attempt to keep stocking densities equal, replaced with tagged abalone of a similar weight and size that had been held at the same treatment water temperature and fed their respective diets.

2.5. Diets and feeding

The nutrient composition of the diets used in Experiments 1 and 2 is presented in Table 1. *U. lactuca* were collected from the Outer Harbour area of Gulf St Vincent, South Australia and cultured in sand-filtered seawater supplied with aeration in 4000-L parabolic tanks under ambient photoperiod and temperature at SARDI SAASC. In Experiment 1 natural, non-enriched *U. lactuca* was used, whereas, in Experiment 2 the *U. lactuca* was nitrogen enriched using an altered Guillard's f/2 nutrient medium (Guillard, 1975; Guillard and Ryther, 1962) [sodium nitrate component substituted with ammonium chloride (4.75%)] prior to feeding. Previous work in our laboratory has shown that enriched *U. lactuca* provides a superior nutritional source to non-enriched *U. lactuca*, and this represents a refinement of our experimental design. The same 5 mm EPA commercial negative control diet was used in both experiments. Feed rates for the commercial diet were 4.0% body weight day^{-1} (% bw d^{-1}) for the 2-year-olds and up to 1.2% bw d^{-1} for the 3-year-olds in Experiments 1 and 2. *U. lactuca* feed rates were 6.0% bw d^{-1} for Experiment 1 and 2.5% bw d^{-1} for Experiment 2. Rations were based on stocking biomass and adjusted on mortality weight, and were in excess of the animal's daily intake. Feeding was carried out at 4:00 pm daily until the end of each trial. Cleaning and collection of food waste occurred at 8:30 am daily and were done by sieving the entire tank contents through a fine mesh. The wet uneaten feed was weighed and stored frozen at −20 °C. Uneaten commercial diet was dried in an oven at 105 °C for 16 h. Uneaten *U. lactuca* was dried in an oven at 60 °C for 48 h. The proportion of uneaten feed that was lost through leaching and the collection net, without animals in the tank was determined; and the correction factor was used to calculate the corrected apparent feed intake. Feed intake was determined daily over an 8 day period prior to the end of Experiment 1. In Experiment 2, daily feed intake (reported as g kg^{-1} abalone day^{-1}) and the proportion of days where no food consumption occurred in each tank (%) were determined.

Table 1
The biochemical composition of the experimental diets fed to greenlip abalone in Experiments 1 and 2.[f]

Item	Exp. 1. *Ulva lactuca* non-enriched	Exp. 2. *Iva lactuca* enriched	Exp. 1 & 2. commercial diet
Analysed proximate composition (dry basis)			
Dry matter (g kg^{-1})	159.0	220.0	900.0
Crude protein (g kg^{-1})	113.3	271.8	306.0
Crude lipid (g kg^{-1})	21.1	42.1	45.0
Ash (g kg^{-1})	243.4	166.7	62.0
Carbohydrate	459.9	195.2	518.7
NFE (g kg^{-1})[a]	622.2	519.4	587.0
Gross energy (MJ kg^{-1})[b]	14.2	17.0	15.2
FRAP value (μmol Fe^{2+} g^{-1} diet)[c]	na	1.81	0.92
Calculated essential amino acids (g kg^{-1} dry basis)[d]			
Arginine	4.3	10.3	16.5
Histidine	1.7	4.1	6.6
Isoleucine	2.5	5.9	11.3
Leucine	5.1	12.3	19.2
Lysine	2.9	6.9	17.6
Methionine	0.7	1.6	2.8
Phenylalanine	3.2	7.7	12.8
Threonine	3.5	8.5	9.7
Valine	4.4	10.7	12.5
Σ EAA[e]	28.3	67.9	109.0

[a] NFE = nitrogen-free extract was calculated by difference = 100% − (crude protein% + total fat% + ash%).
[b] The gross energy content of the enriched and non-enriched *U. lactuca* was determined using the values of 17.2, 23.6 and 39.5 MJ kg^{-1} for carbohydrate, protein and lipid, respectively (NRC, 2011).
[c] FRAP = ferric-reducing antioxidant potential.
[d] Amino acid values for the non-enriched and enriched *U. lactuca* were calculated using book values (Tabarsa et al., 2012) and the respective analysed protein content of each diet.
[e] EAA = essential amino acids.
[f] na = not analysed.

2.6. Sampling, calculation of performance indices and phagocytic rate analysis

At the completion of the experiments abalone from each tank were weighed and measured. Performance indices were calculated using the following formulae for whole, live abalone (Britz and Hecht, 1997):

$$\text{Daily growth rate}\left(\text{DGR, g day}^{-1}\right) = (\text{final weight–initial weight})/\text{days};$$

$$\text{Shell growth rate}\left(\text{mm day}^{-1}\right) = (\text{final shell length–initial shell length})/\text{days; and}$$

$$\text{Condition factor (CF)} = 5575 \times [(\text{weight (g)}/\text{length (mm)}^{2.99}].$$

All data reported for individual animal performance was based on the individual data recorded from each tank, whereas daily feed rates have the weight of mortalities taken into account.

At the end of Experiment 2, haemolymph samples were obtained from the cephalic sinus of abalone from each tank using a 23-guage needle and 3 mL syringe. A proportion of the haemolymph samples was used either directly for the determination of haemocyte phagocytic rate, or centrifuged for 5 min at 4 °C and 2000 *g* and serum was transferred into cryotubes and stored at −80 °C until analysed for superoxide dismutase (SOD) activity and ferric-reducing antioxidant potential (FRAP). The haemocyte phagocytic rate in haemolymph samples from three abalone per tank was measured using the method of Chen et al. (2005) and Dang et al. (2011b) with some modifications. Briefly, a yeast solution for phagocytosis assay was prepared by autoclaving 2.5% baker's yeast (*Saccharomyces cerevisiae*) (Tandaco, Cerebos Foods, Seven Hills, NSW, Australia) in 4% Congo red (Sigma) in filtered seawater (FSW). The stained yeast cells were centrifuged at 1500 *g* for 10 min, washed three times with FSW and resuspended in FSW (0.2 μm) at 10^7 cells mL^{-1}. Fresh haemolymph (150 μL) was added to an Eppendorf tube at room temperature with 40 μL yeast suspension, lightly vortexed then rested for 10 min in the dark. At the end of this time, tubes were vortexed and two drops placed onto a glass slide with a coverslip. Phagocytic rate was determined in triplicates as percentage of phagocytic haemocytes in 50 haemocytes under a light microscope at 400× magnification.

2.7. Biochemical analyses

Both the moisture, ash, crude lipid, gross energy, crude protein and amino acid composition of the commercial diet and the proximate composition of the *U. lactuca* were analysed according to the methods of the AOAC International (1995). The commercial control diet and the *U. lactuca* diets were oven dried to a constant weight at 105 °C for 16 h and 60 °C for 48 h, respectively, 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 of the commercial diet was determined using a bomb calorimeter calibrated with benzoic acid. The gross energy content of the enriched and non-enriched *U. lactuca* was determined using the values of 17.2, 23.6 and 39.5 MJ kg^{-1} for carbohydrate, protein and lipid, respectively (NRC, 2011). Carbohydrate was evaluated using the Molisch test (Lampman et al., 2010) and a glucose standard curve. Amino acids in the commercial diet were analysed using the methods of Bosch et al. (2006). The amino acid composition of the enriched and non-enriched *U. lactuca* was determined from book values (Tabarsa et al., 2012). Feed and serum samples were assayed for FRAP using the methods of Cheah (2011), slightly modified from Xu et al. (2010). Serum samples were assayed for SOD activity using a superoxide dismutase activity assay kit (Cayman Chemical, Ann Arbor, MI, USA).

2.8. Water quality

Water quality data is presented in Tables 2 and 3. Water temperature, dissolved oxygen and pH were measured daily (Exp. 1, 12:00 pm; Exp. 2,

Table 2
Water quality parameters measured at 12:00 pm daily during the light period for Experiment 1.[a–d]

Animal age	2-Year-old						3-Year-old					
Water temperature	18 °C		22 °C		26 °C		18 °C		22 °C		26 °C	
Diet type	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet
Water temperature												
Mean ± SD	18.0 ± 0.5	18.0 ± 0.5	22.0 ± 0.4	22.0 ± 0.4	25.8 ± 0.2	25.8 ± 0.3	18.0 ± 0.6	18.0 ± 0.6	21.9 ± 0.3	22.0 ± 0.4	25.6 ± 0.3	25.8 ± 0.3
Range	16.6–18.7	16.8–18.8	21.4–22.5	21.3–22.6	25.4–26.2	25.0–26.2	16.2–18.8	16.5–18.9	21.3–22.5	21.3–22.6	25.0–26.0	25.1–26.5
Dissolved oxygen (% saturation)												
Mean ± SD	101.0 ± 1.7	96.2 ± 4.0	98.7 ± 4.7	94.8 ± 2.3	99.7 ± 2.0	97.2 ± 2.4	94.5 ± 3.1	87.2 ± 4.8	89.3 ± 3.6	78.7 ± 6.8	91.3 ± 4.0	87.4 ± 6.0
Range	97.4–103.6	77.1–100.1	74.1–101.7	91.4–101.2	95.7–102.6	92.7–101.5	84.7–101.6	79.6–101.0	81.0–101.8	69.4–101.0	83.9–100.9	74.3–100.7
Dissolved oxygen (mg L^{-1})												
Mean ± SD	7.8 ± 0.2	7.4 ± 0.5	7.2 ± 0.4	7.0 ± 0.4	7.0 ± 0.3	6.8 ± 0.4	7.3 ± 0.3	6.8 ± 0.5	6.6 ± 0.5	5.8 ± 0.7	6.4 ± 0.5	6.1 ± 0.6
Range	7.5–8.1	5.2–8.1	5.4–7.9	6.5–8.4	6.5–7.9	6.4–7.9	6.4–8.5	6.1–8.4	6.1–8.4	5.0–8.4	5.8–8.4	5.1–8.4
pH												
Mean ± SD	8.36 ± 0.02	8.33 ± 0.02	8.40 ± 0.03	8.37 ± 0.03	8.39 ± 0.04	8.38 ± 0.03	8.33 ± 0.03	8.26 ± 0.05	8.35 ± 0.03	8.29 ± 0.05	8.36 ± 0.03	8.33 ± 0.05
Range	8.32–8.41	8.28–8.38	8.34–8.45	8.30–8.41	8.29–8.47	8.33–8.46	8.29–8.40	8.19–8.41	8.29–8.41	8.21–8.41	8.29–8.43	8.24–8.42

[a] Data from the seven day acclimatisation period at the start of the trial is excluded from the table.
[b] Values are mean ± SD, water temperature (n = 25), dissolved oxygen (% saturation) (n = 36), dissolved oxygen (mg L^{-1}) (n = 36), pH (n = 36).
[c] Values for 'range' are the lowest and highest values recorded in that treatment.
[d] Ammonia values are not reported as they were always below the limits of detection (<0.25 mg TAN L^{-1}).

6:45 am (before lights on) and 3:30 pm (prior to feeding) using an OxyGuard™ Handygamma dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark) and a pH meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, IL, USA). The change in measurement times represents a refinement of technique, as we wanted to go beyond a standardised time for measurement used in Experiment 1 to examine the time where DO is at its lowest level due the respiration of live macroalgae in the dark period. The measurement of water quality at 3.30 pm was also done to determine water quality prior to the addition of food to each tank. Salinity and ammonia (NH_4^+/NH_3) levels were determined weekly using a salinity refractometer, model RF20 (Extech Instruments, Nashua, NH, USA), and a saltwater ammonia test kit (API, Mars Fishcare Inc., Chalfont, PA, USA), respectively. Light intensity was measured using a LI-COR 1400 Quantum light meter (LI-COR Environmental, Lincoln, NE, USA).

2.9. Statistical analyses

Homogeneity of variances among means was assessed using Levene's test for equality of variance errors. One-factor ANOVA was used to assess differences for each age class separately in initial treatment means for weight, shell length and condition index values. Multifactor ANOVA (Experiment 1: age × water temperature × diet type; Experiment 2: water temperature × diet type) was used to assess the main effects for water quality, growth rates, feed intake, phagocytic rate and SOD and FRAP data. Where significant interactions were observed, the data were analysed using one-factor ANOVA and Student–Newman–Keuls (SNK) multiple range test. The survival patterns were analysed using Kaplan–Meier survival curves, Log-rank and Wilcoxon tests; and Cox proportional hazards regression analysis. In Experiment 2, Two-factor 'split-plot' repeated measures ANOVA were used to

Table 3
Water quality parameters measured at 6:45 am (dark period) and 3:30 pm (light period prior to feeding) for Experiment 2.[a–d]

Water temperature		22 °C		26 °C		TWO-factor ANOVA		
Diet type		*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	Water temperature (A)[e]	Diet type (B)[f]	Interaction (A × B)
Dissolved oxygen (% sat) 6:45 am	Mean	90.7 ± 1.81	82.5 ± 0.19	89.5 ± 1.50	83.3 ± 1.12	0.888	(>) 0.001	0.482
	Range	80–100	70–96	74–101	68–94			
Dissolved oxygen (% sat) 3:30 pm	Mean	92.7 ± 0.41	87.5 ± 0.55	92.6 ± 0.56	88.3 ± 0.37	0.542	(>) <0.001	0.390
	Range	82–98	78–95	85–101	75–99			
Dissolved oxygen (mg L^{-1}) 6:45 am	Mean	6.49 ± 0.12	5.87 ± 0.02	6.04 ± 0.10	5.60 ± 0.09	(>) <0.001	(>) <0.001	0.349
	Range	5.7–7.1	4.9–6.8	5.1–6.9	4.6–6.4			
Dissolved oxygen (mg L^{-1}) 3:30 pm	Mean	6.55 ± 0.08	6.20 ± 0.02	6.20 ± 0.08	5.92 ± 0.05	(>) 0.001	(>) 0.001	0.564
	Range	5.9–7.1	5.5–6.8	5.4–6.9	5.1–6.8			
pH 6:45 am	Mean	8.18 ± 0.01	8.11 ± 0.01	8.21 ± 0.01	8.16 ± 0.01	(<) 0.002	(>) <0.001	0.347
	Range	8.03–8.24	7.96–8.17	8.09–8.36	8.02–8.24			
pH 3:30 pm	Mean	8.16 ± 0.00	8.12 ± 0.01	8.19 ± 0.01	8.16 ± 0.00	(<) <0.001	(>) <0.001	0.282
	Range	8.03–8.22	7.97–8.19	8.07–8.24	8.03–8.25			
Water temperature (°C) 6:45 am	Mean	21.9 ± 0.01	21.9 ± 0.04	25.4 ± 0.08	25.5 ± 0.03	na	na	na
	Range	21.0–22.5	21.1–22.9	24.9–26.5	25.2–26.5		–	–
Water temperature (°C) 3:30 pm	Mean	21.7 ± 0.01	21.7 ± 0.10	25.5 ± 0.10	25.5 ± 0.03	na	na	na
	Range	20.6–22.1	20.6–22.4	25.0–26.5	24.5–26.6	–	–	–

[a] Table excludes data from the seven day acclimatisation period at the start of the experiment.
[b] Values are mean ± SE. n = 3. na = not analysed.
[c] Values for 'range' are the lowest and highest values recorded in that treatment.
[d] Ammonia values are not reported as they were always below the limits of detection (<0.25 mg TAN L^{-1}).
[e] For water temperature (>) denotes that 22 °C was significantly greater than 26 °C, or (<) denotes that 22 °C was significantly less than 26 °C.
[f] For diet type (>) denotes that *U. lactuca* was significantly greater than the commercial diet, or (<) denotes that *U. lactuca* was significantly less than the commercial diet.

analyse feed intake rates and the proportion of days that abalone consumed part of their daily ration. A significance level of $P < 0.05$ was used. All statistical analyses were done using IBM SPSS, Version 20 for Windows (IBM SPSS Inc., Chicago, IL, USA). Unless otherwise stated, all values are presented as means ± standard error of the mean.

3. Results

3.1. Water quality

3.1.1. Experiment 1

Following acclimatisation, water temperatures were kept within ± 1.0 °C of the targeted treatment temperatures of 18, 22 and 26 °C (Table 2). Dissolved oxygen saturation and concentration levels tended to be lower in all treatments fed the commercial diet. Regardless of diet or temperature, lower dissolved oxygen levels were also consistently observed in treatment containing the larger, 3-year-old abalone. pH tended to be lower in treatments fed the commercial diet, and also in treatments containing 3-year-olds. Salinity ranged from 35.0 to 35.5 g L^{-1} and did not differ appreciably between treatments. Ammonia (NH_4^+/NH_3) was always <0.25 mg L^{-1} (i.e. <lowest detectable level) (Table 2).

3.1.2. Experiment 2

Following acclimatisation, water temperatures were kept within ± 1.4 °C of the treatment temperatures of 22 and 26 °C (Table 3). Dissolved oxygen saturation levels at 6:45 am ($P = 0.001$) and 3:30 pm ($P < 0.001$) were significantly affected by diet type (*U. lactuca* > commercial; Two-factor ANOVA), but not by water temperature ($P = 0.888$; $P = 0.542$), and there were no significant interactions for either variable ($P = 0.482$; $P = 0.390$). Dissolved oxygen concentration levels at 6:45 am ($P < 0.001$) and 3:30 pm ($P = 0.001$) were significantly affected by diet type (*U. lactuca* > commercial), and by water temperature ($P < 0.001$; $P = 0.001$; 22 > 26 °C), and there were no interactions ($P = 0.349$; $P = 0.564$). There were also significant effects of diet type ($P < 0.001$; *U. lactuca* > commercial) and water temperature ($P = 0.002$; $P < 0.001$; 26 > 22 °C) at 6:45 am and 3:30 pm on pH levels, and there were no interactions ($P = 0.347$; $P = 0.282$; Table 3). Salinity was similar between treatments ranging from 33 to 37 g L^{-1}, while ammonia (NH_4^+/NH_3) levels were always <0.25 mg L^{-1}.

3.2. Abalone behaviour

No visual symptoms of disease were observed in abalone during either experiment. Due to the nocturnal feeding behaviour of abalone it was difficult to visually determine differences in feeding behaviour during either experiment. However, in both experiments, at all water temperatures, during the light period, a number of abalone were observed to feed on the *U. lactuca* within 5 min of feed introduction to the tanks. In contrast, abalone were not observed to consume the commercial diet during the light period in either experiment.

3.2.1. Experiment 1

Abalone of both age classes, fed either diet, at 18 or 22 °C were observed to exhibit normal movement and resting behaviour during the light period over the course of Experiment 1. However, at 26 °C, the larger 3-year-old abalone, fed either diet, were observed to intermittently raise their shells and exhibited rapid torsional rotation. Occasionally, they would also detach from the tank walls and land upside down on the base of the tank. At this point, they were able to right themselves without assistance. Additionally, during tank cleaning and feed collection, the 3-year-olds, held at 26 °C, occasionally lost their attachment to the tank and would fall into the collection screen. These animals were returned to the tank immediately, however, they appeared unhealthy and died a few days later. These behaviours were not observed in 2-year-old abalone, fed either diet, at 26 °C, or in abalone from any other treatment.

3.2.2. Experiment 2

Abalone were observed to exhibit normal movement and resting behaviour during the daylight period in the 22 °C treatments when fed either diet in Experiment 2. However, when the abalone were held at 26 °C and fed either diet, they exhibited similar physical behaviour as described for the 3-year-old abalone held at 26 °C in Experiment 1.

3.3. Survival

3.3.1. Experiment 1

At 26 °C, the survival of 2-year-old abalone fed the commercial or *U. lactuca* diets was 90% and 92.5%, respectively (Fig. 1a). The survival of large 3-year-old abalone fed the commercial or *U. lactuca* diets was 65 and 90.2%, respectively (Fig. 1b). Analysis of the Kaplan–Meier survival curves showed that the survival of abalone held at 26 °C, and fed the commercial diet, was significantly lower compared to that of abalone fed the *U. lactuca* (Fig. 1a and b; Log-rank and Wilcoxon

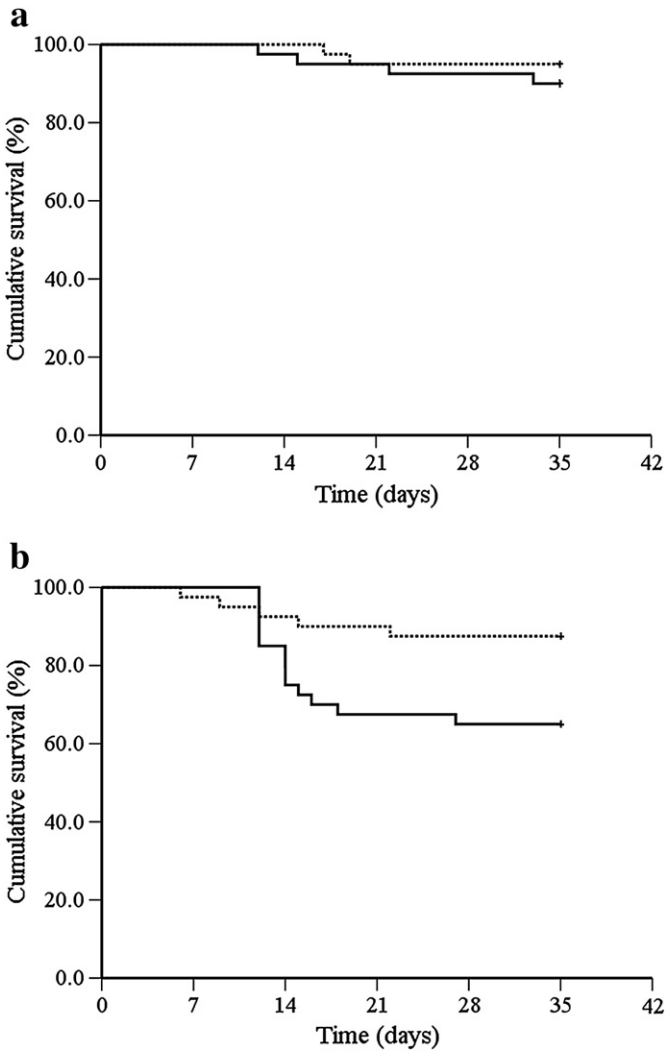

Fig. 1. *a.* Kaplan–Meier survival curves for small 2-year-old greenlip abalone fed the *Ulva lactuca* (·····) versus the commercial diet (——) at 26 °C for 36 days in Experiment 1 (Survival of 2-year-olds fed the *Ulva lactuca* = commercial diet at 26 °C; $P = 0.399$; Log-rank and Wilcoxon tests). *b.* Kaplan–Meier survival curves for large 3-year-old greenlip abalone fed the *Ulva lactuca* (·····) versus the commercial diet (——) at 26 °C for 36 days in Experiment 1 (Survival of 3-year-olds fed the *Ulva lactuca* > commercial diet at 26 °C; $P = 0.023$; Log-rank and Wilcoxon tests).

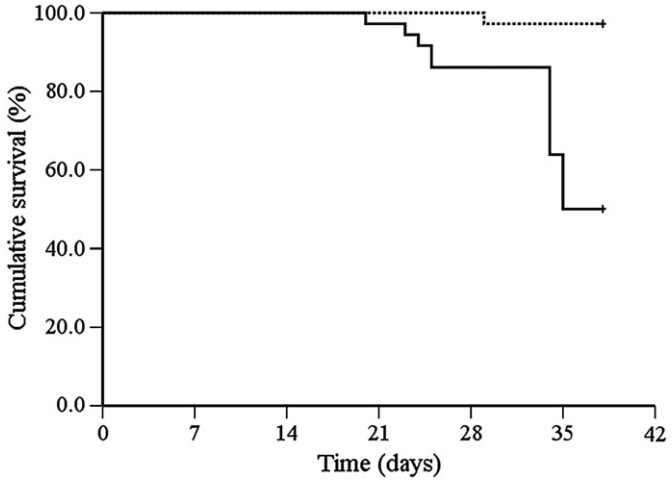

Fig. 2. Kaplan–Meier survival curves for greenlip abalone fed the *Ulva lactuca* (·····) versus the commercial diet (——) at 26 °C for 38 days in Experiment 2 (Survival of 3-year-olds fed the *Ulva lactuca* > commercial diet at 26 °C; $P < 0.001$; Log-rank and Wilcoxon tests). Data for 22 °C treatments are not shown as there were no mortalities recorded over the course of the experiment at this temperature for abalone fed either dietary treatment.

tests; $P = 0.023$), however, diet type had no significant effect on the survival of small 2-year-old abalone ($P = 0.399$). In addition, the survival of 3-year-old abalone was significantly lower than that of smaller 2-year-old abalone when fed the commercial diet (Fig. 1a and b; $P = 0.006$), and when compared to the 2-year-olds, the 3-year-olds were 2.6 times more likely to die over the course of the experiment ($P = 0.006$; Cox proportional hazards regression). In contrast, no effect on survival was observed between either age classes of abalone fed the *U. lactuca* (Fig. 1a and b; $P = 0.227$). At 26 °C, mortality commenced in the second week of the experiment in the 3-year-old abalone fed the commercial diet, and increased thereafter. While at 26 °C, 3-year-old abalone were 2 times more likely to die over the course of the experiment if they were fed the commercial diet compared to the *U. lactuca* ($P = 0.034$; Cox proportional hazards regression). At 22 °C, the survival of 2- or 3-year-old abalone fed the commercial diet or the *U. lactuca* was 100%. Kaplan–Meier survival curve analysis was not used at this temperature. At 18 °C, the survival of 2-year-old abalone fed the commercial or *U. lactuca* diets was 100% and 97.5%, respectively. Survival of 3-year-old abalone at 18 °C was 95.5% and 100% for the *U. lactuca* and commercial diet, respectively. Using Kaplan–Meier survival curve analysis, diet type had no significant effect on survival for 2-year-old or 3-year-old abalone at 18 °C ($P = 0.317$ and 0.993, respectively). Additionally, at 18 °C, age class had no significant effect on the survival of abalone fed the commercial or *U. lactuca* diets ($P = 0.993$ and 0.317).

3.3.2. Experiment 2

The survival of abalone fed either diet at 22 °C was 100%. However, at 26 °C Kaplan–Meier survival curves showed a significant difference between the survival of abalone fed the *U. lactuca* and commercial diet ($P < 0.001$; Log-rank and Wilcoxon tests; Fig. 2). At 26 °C, survival was 97.2 ± 2.8% for abalone fed the *U. lactuca*, whereas, the survival of abalone fed the commercial diet was significantly ($P < 0.001$) reduced to 50.0 ± 26.8%. Mortality commenced in abalone fed the commercial diet at 26 °C in the third week of the experiment and increased thereafter. These abalone were 21 times more likely to die over the course of the experiment than abalone fed *U. lactuca* ($P = 0.003$; Cox proportional hazards regression).

3.4. Growth

3.4.1. Experiment 1

At stocking, there were no significant differences in the initial weights ($P = 1.00$), shell lengths ($P = 1.00$) or condition factors ($P = 1.00$; $P = 0.981$) of 2- or 3-year-old abalone between treatments (Table 5; One-factor ANOVA), respectively. Daily growth rate was also significantly affected by diet type ($P < 0.001$; commercial > *U. lactuca*) and water temperature ($P < 0.001$; 18 = 22 > 26 °C), but not by age class ($P = 0.677$), and there were no significant interactions. There were significant three-way interactions for shell growth rate ($P < 0.001$) and final condition factor ($P = 0.020$; Table 4). For the interaction for shell growth rate, the 2-year-old abalone fed the commercial diet had a significantly higher response (One-factor ANOVA; SNK; $P < 0.05$; Table 5) than those when fed the *U. lactuca* at the corresponding temperatures; whereas, for the 3-year-old abalone, apart from the significantly higher shell growth rate when fed the commercial diet at 26 °C (One-factor ANOVA; SNK; $P < 0.05$; Table 5), there were no significant differences between any other treatments ($P > 0.05$). The 3-way interaction for final condition factor was due to the opposing responses of the 2- and 3-year-old abalone fed the commercial and *U. lactuca* diets at each water temperature (Table 5). At 26 °C, 2-year-olds tended to have a lower condition factor when fed the *U. lactuca* compared to the commercial diet, whereas, 3-year-olds exhibited the opposite response. The opposite response was observed for each year class at 18 and 22 °C (Table 5). Additionally, the condition factor of the 3-year-old abalone tended to be slightly higher compared to the 2-year-olds.

3.4.2. Experiment 2

At the commencement of this experiment there were no significant differences in the initial weights ($P = 0.652$), shell lengths ($P = 0.632$)

Table 4
Three-factor ANOVA table for variables measured in greenlip abalone in Experiment 1.[a]

Item	Age (A) (*P*)[b]	Diet type (B) (*P*)[c]	Water temperature (C)[d]				Interactions (*P*)[e]			
	2-Year-old vs. 3-year-old	*Ulva lactuca* vs. commercial	18 °C	22 °C	26 °C	*P*	A × B	A × C	B × C	A × B × C
Daily growth rate (mg d^{-1})	0.677	(<) <0.001	A	A	B	<0.001	0.450	0.103	0.530	0.455
Shell growth rate (μm d^{-1})	0.350	<0.001	na	na	na	0.008	<0.001	<0.001	0.186	<0.001
Final condition factor[b]	<0.001	0.525	na	na	na	0.017	0.354	0.239	0.775	0.020
Feed intake (g kg abalone^{-1} d^{-1})	<0.001	<0.001	na	na	na	<0.001	<0.001	0.001	<0.001	0.951

[a] na = not analysed due to statistically significant interactions.
[b] For variables with a significant effect of age ($P < 0.05$) and no interaction, (<) or (>) indicates whether measured values for 2-year-olds were less than or greater than measured for 3-year-olds.
[c] For variables with a significant effect of diet type ($P < 0.05$) and no interaction, (<) or (>) indicates whether measured values for *U. lactuca* were less than or greater than measured for the commercial diet.
[d] For variables with a significant effect of water temperature and no interaction, values without a common letter are significantly different (*A* indicated the highest value; $P < 0.05$).
[e] For variables with a significant interaction ($P < 0.05$), differences for each variable are compared across all treatment combinations in Table 5 (One-factor ANOVA, SNK test), values without a common superscript are significantly different (*a* indicated the highest value $P < 0.05$).

Table 5
Mean growth performance and feed intake values for greenlip abalone in Experiment 1.[1,2,3]

Animal age	2-Year-old						3-Year-old					
Water temperature	18 °C		22 °C		26 °C		18 °C		22 °C		26 °C	
Diet type	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet
Initial weight (g)[4,5]	5.96 ± 0.24	5.95 ± 0.27	5.96 ± 0.21	5.94 ± 0.27	5.93 ± 0.30	5.96 ± 0.29	36.24 ± 1.60	36.33 ± 1.67	36.25 ± 1.35	36.17 ± 1.64	36.28 ± 1.74	36.33 ± 1.66
Daily growth rate (mg d^{-1})	71.2 ± 15.99	123.1 ± 5.57	49.1 ± 6.72	145.9 ± 8.61	24.8 ± 16.52	72.5 ± 18.74	61.1 ± 27.14	165.9 ± 7.77	86.2 ± 24.35	164.4 ± 34.78	−13.6 ± 28.90	51.80 ± 17.14
Initial shell length (mm)[4,5]	38.6 ± 0.66	38.5 ± 0.63	38.6 ± 0.89	38.5 ± 0.65	38.5 ± 0.79	38.6 ± 0.69	63.4 ± 0.79	63.2 ± 0.95	63.2 ± 1.13	63.2 ± 0.78	63.2 ± 0.76	63.3 ± 0.89
Shell growth rate (μm d^{-1})	54.1 ± 13.68^{b}	149.8 ± 12.78^{a}	5.80 ± 2.71^{c}	171.3 ± 27.87^{a}	2.7 ± 0.64^{c}	58.0 ± 6.51^{b}	67.7 ± 12.67^{b}	70.7 ± 2.28^{b}	84.2 ± 11.81^{b}	86.9 ± 13.89^{b}	43.5 ± 12.51^{bc}	132.2 ± 16.9^{97}
Initial condition factor[4,5]	0.60 ± 0.04	0.60 ± 0.04	0.60 ± 0.03	0.61 ± 0.05	0.60 ± 0.05^{c}	0.60 ± 0.04	0.83 ± 0.01	0.84 ± 0.05	0.84 ± 0.03	0.83 ± 0.03	0.83 ± 0.02	0.83 ± 0.05
Final condition factor[6]	0.74 ± 0.01^{bc}	0.71 ± 0.03^{bc}	0.76 ± 0.03^{abc}	0.72 ± 0.01^{bc}	0.68 ± 0.01^{c}	0.73 ± 0.02^{bc}	0.79 ± 0.01^{abc}	0.87 ± 0.05^{a}	0.79 ± 0.01^{abc}	0.84 ± 0.05^{ab}	0.77 ± 0.01^{abc}	0.71 ± 0.04^{bc}
Feed intake rate[7] (g kg abalone $^{-1}$ d^{-1})	2.55 ± 0.07^{d}	7.11 ± 0.19^{b}	3.00 ± 0.06^{d}	8.28 ± 0.24^{a}	1.40 ± 0.23^{e}	4.21 ± 0.43^{c}	0.78 ± 0.12^{e}	3.17 ± 0.26^{d}	0.94 ± 0.04^{e}	3.91 ± 0.25^{c}	0.53 ± 0.01^{e}	1.32 ± 0.43^{e}

[1]Values are mean ± SE. n = 3.
[2]Refer to Table 4 for three-factor ANOVA results.
[3]For variables with a significant interaction ($P < 0.05$) from Table 4, differences for each variable are compared across all treatment combinations in this table (One-factor ANOVA, SNK test), values without a common superscript are significantly different (the superscript *a*, indicated the highest value; $P < 0.05$).
[4]There were no significant differences in initial weights ($P = 1.00$), shell lengths ($P = 1.00$) or condition factors ($P = 1.00$) of 2-year-old abalone between treatments (One-factor ANOVA).
[5]There were no significant differences in initial weights ($P = 1.00$), shell lengths ($P = 1.00$) or condition factor ($P = 0.981$) of 3-year-old abalone treatments (One-factor ANOVA).
[6]Condition factor (CF) = 5575 × (weight [g] / length [mm]$^{2.99}$) (Britz and Hecht, 1997).
[7]Feed intake values were determined for each treatment over an 8 day period prior to the end of the experiment.

or condition factors ($P = 0.080$) of 3-year-old abalone between treatments (One-factor ANOVA; Table 6). Daily growth rate and shell growth rate were significantly affected by diet type (commercial > *U. lactuca*; Two-factor ANOVA; Table 6) and by water temperature (22 > 26 °C), and there was no significant interactions between the two factors for any of the variables. The final condition factor of abalone was affected by water temperature ($P = 0.011$; 22 > 26 °C; Table 6), but not by diet type ($P = 0.113$), and there was no significant interaction between the two factors ($P = 0.506$).

3.5. Feed intake

3.5.1. Experiment 1

Significant interactions were observed for feed intake ($P < 0.001$; Table 4). The interactions may be explained by the higher feed intake, on a g feed kg bw^{-1} day^{-1} basis, for the smaller 2-year-old abalone compared to the larger 3-year-olds, combined with the larger negative effect of 26 °C on the intake of the commercial diet by the larger 3-year-old abalone compared to treatments at other temperatures (Table 5).

3.5.2. Experiment 2

Feed intake was measured daily throughout the experiment and was significantly different between temperatures over time ($P < 0.05$; Two-factor 'split-plot' repeated measures ANOVA; Fig. 3), but not significantly different between diet type over time ($P > 0.05$). The feed intake for the 22 °C treatments significantly increased ($P < 0.05$) from week one to week two, but did not significantly change ($P > 0.05$) over the course of the remaining weeks. For the 26 °C treatments, feed intake significantly progressively decreased ($P < 0.05$) from week one to week four. From week four onwards, there were no significant changes ($P > 0.05$) in feed intake ($P > 0.05$; Fig. 3). The proportion of days that abalone actually consumed part of their daily feed ration was determined daily throughout the course of the experiment (Fig. 4). Over the duration of the experiment, abalone at 22 °C consumed food every day. There was a significant difference between water temperature over time for the proportion of days that abalone consumed part of their daily feed ration (22 > 26 °C; $P < 0.05$; Two-factor 'split-plot' repeated measures ANOVA; Fig. 4). There was no significant difference ($P > 0.05$) over time between diet types. When comparing diet types at 26 °C, there was no significant difference in the proportion of days that abalone consumed part of their daily ration ($P > 0.05$; Fig. 4). At 26 °C, the proportion of days that abalone consumed part of their daily ration began to decline by week three and was significantly reduced by week four, and remained so until the end of the experiment (Fig. 4).

3.6. Haemocyte phagocytic rate and haemolymph constituents

For Experiment 2, haemocyte phagocytic rate was significantly affected by water temperature ($P = 0.006$; Two-factor ANOVA; 22 > 26 °C) and diet type ($P = 0.012$; *U. lactuca* > commercial), and there was no significant interaction between the two factors ($P = 0.481$; Table 6). Serum FRAP values were also significantly affected by water temperature ($P = 0.033$; Two-factor ANOVA; 22 > 26 °C) and diet type ($P = 0.047$; *U. lactuca* > commercial), and there was no interaction between the two factors ($P = 0.652$; Table 6); whereas, serum SOD activities were not significantly affected by water temperature ($P = 0.641$; Two-factor ANOVA; Table 6), diet type ($P = 0.150$), or interaction between the two factors ($P = 0.374$).

4. Discussion

During the present study at 26 °C, we repeatedly induced significant levels of mortalities in larger 3-year-old greenlip abalone when fed commercial diets. Concomitantly, we were able to ensure a relatively high level of survival at 26 °C by feeding live *U. lactuca*. The mortality

Table 6
Growth performance, feed intake, serum superoxide dismutase (SOD) and ferric ion reducing antioxidant power (FRAP) for green lip abalone in Experiment 2.[a]

Water temperature	22 °C		26 °C		Two-factor ANOVA (*P*)		
Diet type	*Ulva lactuca*	Commercial diet	*Ulva lactuca*	Commercial diet	Water temperature (A)[b]	Diet type (B)[c]	Interaction (A × B)
Initial weight (g)[d]	27.3 ± 0.69	26.6 ± 0.38	25.5 ± 0.75	27.9 ± 2.45	na	na	na
Daily growth rate (mg day^{-1})	91.8 ± 15.8	220.6 ± 23.8	26.4 ± 11.1	85.6 ± 18.3	(>) 0.002	(<) 0.002	0.117
Initial shell length (mm)[d]	57.8 ± 0.58	57.1 ± 0.34	57.3 ± 0.58	58.6 ± 1.56	na	na	na
Shell growth rate (μm day^{-1})	49.6 ± 7.2	120.3 ± 13.0	13.5 ± 1.2	44.6 ± 7.5	(>) <0.001	(<) 0.001	0.063
Initial condition factor (CF)[d,e]	0.82 ± 0.01	0.83 ± 0.02	0.79 ± 0.01	0.80 ± 0.01	na	na	na
Final condition factor (CF)[e]	0.83 ± 0.01	0.87 ± 0.04	0.80 ± 0.01	0.81 ± 0.10	(>) 0.011	0.113	0.506
Haemocyte phagocytic rate (%)	62.96 ± 2.14	51.48 ± 0.64	50.25 ± 3.84	43.24 ± 4.12	(>) 0.006	(>) 0.012	0.481
Serum FRAP value (μmol Fe^{2+} equivalent)	0.136 ± 0.01	0.120 ± 0.01	0.118 ± 0.00	0.096 ± 0.01^	(>) 0.033	(>) 0.047	0.652
Serum SOD activity (U mL^{-1})	0.132 ± 0.02	0.090 ± 0.01	0.108 ± 0.02	0.098 ± 0.01^	0.641	0.150	0.374

[a] Values are mean ± SE. n = 3, except for superscript ^, where n = 2.
[b] For water temperature (>) denotes that 22 °C was significantly greater than 26 °C, or (<) denotes that 22 °C was significantly less than 26 °C.
[c] For diet type (>) denotes that *U. lactuca* was significantly greater than the commercial diet, or (<) denotes that *U. lactuca* was significantly less than the commercial diet.
[d] There were no significant differences in initial weight ($P = 0.652$), initial shell length ($P = 0.632$) and initial condition factor ($P = 0.080$) between treatments at stocking (One-factor ANOVA).
[e] Condition factor (CF) = 5575 × (weight [g] / length [mm]$^{2.99}$) (Britz and Hecht, 1997).

patterns were also similar to those experienced on-farm during SM events, in that larger 3-year-old abalone were affected at 26 °C, whereas the smaller 2-year-old abalone were not (Figs. 1a, b and 2). In the summer of 2012/2013, abalone farms in South Australian reported significant financial losses due to high mortality of large greenlip abalone, associated with SM, ranging from 15 to 50% (Shane McLinden, Manager, Southseas Abalone Pty Ltd., personal communication). These farm mortality rates are consistent with those previously reported for SM on-farm for greenlip abalone by Vandepeer (2006) and Dang et al. (2011a). Survival was the main factor of interest in the present study as it is particularly relevant to the abalone industry.

In South Australia, peaks in water temperature during summer (>22 °C) are associated with periods of low tidal movement, particularly during "dodge tides" on extremely hot windless and cloudless days. Our mortality events commenced after 2 to 3 weeks of extended exposure to 26 °C (Figs. 1a, b and 2). The mortality patterns on farms may be caused by an acute temperature spike, but alternatively may also be from the chronic compromises to their metabolism from inadequate diets, as we have demonstrated in this study, and the subsequent consequences of chronic oxidative stress, brought about by extended periods of high water temperature in addition to other husbandry stressors. Additionally, even though the present study had higher water temperatures, differences in the onset-time of mortality between the laboratory and on-farm may also have been due to the cleaner, less stressful culture environment of the laboratory system compared to on-farm conditions. Stressors associated with husbandry practices have been demonstrated to be compounding on the stress response, and are capable of eliciting a greater challenge to the survival of fish (Stone et al., 2008; Wendelaar Bonga, 1997) and abalone (Cardinaud et al., 2014; Hooper et al., 2011).

Typically, acute short-term temperature or bacterial challenges are used to test the immunological competency and survival of abalone following extended periods of experimental treatment under normal environmental conditions (Dang et al., 2011b; Hooper et al., 2007). In contrast, we have demonstrated repeated mortality patterns for abalone over an extended time period at elevated water temperature.

This study also built on the previous research carried out by Vandepeer (2006) at the SARDI SAASC. In order to develop this work further, we considered that the causal factors of SM in abalone were a combination of biological, nutritional, anthropogenic and environmental factors (Cardinaud et al., 2014; Travers et al., 2008, 2009a). Combined, we assumed that these factors would cause oxidative stress and ultimately death, due to an increased biological oxygen demand in an already hypoxic culture system. In retrospect, Vandepeer (2006) used smaller 30 mm SL greenlip abalone, which based on the results of the present study, are not susceptible to the 26 °C temperature challenge. The incoming seawater in the present study was filtered to 30 μm, whereas, Vandepeer (2006) used 10 μm filtration which would have resulted in a reduced load of suspended particulate matter and microbes. In addition, despite higher mortality levels at 26 °C across all treatments, a control diet was not used to ensure survival of abalone (Vandepeer, 2006). These factors combined may have transpired to produce environmental conditions below the threshold required to induce

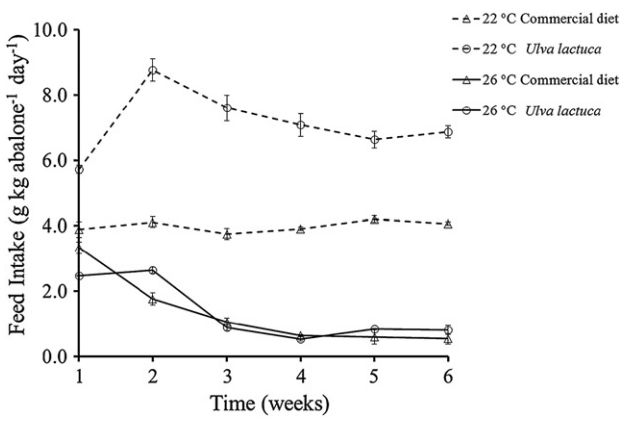

Fig. 3. Daily feed intake rates for greenlip abalone fed *Ulva lactuca* or the commercial diet at waters temperatures of 22 or 26 °C over the course of Experiment 2. (Daily feed intake at 22 °C > 26 °C, $P < 0.05$; Daily feed intake of *Ulva lactuca* = commercial diet, $P > 0.05$; Two-factor 'split-plot' repeated measures ANOVA; Values are mean ± SE, n = 3).

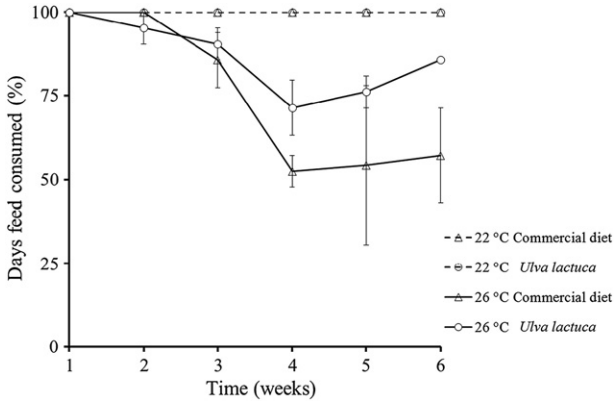

Fig. 4. The proportion of days that abalone consumed part of their daily feed ration when fed *Ulva lactuca* or the commercial diet at water temperatures of 22 or 26 °C over the course of Experiment 2. The proportion of days that abalone consumed part of their daily feed ration was significantly different over time between water temperatures (22 > 26 °C; $P < 0.05$; Two-factor 'split-plot' repeated measures ANOVA), but not significantly different ($P > 0.05$) over time between diet types. (Values are mean ± SE, n = 3).

symptoms of SM, and circumvented the successful development of their challenge model (Vandepeer, 2006).

The commercial diet was selected as a negative control, as it has been used routinely in a commercial setting when SM had occurred. In contrast, live *U. lactuca* was selected as a positive control diet, as it has been reported to possess a range of attributes that are not only beneficial to the environment, but are also potentially nutritionally therapeutic to abalone (Dang et al., 2011b). *U. lactuca* may act as a bio-filter via the process of photosynthesis through carbon dioxide uptake from the environment in conjunction with the addition of oxygen to the environment. *U. lactuca* may also reduce the level of nitrogenous products, and other potentially harmful products, in the surrounding environment. The main benefit of using *U. lactuca* may lie in its use as a therapeutic agent when consumed, as it contains a range of bioactive compounds that may exhibit antimicrobial and antioxidant properties (see FRAP values, Table 1) (Abirami and Kowsalya, 2011; Silva et al., 2013; Tierney et al., 2010). These bioactive compounds include: peptides, sulphated polysaccharides such as ulvan, sterols, polyphenols, omega-3 PUFAs, phospholipids, vitamins such as ascorbic acid and vitamin precursors including α-tocopherol, carotenoids including β-carotene and chlorophylls (Tierney et al., 2010; Abirami and Kowsalya, 2011). Supporting this, Dang et al. (2011b) measured an enhanced antimicrobial response in greenlip abalone fed *U. lactuca*, compared to a commercially formulated diet.

During periods of prolonged exposure to high summer water temperatures, dietary energy will be partitioned preferentially to support essential metabolic functions such as respiration, while growth, oxidative status and immune competence will be compromised (Kaushik, 1986; Tomanek, 2010). FRAP values and SOD activities may both be considered measures of the oxidative status of a system (Hooper et al., 2007; Xu et al., 2010). The FRAP value is considered to be a measure of the antioxidant power within a system, or substrate, with higher FRAP values being indicative of a greater antioxidant status (Xu et al., 2010). Interestingly, the FRAP values measured in the serum of the greenlip abalone fed the *U. lactuca* were significantly elevated, indicating an improved oxidative status, compared to those of the abalone fed the commercial diet. FRAP values were also significantly reduced in abalone at 26 °C, indicating a reduction in antioxidant status (Table 6). The FRAP values in the diets also differed, and appeared to correlate with serum values, with values in the *U. lactuca* two-fold those measured in the commercial diet (Table 1). SODs are enzymes that catalyse the dismutation of superoxide into oxygen and hydrogen peroxide. The activity of serum SOD, although not significantly elevated, displayed a tendency to be higher in the *U. lactuca* fed abalone and to be reduced by increasing temperature (Table 6). It is reported that during periods of acute oxidative stress, in vivo, serum SOD activity increases in response to increasing levels of oxidative stress. However, in vivo, serum SOD activities have also been reported to diminish in response to chronic stress (Vosloo et al., 2013). These phenomena are common to acute and chronic stress models in most organisms (Wendelaar Bonga, 1997).

Based on the mortality responses of abalone at 26 °C, combined with the serum FRAP and SOD responses for the corresponding abalone, it appeared that the temperatures used in this study induced the condition of chronic oxidative stress in the abalone fed the commercial diet. In contrast, certain compounds present in *U. lactuca* were able to ameliorate the negative impacts of high water temperature on oxidative stress and survival of large greenlip abalone. Extended periods of excessive oxidative stress have been reported to have negative impact on the immune system of abalone (Hooper et al., 2007).

Dang et al. (2012) reported a differential alteration to the immune response of blacklip abalone (*Haliotis rubra* Leach) as the water temperatures rose from 20 to 24 °C. Antiviral properties remained constant as the temperature rose while antibacterial properties diminished. In the present study, although not measured, it appears that the antibacterial activity of greenlip abalone was also compromised. The haemocyte phagocytic rates were significantly reduced at 26 °C, and also by feeding the commercial diet (Table 6). Hooper et al. (2011) reported phagocytic rates ranging from 62 to 79% in healthy farmed 31 g hybrid abalone (*H. laevigata* × *H. rubra*) fed a commercial diet at 13 to 15.5 °C. Travers et al. (2008) also reported a phagocyte rate of 61% for 70 mm SL *H. tuberculata* fed *Laminaria digitata* at 15 °C. Baseline levels from both studies are comparable to those observed for greenlip abalone fed *U. lactuca* at 22 °C. Hooper et al. (2011) reported a rapid decline in phagocytic rates, from 62% to 28% or 32%, respectively, on the same day following anaesthesia, or anaesthesia and tank transfer. The temperature and dietary induced alterations in phagocytic rate in the current study would suggest a decline in antibacterial activity and immune status of the abalone. Haemocyte phagocytosis is a major component of the innate immune system of abalone and is considered to be the first line of defence to ward off pathogens (Handlinger et al., 2005; Hooper et al., 2007, 2011; Jiang et al., 2013). Bissett et al. (1998) reported bacterial colonisation, particularly with *Vibrio* spp., of formulated abalone feed following immersion, and this may contribute to the proliferation of disease. Given the complicit link between Vibriosis and SM in greenlip abalone (Hooper et al., 2007), the reduced capacity of the innate immune function in greenlip abalone, measured by the reduced phagocytic rate in response to elevated water temperatures is critical.

Harris et al. (1999) recommended that the dissolved oxygen saturation level was not less than 81% for 10 g juvenile greenlip abalone, as below this level oxygen uptake was limited and growth may be compromised. In both experiments, the DO% saturation levels in the culture environment of abalone fed the commercial diet were reduced (Tables 2 and 3). Additionally, the saturation levels of dissolved oxygen were also reduced at 26 °C. Combined, at 26 °C, the negative effects of feeding the commercial diet on oxygen saturation levels resulted in the abalone being chronically exposed to lower concentrations of dissolved oxygen (Harris et al., 1999). The dissolved oxygen levels in the low and intermediate treatments in the study of Vosloo et al. (2013) were similar to those reported in the present study (Tables 2 and 3). Vosloo et al. (2013) assessed organismal and cellular responses of juvenile (41 mm SL) and adult (65 mm SL) *H. midae* exposed to low (83% saturation; 6.5 mg L^{-1}) and intermediate (95% saturation; 7.7 mg L^{-1}) oxygen saturation levels at 16 °C for one month and reported DNA fragmentation and protein damage in adults, while juvenile were unaffected by the moderately hypoxic conditions. SOD activities were also reported to decrease in both juveniles and adults in response to hypoxic conditions. Vosloo et al. (2013) observed elevated basal rates of superoxide dismutase and catalase in juvenile compared to adults. Vosloo et al. (2013) suggested that these levels were sufficient to prevent DNA fragmentation and protein damage in the juveniles exposed to hypoxia, whereas, the lower basal rate of antioxidant enzymes in adults was not. Vosloo et al. (2013) also hypothesised that the insensitivity of juveniles to decreased oxygen levels might be related to their life history where they are exposed to extreme diurnal fluctuations in oxygen levels. These phenomena, combined with the lower tank stocking density for the smaller 2-year-old greenlip abalone in the current study may explain the improved survival compared to the 3-year-olds in Experiment 1, and also the survival differences observed between diet types for the larger 3-year-olds at 26 °C in both experiments.

Growth rate and feed intake were suppressed in greenlip abalone grown at 26 °C. This may be expected, as they are operating at the extremes of their temperature and oxygen tolerance limits. Optimal water temperatures for growth in this species is size dependent, and has been reported to be 22 °C for 25 mm SL animals (Stone et al., 2013). The 50% critical thermal maxima was 27.5 °C for 82 mm SL greenlip abalone (Gilroy and Edwards, 1998); whereas, Madigan et al. (2000) reported the 50% critical thermal maxima to be 29.5 °C for 83 mm SL greenlip abalone. Differences may have been due to genetic differences as Gilroy and Edwards (1998) worked with a Tasmanian strain while Madigan et al. (2000) used a more heat tolerant South

Australian strain. The more heat tolerant South Australian strain was used in the current study. In terms of surface area coverage, the stocking density of animals in this study was at most 308 cm^2 of abalone in a 1129 cm^2 tank for the 3-year-old animals; according to earlier work by Koike et al. (1979), density has an impact on growth when the area of abalone exceeds that of 50% of the tank surface area, and more recently Huchette et al. (2003) used stocking densities (85 abalone m^{-2}) comparable to ours (88 abalone m^{-2}) and demonstrated excellent growth. The only other potential impacts on survival in this system could come from insufficient flow leading to inadequate dissolved oxygen or accumulated ammonia, however, as our system employed 3.2 water exchanges per hour (flow of 18 L h^{-1}, water volume 5.6 L $tank^{-1}$), we were unable to detect any ammonia (Tables 2 and 3), and the dissolved oxygen saturation level for all treatments (min 79%) was above that demonstrated to affect survival of greenlip abalone (63%) by Harris et al. (1999), using animals within the size range used in the present study.

In this study, the feed intake was reduced in abalone at 26 °C (Tables 4, 5; Fig. 3). This would have detrimental effects on the intake of essential nutrients such as vitamins and minerals for immunological function, and protein, lipid and energy intake for growth. Given that the abalone were under severe temperature stress, and immunologically challenged, one would expect that abalone would have a higher requirement for essential nutrients. Interestingly, in Experiment 2, abalone held at 26 °C progressively went off their food as the experiment progressed (Fig. 4). While there was no statistical difference in meals missed due to diet type, there was a trend for abalone fed the commercial diet to miss a larger proportion of meals. Additionally, some tanks of abalone fed the commercial diet at 26 °C were also observed to have missed meals for up to four days in a row, whereas, the abalone fed *U. lactuca* at the same water temperature tended to alternate daily between eating and not eating. Loss of appetite is considered to be one of the first symptoms of stress and the onset of disease in aquatic animals, and will ultimately contribute to reduced growth performance (Kaushik, 1986; Wendelaar Bonga, 1997).

In a previous study utilising the same facility, the growth rate and feed efficiency of larger 2-year-old greenlip abalone were significantly lower compared to 1-year-old greenlip abalone at 18 and 22 °C (Stone et al., 2013). Additionally, in another study, smaller 1-year-old greenlip abalone exhibited significantly better growth and feed efficiency when fed the commercial diet compared to non-enriched or nitrogen-enriched *U. lactuca* (Bansemer et al., unpublished data). However, in the present study, due to the relatively slow growth rate of larger 2- and 3-year-old greenlip abalone, and the short duration of the current experiments, caution must be exercised when interpreting the treatment differences in growth performance and feed efficiency.

In conclusion, using a negative control diet, we demonstrated that dietary intervention reduced mortality in larger abalone at 26 °C. We also demonstrated a pattern of mortality in response to high water temperatures that differed for age classes. This information is valuable for systematic research to understand and alleviate the occurrence of mortality on-farm with greenlip abalone at high water temperatures, particularly in the areas of nutritional or therapeutic intervention. Although we did not investigate the effect of reproductive state on this mortality, it would be an area worth investigating using dietary intervention in the future. Other areas of potential application include: investigation into other algal species that may produce the same survival results with greenlip abalone; the potential of dried *U. lactuca* or other active constituent, as dietary additives; and the potential to use dietary antioxidants to alleviate this mortality.

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References

Abirami, R.G., Kowsalya, S., 2011. Nutrient and nutraceutical potentials of seaweed biomass *Ulva lactuca* and *Kappaphycus alvarezii*. J. Agric. Sci. Technol. 5, 1939-1250.
AOAC International, 1995. Official Methods of Analysis of AOAC International, 16th edition. vol. 2. Association of Analytical Communities, Arlington, VA, USA.
Bissett, A., Burke, C., Dunstan, G.A., Maguire, G.B., 1998. Bacterial colonization of a formulated abalone diet during extended immersion. J. Shellfish Res. 17, 995–1002.
Bosch, L., Amparo, A., Farre, R., 2006. Application of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent to the RP-HPLC determination of amino acids in infant foods. J. Chromatogr. B 831, 176–183.
Britz, P.J., Hecht, T., 1997. Effect of dietary protein and energy level on growth and body composition of South African abalone, *Haliotis midae*. Aquaculture 156, 195–210.
Cardinaud, M., Offret, C., Huchette, S., Moraga, D., Paillard, C., 2014. The impacts of handling and air exposure on immune parameters, gene expression, and susceptibility to vibriosis of European abalone *Haliotis tuberculata*. Fish Shellfish Immunol. 36, 1–8.
Cheah, K.Y., 2011. Grape Sourced Bioactives: A Potential New Treatment Strategy for Intestinal Mucositis and Colon Cancer. (Ph.D thesis) The University of Adelaide (131 pp.).
Chen, H., Mai, K., Zhang, W., Liufu, Z., Xu, W., Tan, B., 2005. Effects of dietary pyridoxine on immune responses in abalone, *Haliotis discus hannai* Ino. Fish Shellfish Immunol. 19, 241–252.
Dang, V.T., Speck, P., Benkendorff, K., 2011a. Effects of micro and macroalgal diet supplementations on growth and immunity of greenlip abalone, *Haliotis laevigata*. Aquaculture 320, 91–98.
Dang, V.T., Speck, P., Doroudi, M., Smith, B., Benkendorff, K., 2011b. Variations in the antiviral and antibacterial activity of *Haliotis laevigata*, *H. rubra* and their hybrid in South Australia. Aquaculture 315, 242–249.
Dang, V.T., Speck, P., Benkendorff, K., 2012. Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*. Fish Shellfish Immunol. 32, 732–740.
Gilroy, A., Edwards, J.S., 1998. Optimum temperature preference for growth of Australian abalone: preferred temperature and critical thermal maximum for blacklip abalone, *Haliotis rubra* (Leach), and the greenlip abalone, *Haliotis laevigata* (Leach). Aquac. Res. 29, 481–485.
Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York, NY, USA, pp. 26–60.
Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. Can. J. Microbiol. 8, 229–239.
Handlinger, J., Carson, J., Donachie, L., Gabor, L., Taylor, D., 2005. Bacterial infection in Tasmanian farmed abalone: causes, pathology, farm factors and control options. In: Walker, P., Lester, R., Bondad-Reantaso, M.G. (Eds.), Diseases in Asian aquaculture V. Fish health section. Asian Fisheries Society, pp. 289–299 (Manila).
Harris, J.O., Maguire, G.B., Edwards, S.J., Johns, D.R., 1999. Low dissolved oxygen reduces growth rate and oxygen consumption rate of juvenile greenlip abalone, *Haliotis laevigata* Donovan. Aquaculture 174, 265–278.
Hooper, C., Day, R., Slocombe, R., Handlinger, J., Benkendorff, K., 2007. Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. Fish Shellfish Immunol. 22, 363–379.
Hooper, C., Day, R., Slocombe, R., Benkendorff, K., Handlinger, J., 2011. Effect of movement stress on immune function in farmed Australian abalone (hybrid *Haliotis laevigata* and *Haliotis rubra*). Aquaculture 315, 348–354.
Huchette, S.M.H., Koh, C.S., Day, R.W., 2003. Growth of juvenile blacklip abalone (*Haliotis rubra*) in aquaculture tanks: effects of density and ammonia. Aquaculture 219, 457–470.
Jiang, H.F., Liu, X.L., Chang, Y.Q., Liu, M.T., Wang, G.X., 2013. Effects of dietary supplementation of probiotic *Shewanella colwelliana* WA64, *Shewanella olleyana* WA65 on the innate immunity and disease resistance of abalone, *Haliotis discus hannai* Ino. Fish Shellfish Immunol. 35, 86–91.
Kaushik, S.J., 1986. Environmental effects on feed utilization. Fish Physiol. Biochem. 2, 131–140.
Koike, Y., Flassch, J., Mazurier, J., 1979. Biological and ecological studies on the propagation of the ormer, *Haliotis tuberculata* Linnaeus: II. Influence of food and density on the growth of juveniles. 17. La Mer (43Mer).
Lampman, G.M., Kriz, G.G., Engel, R.G., 2010. A Small Scale Approach to Organic Laboratory Techniques, 3rd edition. Brooks Cole, Cengage Learning, Belmont, CA. USA (1024 pp.).
Madigan, S., Lehmann, D., Clarke, S., 2000. Determination of critical thermal maximum for four species of South Australian abalone; *Haliotis laevigata*, *H. scalaris*, *H. roei* and *H. cyclobates*. A Report by the South Australian Research and Development Institute (13 pp.).
Nicolas, J.L., Basuyaux, O., Mazurié, J., Thébault, A., 2002. *Vibrio carchariae*, a pathogen of the abalone *Haliotis tuberculata*. Dis. Aquat. Org. 50, 35–43.
NRC, 2011. Nutrient Requirements of Fish and Shrimp. National Academies Press, Washington D.C., USA (376 pp.).
Shepherd, S.A., 1973. Studies on southern Australian abalone (genus *Haliotis*). 1. Ecology of 5 sympatric species. Aust. J. Mar. Freshwat. Res. 24, 217–258.
Silva, M., Vieira, L., Almeida, A.P., Kijjoa, A., 2013. The marine macroalgae of the genus *Ulva*: chemistry, biological activities and potential applications. Oceanography 1, 101. http://dx.doi.org/10.4172/ocn.1000101.
Stone, D.A.J., Gaylord, G.T., Johansen, K.A., Overturf, K., Sealey, W.M., Hardy, R.W., 2008. Evaluation of the effects of repeated fecal collection by manual stripping on the plasma cortisol levels, TNF-α gene expression, and digestibility and availability of

nutrients from hydrolyzed poultry and egg meal by rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture 275, 250–259.
Stone, D.A.J., Harris, J.O., Mercer, G.J., Wang, H., Schaefer, E.N., Bansemer, M.S., 2013. Dietary protein level and water temperature interactions for greenlip abalone, *Haliotis laevigata*. J. Shellfish Res. 32, 119–130.
Tabarsa, M., Rezaei, M., Ramezanpour, Z., Waaland, J.W., 2012. Chemical compositions of the marine algae *Gracilaria salicornia* (Rhodophyta) and *Ulva lactuca* (Chlorophyta) as a potential food source. J. Sci. Food Agric. 92, 2500–2506.
Tierney, M.S., Croft, A.K., Hayes, M., 2010. A review of antihypertensive and antioxidant activities in macroalgae. Bot. Mar. 53, 387–408.
Tomanek, L., 2010. Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. J. Exp. Biol. 213, 971–979.
Travers, M.A., Le Goïc, N., Huchette, S., Koken, M., Paillard, C., 2008. Summer immune depression associated with increased susceptibility of the European abalone, *Haliotis tuberculata* to *Vibrio harveyi* infection. Fish Shellfish Immunol. 25, 800–808.
Travers, M.A., Basuyaux, O., Le Goïc, N., Huchette, S., Nicolas, J.L., Koken, M., Paillard, C., 2009a. Influence of temperature and spawning effort on *Haliotis tuberculata* mortalities caused by *Vibrio harveyi*: an example of emerging vibriosis linked to global warming. Glob. Chang. Biol. 15, 1365–1376.
Travers, M.A., Le Bouffant, R., Friedman, C.S., Buzin, F., Cougard, B., Huchette, S., Koken, M., Paillard, C., 2009b. Pathogenic *Vibrio harveyi*, in contrast to non-pathogenic strains, intervenes with the p38 MAPK pathway to avoid an abalone haemocyte immune response. J. Cell. Biochem. 106, 152–160.
Vandepeer, M., 2006. SARDI Publications, Abalone Aquaculture Subprogram: Preventing Summer Mortality of Abalone in Aquaculture Systems by Understanding Interactions Between Nutrition and Water Temperature. South Australian Research and Development Institute (Aquatic Sciences), Adelaide, SA, Australia (85 pp. SARDI Publication Number RD02/0035-2).
Vosloo, A., Laas, A., Vosloo, D., 2013. Differential responses of juvenile and adult South African abalone (*Haliotis midae* Linnaeus) to low and high oxygen levels. Comp. Biochem. Physiol. A 164, 192–199.
Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.
Xu, X., Xie, H., Wang, Y., Wei, X., 2010. A-type proanthocyanidins from lychee seeds and their antioxidant and antiviral activities. J. Agric. Food Chem. 58, 11667–11672.